Douglas L. Mayers
Jack D. Sobel · Marc Ouellette
Keith S. Kaye · Dror Marchaim
Editors

Antimicrobial Drug Resistance

Mechanisms of Drug Resistance, Volume 1

Second Edition

Springer

Douglas L. Mayers • Jack D. Sobel
Marc Ouellette • Keith S. Kaye • Dror Marchaim
Editors

Antimicrobial Drug Resistance

Mechanisms of Drug Resistance, Volume 1

Second Edition

Springer

Editors
Douglas L. Mayers, M.D.
Chief Medical Officer
Treiber Therapeutics
Cambridge, MA, USA

Marc Ouellette, Ph.D.
Professor
Canada Research Chair in Antimicrobial Resistance
Centre de recherche en Infectiologie
University of Laval
Quebec City, Canada

Dror Marchaim, M.D.
Infection Control and Prevention
Unit of Infectious Diseases
Assaf Harofeh Medical Center
Sackler Faculty of Medicine
Tel-Aviv University
Tel Aviv, Israel

Jack D. Sobel, M.D.
Professor of Medicine
Dean, Wayne State University School
of Medicine
Detroit Medical Center
Detroit, MI, USA

Keith S. Kaye, M.D., M.P.H.
Professor of Internal Medicine
Director of Clinical Research, Division
of Infectious Diseases
University of Michigan Medical School
Ann Arbor, MI, USA

ISBN 978-3-319-46716-0 ISBN 978-3-319-46718-4 (eBook)
DOI 10.1007/978-3-319-46718-4

Library of Congress Control Number: 2017930150

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Antimicrobial drug resistance is a global health problem that continues to expand as microorganisms adapt to the antibiotics we use to treat them and as new classes of antimicrobial agents have been harder to discover and advance into the clinic. The second edition of *Antimicrobial Drug Resistance* grew out of a desire by the editors and authors to provide an updated, comprehensive resource of information on antimicrobial drug resistance that would encompass the current information available for bacteria, fungi, protozoa, and viruses. The two volumes have been extensively revised with many new authors and chapters as the field of drug resistance has evolved. We believe that this information will be of value to clinicians, epidemiologists, microbiologists, virologists, parasitologists, public health authorities, medical students, and fellows in training. We have endeavored to provide this information in a style that is accessible to the broad community of persons who are concerned with the impact of drug resistance in our clinics and across broader global communities.

Antimicrobial Drug Resistance is divided into two volumes. Volume 1 has sections covering a general overview of drug resistance and mechanisms of drug resistance, first for classes of drugs and then by individual antimicrobial agents, including those targeting bacteria, fungi, protozoa, and viruses. Volume 2 addresses clinical, epidemiologic, and public health aspects of drug resistance, along with an overview of the conduct and interpretation of specific drug resistance assays. Together, these two volumes offer a comprehensive source of information on drug resistance issues by the experts in each topic.

We are very grateful to the 197 international experts who have contributed to this textbook for their patience and support as the work came together. The editors would like to especially thank Michelle Feng He for her exceptional support and encouragement to the editors in bringing this revised textbook to print. Finally, the book would never have been completed without the patience and support of our wives and families.

Cambridge, MA, USA — Douglas L. Mayers, M.D.
Detroit, MI, USA — Jack D. Sobel, M.D.
Québec, Canada — Marc Ouellette, M.D.
Ann Arbor, MI, USA — Keith S. Kaye, M.D., M.P.H.
Tel Aviv, Israel — Dror Marchaim, M.D.

Contents

Contributors

Kamilia Abdelraouf Department of Pharmaceutics, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

Sabeena Ahmed, M.Sc. Senior Research Officer, Infectious Disease Division, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh

Robert A. Akins, Ph.D. Professor of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, MI, USA

Barbara D. Alexander, M.D. Professor of Medicine and Pathology, Duke University, Durham, NC, USA

Elizabeth R. Andrews, Pharm.D. Clinical Scientist, G1 Therapeutics, Research Triangle Park, NC, USA

Sevtap Arikan-Akdagli, M.D. Professor of Microbiology and Clinical Microbiology, Hacettepe University Medical School, Ankara, Turkey

Director, Mycology Laboratory, Hacettepe University Medical School, Ankara, Turkey

Eric J. Arts, Ph.D. Professor of Microbiology and Immunology, Canada Research Chair on HIV Pathogenesis and Viral Control, University of Western Ontario, London, ON, Canada

Dominique Aubert, Ph.D. Laboratory Parasitology-Mycology, Hospital Maison Blanche and EA 3800, University of Reims Champagne-Ardenne, Reims, France

Fernando Baquero, M.D., Ph.D. Research Professor, Biology and Evolution of Microorganisms, Ramón y Cajal Institute for Health Research (IRYCIS), CIBERESP, Ramón y Cajal University Hospital, Madrid, Spain

Margaret C. Bash, M.D., M.P.H. Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, USA

Department of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

John D. Baxter, M.D. Division of Infectious Diseases, Cooper University Hospital, Cooper Medical School of Rowan University, Camden, NJ, USA

Gonzalo M.L. Bearman, M.D., M.P.H. Chair, Division of Infectious Diseases, Epidemiology and Community Medicine, Richmond, VA, USA

Professor of Internal Medicine, Epidemiology and Community Medicine, Richmond, VA, USA

Kirthana R. Beaulac, Pharm.D. Tufts Medical Center, Boston, MA, USA

Apostolos Beloukas, M.Sc., Ph.D. Research Associate, Institute of Infection and Global Health, University of Liverpool, Liverpool, UK

Thomas Benfield, M.D., D.M.Sci. Department of Infectious Diseases, Hvidovre University Hospital, Copenhagen, Denmark

Michael L. Bennish, M.D. Executive Director, Mpilonhle, Mtubatuba, South Africa

Michel G. Bergeron, O.Q., M.D., F.R.C.P.C. Founder and Director, Centre de recherche en infectiologie, CHU de Quebec-Université Laval, CHUL, Québec City, QC, Canada

Hiranmoy Bhattacharjee, Ph.D. Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

Giancarlo A. Biagini, Ph.D. Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK

John P. Bilello, Ph.D. Principal Scientist, Infectious Diseases Biology-Discovery, Merck, West Point, PA, USA

John S. Blanchard, Ph.D. Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, USA

Guy Boivin, M.D. Professor of Microbiology, Canada Research Chair on Emerging Viruses and Antiviral Resistance Research Center in Infectious Diseases, Laval University, Quebec City, QC, Canada

Robert A. Bonomo, M.D. Professor of Medicine, Pharmacology, Molecular Biology and Microbiology, University Hospitals Case Medical Center, Cleveland, OH, USA

Chief, Medical Service, Louis Stokes Cleveland Department of Veteran Affairs Medical Center, University Hospitals Case Medical Center, Cleveland, OH, USA

Vice Chair for Veteran Affairs, Department of Medicine, University Hospitals Case Medical Center, Cleveland, OH, USA

Michelle D. Brazas, Ph.D. Ontario Institute for Cancer Research, MaRS Centre, Toronto, ON, Canada

Itzhak Brook, M.D., M.Sc. Professor of Pediatrics, Georgetown University School of Medicine, Washington, DC, USA

Robert W. Buckheit Jr. , Ph.D. ImQuest BioSciences, Inc., Frederick, MD, USA

Joseph Adrian L. Buensalido, M.D. Clinical Associate Professor, Section of Infectious Diseases, Department of Medicine, University of the Philippines—Philippine, General Hospital Manila, Metro Manila, Philippines

Karen Bush, Ph.D. Professor of Practice, Biotechnology Program, Biology Department, Indiana University, Bloomington, IN, USA

Gerard Cangelosi, Ph.D. Professor, Department of Environmental and Occupational Health Sciences, School of Public Health, University of Washington, Seattle, WA, USA

Lilia López Cánovas, Ph.D. Professor, Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México, México City, Mexico

Rafael Cantón, Ph.D. Director, Department of Microbiology, Ramón y Cajal University Hospital, Madrid, Spain

Department of Microbiology, Faculty of Pharmacy Complutense, University Madrid, Madrid, Spain

Jelena Catania, M.D. Infectious Diseases Fellow, Duke University Medical Center, Durham, NC, USA

Vincent Cattoir, Pharm.D., Ph.D. Professor of Clinical Microbiology, Department of Clinical Microbiology, School of Medicine, Caen University Hospital, University of Caen Normandie, Caen, France

Jaya Chakravarty, M.B.B.S., M.D. Associate Professor of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Jyotsna Chandra, Ph.D. Senior Research Associate, Center for Medical Mycology, Department of Dermatology, University Hospitals of Cleveland, Case Western Reserve University, Cleveland, OH, USA

P.H. Chandrasekar, M.D. Division of Infectious Diseases, Department of Internal Medicine, Wayne State University School of Medicine, Harper University Hospital, Detroit, MI, USA

Kimberly C. Claeys, Pharm.D. Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy, Wayne State University, Detroit, MI, USA

Gerald C. Coles, M.A. Ph.D., Sc.D. School of Veterinary Sciences, University of Bristol, Bristol, UK

Lynn E. Connolly, M.D., Ph.D. Achaogen, Inc., San Francisco, CA, USA

Department of Medicine, Division of Infectious Diseases, University of California, San Francisco, CA, USA

A.J. Cornell School of Animal and Veterinary Sciences and Graham Centre for Agricultural Innovation, Charles Sturt University, Wagga Wagga, NSW, Australia

Patrice M. Courvalin, M.D., F.R.C.P. Unité des Agents Antibactériens, Institut Pasteur, Paris, France

Mathieu Coutu Department of Microbiology, Infectiology and Immunology, Université de Montréal, Montreal, QC, Canada

Clyde S. Crumpacker II , M.D. Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Professor of Medicine, Harvard Medical School, Boston, MA, USA

Sarah L. Cudmore, B.Sc. Infection Prevention and Control, Public Health Ontario, Ottawa, ON, Canada

Vanessa M. D'Costa, Ph.D. Cell Biology Program, The Hospital for Sick Children, Toronto, ON, Canada

Florence Depardieu Unité des Agents Antibactériens, Institut Pasteur, Paris, France

Nainee Desai, Pharm.D. Medical Affairs Department, Cubist Pharmaceuticals, Lexington, MA, USA

Abhay Dhand, M.D. Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Boston, MA, USA

Carlos A. Diaz Granados, M.D., M.S.C.R. Director of Clinical Sciences, Clinical Department, Sanofi Pasteur, Swiftwater, PA, USA

Michael J. Doenhoff, B.Sc., Ph.D. School of Life Sciences, University of Nottingham, University Park, Nottingham, UK

Yohei Doi, M.D., Ph.D. Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Robert A. Domaoal, Ph.D. Department of Pediatrics, Laboratory of Biochemical Pharmacology, Center for AIDS Research, Emory University School of Medicine, Atlanta, GA, USA

Summer Donovan, M.D. Fellow, Division of Pediatric Infectious Diseases, Virginia Commonwealth University Medical Center, Richmond, VA, USA

Shira I. Doron, M.D. Associate Professor of Medicine, Tufts University School of Medicine, Boston, MA, USA

Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Boston, MA, USA

Jacques Dumas, Ph.D. Chief Scientific Officer, Tetraphase Pharmaceuticals, Watertown, MA, USA

Herbert L. DuPont, M.D. University of Texas School of Public Health, Houston, TX, USA

McGovern Medical School, Baylor College of Physicians and Kelsey Research Foundation, Houston, TX, USA

Noton K. Dutta, Ph.D. Research Associate of Medicine, Division of Infectious Diseases, Department of Medicine, Center for Tuberculosis Research, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Maryam Ehteshami, Ph.D. Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA

Matthew E. Falagas, M.D., M.Sc., D.Sc. Alfa Institute of Biomedical Sciences (AIBS), Athens, Greece

Department of Medicine, Tufts University School of Medicine, Boston, MA, USA

Loïc Favennec, M.D., Ph.D. Professor of Pharmacy, Chief Medical Officer, Laboratory Parasitology-Mycology, Hospital Charles Nicolle and EA 3800, University of Rouen, Rouen, France

Lucía Fernández Instituto de Productos Lacteos de Asturias (IPLA), Consejo Superior de Investigaciones Cientificas (CSIC), Villaviciosa, Asturias, Spain

Andrés Finzi, Ph.D. Research Assistant Professor, Department of Microbiology, Infectiology and Immunology, Canada Research Chair on Retroviral Entry, Université de Montréal, Montreal, QC, Canada

Gary E. Garber, M.D., F.R.C.P.C., F.A.C.P. Infection Prevention and Control, Public Health Ontario, Ottawa, ON, Canada

Consuelo Gómez García, Ph.D. Professor, Programa Institucional de Biomedicina Molecular, Escuela Nacional de Medicina y Homeopatía (ENMyH), Instituto Politécnico Nacional, México City, Mexico

Anna Maria Geretti, M.D., Ph.D., FRCPath. Professor of Virology and Infectious Diseases, Honorary Consultant in Infectious Diseases, Institute of Infection and Global Health, University of Liverpool, Liverpool, UK

Mahmoud A. Ghannoum, Ph.D. Professor, Center for Medical Mycology, Department of Dermatology, University Hospitals of Cleveland, Case Western Reserve University, Cleveland, OH, USA

Vaidya Govindarajan Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

Fabrice E. Graf Swiss Tropical and Public Health Institute, Parasite Chemotherapy Unit, Basel, Switzerland

Robert M. Greenberg, Ph.D. Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA

Ying-Shan Han, Ph.D. McGill University AIDS Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada

Robert E.W. Hancock, Ph.D. Canada Research Chair and Professor, Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada

Director, Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, BC, Canada

Kimberly E. Hanson, M.D., M.H.S. Division of Infectious Diseases, University of Utah, Salt Lake City, UT, USA

Bianca Heinrich, Ph.D. Senior Scientist, Genomic Assays, Abcam, Inc., Cambridge, MA, USA

David K. Henderson, M.D. Office of the Deputy Director for Clinical Care, National Institutes of Health, Bethesda, MD, USA

Brian D. Herman, Ph.D. Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Veterans Affairs Medical Center, Atlanta, GA, USA

Kathleen L. Horan, M.D. Pulmonary Medicine, Virginia Mason Medical Center, Seattle, WA, USA

Ann Huletsky, Ph.D. Adjunct Professor, Centre de recherche en infectiologie, CHU de Quebec-Université Laval, CHUL, Québec City, QC, Canada

Michael G. Ison, M.D., M.S. Associate Professor, Divisions of Infectious Diseases and Organ Transplantation, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Michael R. Jacobs, M.D., Ph.D. Professor of Pathology and Medicine, Director of Clinical Microbiology, Case Western Reserve University and University Hospitals Case Medical Center, Cleveland, OH, USA

George A. Jacoby, M.D. Associate Professor of Medicine, Part-Time, Harvard Medical School, Boston, MA, USA

Jisha John, M.D. Fellow, Infectious Diseases, Wayne State University, Detroit, MI, USA

Matthew D. Johnson Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia

Glenn W. Kaatz, M.D. Associate Chief of Staff, Research and Development, John D. Dingell VA Medical Center, Professor Medicine, Department of Internal Medicine and Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, MI, USA

Petros C. Karakousis, M.D. Associate Professor of Medicine, Center for Tuberculosis Research, Department of Medicine, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Angela D.M. Kashuba, B.Sc.Phm., Pharm.D., D.A.B.C.P. Professor and Chair, Division of Pharmacotherapy and Experimental Therapeutics, Eshelman School of Pharmacy University of North Carolina, Chapel Hill, NC, USA

David E. Katz, M.D., M.P.H. Director, Internal Medicine Department 'D', Shaare Zedek Medical Center, Hebrew University School of Medicine, Jerusalem, Israel

Wasif A Khan, M.B.B.S., M.H.S. Scientist, Infectious Disease Division, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh

Keith P. Klugman, M.D., Ph.D. Director, Pneumonia, Bill and Melinda Gates Foundation, Seattle, WA, USA

Joseph Kovacs, M.D. Department of Infectious Diseases, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

Sebastian G. Kurz, M.D., Ph.D. Division of Pulmonary and Critical Care, Department of Medicine, Tufts Medical Center, Boston, MA, USA

Jannik-Helweg Larsen, M.D. Department of Infectious Diseases, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

Roland Leclercq, M.D., Ph.D. Professor of Clinical Microbiology, Department of Clinical Microbiology, Caen University Hospital, School of Medicine, University of Caen Normandie, Caen, France

Danielle Légaré, Ph.D. Centre de Recherche en Infectiologie du CHU de Québec, Université Laval, Quebec City, QC, Canada

Donald P. Levine, M.D. Professor of Medicine, Wayne State University, Detroit, MI, USA

Shawn Lewenza, Ph.D. Associate Professor, Faculty of Science and Technology, Athabasca University, Athabasca, AB, Canada

Jennifer Li, B.Sc. (Hons) Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada

Jian Li, Ph.D. Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia

Xian-Zhi Li, M.D., Ph.D Team Leader, Human Safety Division, Veterinary Drugs Directorate, Health Products and Food Branch, Health Canada, Ottawa, ON, Canada

Stephen A. Locarnini, MBBS, BSc (Hon), PhD, FRCPath. Head Research and Molecular Development, Victorian Infectious Diseases Reference Laboratory, Doherty Institute, Melbourne, Australia

Jose L. Lopez-Ribot, Pharm.D., Ph.D. Department of Biology and South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio, San Antonio, TX, USA

R. Dwayne Lunsford, Ph.D. Integrative Biology and Infectious Diseases Branch, Microbiology Program, NIDCR: National Institute of Dental and Craniofacial Research, Bethesda, MD, USA

Joseph D. Lutgring, M.D. Assistant Professor of Medicine, Division of Infectious Diseases, Emory University, Atlanta, GA, USA

Pauline Macheboeuf, Ph.D. Senior Scientist, Institute for Structural Biology, CNRS, CEA, Université Grenoble Alpes, Grenoble, France

Maria L. Magalhães Department of Food and Animal Production State University of Santa Catarina, Lages, SC, Brazil

Elias K. Manavathu, Ph.D. Division of Infectious Diseases, Department of Medicine, Medical College of Georgia, Augusta University, Augusta, GA, USA

Goutam Mandal, Ph.D. Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

Laurence A. Marchat, Ph.D. Professor, Programa Institucional de Biomedicina Molecular, Escuela Nacional de Medicina y Homeopatía (ENMyH), Instituto Politécnico Nacional, México City, Mexico

Pascal Mäser, Ph.D. Swiss Tropical and Public Health Institute, Parasite Chemotherapy Unit, Basel, Switzerland

Henry Masur, M.D. Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Kathryn A. Matthias, Ph.D. Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, USA

Douglas L. Mayers, M.D. Chief Medical Officer, Treiber Therapeutics, Cambridge, MA, USA

Matthew McCarthy, M.D. Transplantation-Oncology Infectious Diseases Program, Weill Cornell Center, New York, NY, USA

Patrick F. McDermott, M.S., Ph.D. Center for Veterinary Medicine, Office of Research, U. S. Food and Drug Administration, Laurel, MD, USA

Lesley McGee, Ph.D. Microbiologist, Respiratory Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA

John E. McGowan Jr. , M.D. Professor, Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA, USA

Joseph B. McPhee, Ph.D. Assistant Professor, Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada

Francis Mégraud, M.D. National Reference Center for Campylobacters and Helicobacters, University Bordeaux Segalen, Bordeaux, France

Thibault Mesplède, Ph.D. McGill University AIDS Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada

Melissa B. Miller, Ph.D., D.(A.B.M.M.) Department of Pathology and Lab Medicine, UNC School of Medicine, Chapel Hill, NC, USA

Greg Moeck, Ph.D. Vice President, Biology, The Medicines Company, Saint-Laurent, QC, Canada

Stephen A. Morse, M.S.P.H., Ph.D. Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging, Zoonotic and Infectious Diseases Centers for Disease Control and Prevention (Retired), Atlanta, GA, USA

Rita Mukhopadhyay, Ph.D. Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

Alexandre Mzabi, M.D. Laboratory Parasitology-Mycology, Hospital Maison Blanche and EA 3800, University of Reims Champagne-Ardenne, Reims, France

Roger L. Nation, Ph.D. Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia

Eleni Ntokou, Ph.D. Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Esther Orozco, Ph.D. Professor, Departamento de Infectómica y Patogénesis Molecular, CINVESTAV, IPN, México City, Mexico

Elizabeth M. O'Shaughnessy, M.D. Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA

Marc Ouellette, Ph.D. Professor, Canada Research Chair in Antimicrobial Resistance, Centre de recherche en Infectiologie, University of Laval, Quebec City, Canada

Tara N. Palmore, M.D. Hospital Epidemiology Service, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Neil T. Parkin, Ph.D. Executive Director, Data First Consulting, Inc., Belmont, CA, USA

Sally R. Partridge, D.Phil. Principal Research Fellow, Centre for Infectious Diseases and Microbiology, The Westmead Institute for Medical Research, The University of Sydney and Westmead Hospital, Sydney, NSW, Australia

David L. Paterson, M.D., Ph.D. University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, Brisbane, Australia

Thomas F. Patterson, M.D., F.A.C.P., F.I.D.S.A. Department of Medicine, Division of Infectious Diseases, University of Texas Health Science, Center at San Antonio and Audie Murphy Division, South Texas Veterans Health Care System, San Antonio, TX, USA

Federico Perez, M.D. Medicine and Research Services, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Case Western Reserve University School of Medicine, Cleveland, OH, USA

D. Guillermo Pérez Ishiwara, Ph.D. Professor, Centro de Investigación en Biotecnología Aplicada (CIBA), Instituto Politécnico Nacional, México City, Mexico

John R. Perfect, M.D. James B. Duke Professor of Medicine, Chief, Division of Infectious Diseases and International Health, Duke University Medical Center, Durham, NC, USA

David S. Perlin, Ph.D. Public Health Research Institute, New Jersey Medical School, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

Jocelyne Piret, Ph.D. Research Center in Infectious Diseases, CHU de Québec and Université, Laval, QC, Canada

Bruno Pradines, Pharm.D., Ph.D. Unité de Parasitologie et d'Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France

Roger K. Prichard, Ph.D. Institute of Parasitology, McGill University, St. Anne de Bellevue, QC, Canada

Michael J. Pucci, Ph.D. Executive Director, Spero Therapeutics, Cambridge, MA, USA

Annette N. Ratcliff, Ph.D. Technology Licensing Manager, Ohio State University, Columbus, OH, USA

Jacqueline D. Reeves, Ph.D. Director, Monogram Biosciences, Laboratory Corporation of America® Holdings, South San Francisco, CA, USA

John H. Rex, M.D. Senior Vice President and Chief Strategy Officer, AstraZeneca Infection Business Unit, Waltham, MA, USA

Katherine Reyes, M.D., M.P.H. Corporate Medical Director, Infection Prevention and Control, Henry Ford Health System, Detroit, MI, USA

Senior Staff Physician, Infectious Diseases, Henry Ford Hospital, Detroit, MI, USA

Louis B. Rice, M.D. Brown University and Rhode Island Hospital, Providence, RI, USA

Marilyn C. Roberts, Ph.D. Department of Environmental and Occupational Health Sciences, School of Public Health, University of Washington, Seattle, WA, USA

Mario Alberto Rodríguez, Ph.D. Professor, Departamento de Infectómica y Patogénesis Molecular, CINVESTAV, IPN, México City, Mexico

Paul H. Roy, Ph.D. Professor Emeritus, Centre de recherche en Infectiologie, Université Laval, Quebec City, QC, Canada

William A. Rutala, Ph.D., M.P.H. Department of Hospital Epidemiology, UNC Health Care System, Chapel Hill, NC, USA

Division of Infectious Diseases, UNC School of Medicine, Chapel Hill, NC, USA

Michael J. Rybak, Pharm.D., M.P.H. Director, Professor of Pharmacy and Adjunct Professor of Medicine, Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, USA

Max Salfinger, M.D. Executive Director, Advanced Diagnostic Laboratories, Mycobacteriology and Pharmacokinetics, National Jewish Health, Denver, CO, USA

Nicholas C. Sangster, B.Sc.(Vrt.), B.F.Sc., Ph.D. School of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia

Celia A. Schiffer, Ph.D. Professor and Director, Institute of Drug Resistance, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA

Raymond F. Schinazi, Ph.D., D.Sc. Frances Winship Walters Professor of Pediatrics, Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Veterans Affairs Medical Center, Atlanta, GA, USA

Bryan D. Schindler, Ph.D. Microbiologist II, NSF International, Ann Arbor, MI, USA

Stefan Schwarz, Ph.D. Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany

Alisa W. Serio, Ph.D. Achaogen, Inc., South San Francisco, CA, USA

Mansi Sharma, Ph.D. Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

Ola E. Sköld, M.D., Ph.D. Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Nicolas Sluis-Cremer, Ph.D. Associate Professor of Medicine, Department of Medicine, Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Jeffrey D. Smith, M.Sc. Infection Prevention and Control, Public Health Ontario, Ottawa, ON, Canada

Jordan R. Smith, Pharm.D. Assistant Professor of Clinical Sciences, High Point University School of Pharmacy, High Point, NC, USA

David R. Snydman, M.D., F.A.C.P. Professor of Medicine, Tufts University School of Medicine and Division of Geographic Medicine and Infectious Diseases, Tufts Medical Center, Boston, MA, USA

Jack D. Sobel, M.D. Professor of Medicine, Dean, Wayne State University School of Medicine, Detroit Medical Center, Detroit, MI, USA

Akos Somoskovi, M.D., Ph.D., D.Sc. Department of Respiratory Medicine, Skaraborg Hospital, Skövde, Sweden

Kathryn A. Stafford, B.Sc., M.Sc., Ph.D. Research Assistant, Department of Clinical Veterinary Science, University of Bristol, Langford, Bristol, UK

Judith N. Steenbergen, Ph.D. Executive Director Clinical Microbiology, Cubist Pharmaceuticals, Lexington, MA, USA

Shyam Sundar, M.D., F.R.C.P., F.N.A. Professor of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Göte Swedberg, Ph.D. Associate Professor, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Vincent H. Tam, Pharm.D. Department of Pharmacy Practice and Translational Research, University of Houston College of Pharmacy, Houston, TX, USA

Sandeep Tamber, Ph.D. Research Scientist, Bureau of Microbial Hazards, Health Canada, Ottawa, ON, Canada

Fred C. Tenover, Ph.D. D.(A.B.M.M.) Vice President, Scientific Affairs, Cepheid, Sunnyvale, CA, USA

Kyriakos K. Trigkidis, M.D. Alfa Institute of Biomedical Sciences (AIBS), Athens, Greece

Liza Valdivia, M.D. Brown University and Rhode Island Hospital, Providence, RI, USA

Jose A. Vazquez, M.D. Chief, Division of Infectious Diseases, Professor of Medicine, Medical College of Georgia at Augusta University, Augusta, GA, USA

Colin M. Venner Department of Microbiology and Immunology, Western University, London, ON, Canada

Thierry Vernet, Ph.D. Group Head, Institute for Structural Biology, CNRS, CEA, Université Grenoble Alpes, Grenoble, France

Birte Vester, Ph.D. Professor, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Isabelle Villena, M.D. Professor of Medicine, Chief Medical Officer, Laboratory Parasitology-Mycology, Hospital Maison Blanche and EA 3800, University of Reims Champagne-Ardenne, Reims, France

Erhard Van der Vries, Ph.D. Research Center for Emerging Infections and Zoonoses, University of Veterinary Medicine, Hannover, Germany

Mark A. Wainberg, Ph.D. McGill University AIDS Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada

Thomas J. Walsh, M.D. Department of Pediatrics and Transplantation-Oncology Infectious Diseases Program, Weill Cornell Center, New York, NY, USA

Stephen A. Ward, Ph.D. Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK

David J. Weber, M.D., Ph.D. Department of Hospital Epidemiology, UNC Health Care System, Chapel Hill, NC, USA

Division of Infectious Diseases, UNC School of Medicine, Chapel Hill, NC, USA

Linda M. Weigel, Ph.D. Principal Investigator, Biodefense Research and Development Laboratory, Centers for Disease Control and Prevention, Atlanta, GA, USA

L. Joseph Wheat, M.D. Medical Director, MiraVista Diagnostics, Indianapolis, IN, USA

Jean M. Whichard, D.V.M., Ph.D. Division of Foodborne, Waterborne and Environmental Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

Nathan P. Wiederhold, Pharm.D. Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

Rob G. Woodgate, BSc, BVMS (Hons), PhD (Murd) Senior Lecturer in Veterinary Parasitology, School of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia

Gerard D. Wright, Ph.D. Professor of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada

Director of Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada

Hui-Ling Yen, Ph.D. School of Public Health, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong

Nese Kurt Yilmaz, Ph.D. Research Assistant Professor, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA

Juwon Yim, Pharm.D. Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy, Wayne State University, Detroit, MI, USA

André Zapun, Ph.D. Senior Scientist, Institute for Structural Biology, CNRS, CEA, Université Grenoble Alpes, Grenoble, France

Evan J. Zasowski, Pharm.D., M.P.H. Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy, Wayne State University, Detroit, MI, USA

Marcus Zervos, M.D. Division Head, Infectious Diseases, Henry Ford Health System, Detroit, MI, USA

Professor of Medicine, Wayne State University School of Medicine, Detroit, MI, USA

Part I

General Overview

History of Drug-Resistant Microbes

1

George A. Jacoby

1 Introduction

Instead of eliminating infectious diseases, as some had predicted, antibiotic use has inevitably led to the emergence of more antibiotic-resistant pathogens. This chapter reviews the history of our understanding of the processes by which resistance arises. Knowledge of the chemistry and genetics of this phenomenon has allowed the development of improved antibiotics and has made major contributions to molecular biology and the biotechnical revolution.

Resistance to antimicrobial agents has been recognized since the dawn of the antibiotic era. Paul Ehrlich, the father of modern chemotherapy, observed that during treatment of trypanosome infections organisms sometimes emerged that were resistant to the agent being used. Resistance was specific in the sense that a fuchsin dye-resistant strain was still susceptible to an arsenic compound while a strain resistant to the arsenic compound retained sensitivity to the dye. He showed that resistance, once acquired, was stably inherited and in 1908 proposed that resistance was due to "reduced avidity of the chemoreceptors so that they are no longer able to take up" drug [1]. Substitute "target" for "chemoreceptor" and one of the major mechanisms for antimicrobial resistance was revealed as was its specificity for particular compounds. Drug inactivation was discovered early as well. In 1919, Neuschlosz reported that *Paramecium caudatum* resistant to quinine and to certain dyes acquired the ability to destroy the toxic agents [2].

Early on resistance was categorized as either natural or acquired. For example, natural resistance to gentian violet was a property of gram-negative as compared to gram-positive organisms. Some agents (sulfonamides, aminoglycosides, chloramphenicol, rifampin, and others) were recognized to have a broad spectrum while other agents had a narrower focus (vancomycin, macrolides, isoniazid). The less susceptible organisms were said to be naturally resistant. The natural resistance of gram-negative bacteria to dyes and many other agents was attributed to an outer membrane barrier, which with our now increased appreciation of efflux pumps is understood to be only part of the story [3]. Acquired resistance properly involved reduced susceptibility of an organism that was previously more sensitive to the drug, and was to be distinguished, if possible, from replacement of a susceptible organism by more resistant but unrelated ones, a process soon appreciated to occur all too readily in hospitals, which became breading grounds for increasingly resistant flora.

How to interpret the emergence of resistance revived a nineteenth century controversy between Nägeli and Koch. Nägeli held that microorganisms were polymorphic and could transform spontaneously in shape and biochemical behavior. Koch believed that they were monomorphic with fixed properties and hence classifiable into species that could be rigidly defined. In the 1920s and 1930s this debate took the form of belief in the influence of bacterial life cycles. The theory of microbial dissociation held that such properties as shape, nutritional requirements, antigenicity, virulence, chemical reactivity, and hence susceptibility were not fixed properties of an organism but varied with the growth phase and life cycle of the bacterial culture [4]. By this line of reasoning the appearance of antibiotic resistance was but another manifestation of dissociation.

In today's terms the issue was adaptation versus mutation. Did acquired resistance represent an adaptive response to the drug, which persisted for many generation after the drug was removed, or selection from the initial population of rare pre-existing resistant mutants? The adaptation hypothesis was championed in the 1940s by Hinshelwood who argued that if a culture was grown in the presence of an inhibitor, the concentration of the substrate for the blocked reaction would accumulate and reverse the inhibition. Serial culturing in successively higher concentrations of drug was interpreted as thus "training" the culture to tolerate the inhibition [5].

G.A. Jacoby, M.D. (✉)
Part-Time, Harvard Medical School, Boston, MA, USA
e-mail: gajacoby50@gmail.com

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_1

The issue was settled in favor of mutation by demonstration that resistance could emerge in the absence of antibiotic and by its transfer with DNA. For example, the Lederbergs showed by replica plating that streptomycin-resistant colonies of *Escherichia coli* were present in a culture never exposed to the drug [6], while Hotchkiss demonstrated that penicillin resistance could be transferred to a susceptible pneumococcus by DNA from a resistant one [7].

Adaptation returned later, however, in the form of adaptive mutations and adaptive antibiotic resistance. Adaptive mutations are defined as mutations formed in response to the environment in which they have been selected [8, 9]. Such mutants occur in nondividing or slowly dividing cells and are specific for events that allow growth in that environment, as, for example, the emergence of ciprofloxacin-resistant mutants in nondividing cultures of *E. coli* exposed for a week to ciprofloxacin in agar [10]. Adaptive resistance is a phenomenon seen with aminoglycosides when bacteria pre-exposed to the antibiotic show less killing on subsequent exposure [11]. A reappreciation of genomic plasticity returned as well as the many mechanisms of horizontal gene transfer were elucidated and again challenged the notion of fixed bacterial species.

Until penicillin became available sulfonamides were widely used for both treatment and prophylaxis, and before long resistance began to appear in several pathogens. Daily administration of sulfadiazine to prevent upper respiratory infections at military bases during World War II was followed by the emergence of resistant β-hemolytic streptococci. The question was whether the resistance was acquired or preexisting. Since the resistant organisms mainly belonged to only a few serotypes, selection of naturally resistant strains was favored although the possibility that only particular serotypes could readily acquire resistance seems not to have been considered [12, 13]. Use of sulfonamides for treatment of gonorrhea was followed by increasing failure rates and the proliferation of sulfonamide-resistant strains of *Neisseria gonorrhoeae* [14]. Increasing sulfonamide resistance was also noted in *Neisseria meningitidis* with corresponding clinical failure [15]. Whether the neisseria truly acquired resistance was unclear since sulfonamide-resistant strains were discovered in cultures of *N. gonorrhoeae* or *N. meningitidis* from the presulfonamide era [15, 16]. Sulfonamide treatment of bacillary dysentery became complicated as well by the isolation of resistant strains, especially of resistant *Shigella sonnei* [17]. Isolated instances were also reported of sulfadiazine resistance in pneumococci recovered after therapy of either pneumococcal pneumonia [18] or pneumococcal meningitis [19]. Knowledge of bacterial biochemistry and metabolism had advanced after the empirical discovery of sulfonamides so that in 1940 *p*-aminobenzoic acid (PABA) was discovered to block the action of sulfonamide. PABA was proposed to be an essential metabolite for bacteria. Sulfonamide was hypothesized to mimic the chemical structure of PABA and to impede bacterial growth by competing with PABA to prevent its utilization [20]. Extracts of resistant pneumococci were soon found to contain increased amounts of a sulfonamide inhibitor [21], which was identified as PABA in extracts of other sulfonamide-resistant bacteria [22], so all seemed consistent with resistance as the result of PABA overproduction. The story took another twist, however, when sulfonamide-resistant *E. coli* were found to make not excess PABA but a sulfonamide-resistant enzyme that utilizes PABA in an early step of folic acid biosynthesis [23]. Such target enzyme insensitivity is now thought to be the main, if not the sole, mechanism for sulfonamide resistance [24].

The major mechanism for resistance to penicillin was much more quickly identified. The dramatic increase in penicillin resistance in *Staphylococcus aureus* that took place in the first decade of the antibiotic's use resulted from the selective advantage provided by an enzyme that inactivated penicillin, which was present initially in only a few isolates. The enzyme, penicillinase, was first described, not in *S. aureus,* but in *E. coli,* in 1940, the same year clinical studies with penicillin began [25]. By 1942 increased resistance was reported in *S. aureus* from patients receiving penicillin [26], and in 1944 penicillinase was extracted from resistant strains of *S. aureus* obtained from patients who had not even been exposed to the drug [27]. At Hammersmith Hospital in London the fraction of *S. aureus* isolates that were penicillin resistant increased rapidly from 14 % in 1946, to 38 % in 1947, and to 59 % in 1948 [28] eventually stabilizing at the 90 % resistance seen today and inspiring the development of semi-synthetic β-lactamase-resistant penicillins, which were the first antibiotics specifically designed to overcome a characterized resistance mechanism [29]. Unfortunately, methicillin-resistant *S. aureus* appeared within a few years and were found to make not a methicillin-degrading enzyme but rather a novel methicillin-resistant protein involved in cell wall biosynthesis [30, 31]. The battle between bacteria and pharmaceutical chemists synthesizing improved β-lactam antibiotics had been joined and would continue [32].

The basis of resistance to streptomycin remained a puzzle for a long time. Streptomycin-resistant mutations arose at low frequency in many kinds of bacteria, including, unfortunately, *Mycobacterium tuberculosis* when the agent was used alone for treatment. Mutation produced not only high-level resistance but also bacteria dependent on streptomycin for growth, a curious type that could even be recovered from patients treated with the drug [33]. A variety of biochemical changes followed exposure to streptomycin, including damage to the cell membrane [34], but it was the observation that growth of a streptomycin-dependent mutant of *E. coli* in a suboptimal concentration of streptomycin resulted in

decreased concentrations of protein and increased amounts of RNA that led Spotts and Stanier to propose that streptomycin blocked protein synthesis in susceptible cells but was required for proper mRNA attachment to the ribosome in dependent ones [35]. Direct demonstration that streptomycin impaired amino acid incorporation in a cell-free system soon followed [36]. Streptomycin at a concentration as low as 10^{-6} M could inhibit polyuridylate directed incorporation of phenylalanine, but a 1000-fold higher concentration was required if the cell-free system was derived from a streptomycin-resistant organism. Furthermore, streptomycin was found to cause misreading of the genetic code so that in its presence polyuridylate catalyzed the misincorporation of isoleucine and other amino acids [37]. So much was learned in studying the interaction of streptomycin and other drugs with the bacterial ribosome [38] that it came as something of a surprise that clinical isolates resistant to streptomycin relied on quite a different strategy, namely modification by adenylation, phosphorylation, and, for other aminoglycosides, acetylation as well [39]. The lesson that resistance selected in the laboratory could be different from that selected in the clinic had to be learned.

Resistance to other antimicrobial agents emerged and was studied, but the next major conceptual advance was the appreciation of the importance of R-plasmids, which led not only to a better understanding of resistance acquisition and dissemination but ultimately to recombinant DNA and the biotechnology revolution. The demonstration of transferable resistance in Japan dated from 1959 but took several more years to attract attention and be accepted [40, 41]. An explosion of discoveries followed. R-plasmids were found around the world not only in *Enterobacteriaceae* but also in pseudomonas, acinetobacter, staphylococci, enterococci, bacteroides, clostridia, and in virtually every bacterial species examined. Some had remarkably wide host ranges while others were limited to gram-positive, gram-negative, anaerobic, or even smaller bacterial subsets. Techniques were developed for plasmid transfer, isolation, and classification [42, 43]. Transposons that allowed resistance genes to jump from one DNA site to another were discovered [44], as were integrons that allowed resistance gene cassettes to be captured on plasmids and efficiently expressed [45], and specialized insertion sequences adept at gene capture [46]. Restriction enzymes, often plasmid-mediated, facilitated analysis of plasmid structure and permitted DNA cloning. The genetics of antibiotic resistance became as tractable as its biochemistry and contributed much to the emerging discipline of molecular biology.

The finding that a β-lactamase (designated TEM) from a clinical isolate of *E. coli* was carried on an R-plasmid [47] led to the realization that this resistance mechanism could spread not only to other *E. coli* but also to other genera. Before long TEM β-lactamase was found in ampicillin-resistant *Haemophilus influenzae* [48] and in penicillin-resistant *N. gonorrhoeae* [49]. Enzymes more active on cephalosporins than penicillins were discovered, functional classification of the growing body of β-lactamases began [50], the technique of isoelectric focusing was added to the repertoire of β-lactamase biochemists [51], introduction of cefamandole led to the recognition that β-lactamase derepression could provide resistance in some organisms [52], and clinical use of expanded-spectrum cephalosporins was followed by an explosion of extended-spectrum and other β-lactamases [32, 53].

Plasmids carry genes for resistance to many other antimicrobial agents. Some genes code for enzymes that modify or inactivate the agents, others for enzymes that alter drug targets in the cell or provide alternate biosynthetic pathways. Genes for antibiotic efflux (chloramphenicol, tetracycline) were also found to be plasmid-determined, but efflux-mediated resistance occurred as well from chromosomal mutations that alter control circuits also involved in expression of outer membrane proteins that form porin channels for antibiotic uptake. Study of bacteria collected in the preantibiotic era indicated that the plasmids that organize, express, and transmit resistance predated the clinical use of antibiotics [54]. R-plasmids resulted from the insertion of resistance genes into previously existing vehicles for their spread. The resistance genes themselves have had a diverse origin. Some have come from organisms producing antibiotics since these organisms needed a mechanism for self-protection [55, 56]. Others are now appreciated to have been present in environmental organisms for millennia to counteract the biological weapons of competing antibiotic producers. Potential reservoirs of resistance genes have been found in ancient permafrost and at the bottom of caves sealed from above for millions of years [57, 58].

Plasmids are not the only vehicle for gene transfer. Naturally transformable pathogens such as *Streptococcus pneumoniae, N. meningitidis, N. gonorrhoeae,* and *H. influenzae* were found to exchange chromosomal genes with members of closely related species, including genes for penicillin-binding proteins and topoisomerases that provide resistance to penicillin or quinolones [59–61]. Mutation plays an important role in resistance to some antimicrobial agents usually by altering enzyme specificity or reducing binding to a lethal target. The notion that resistance was based on infrequent mutational events also led to the concept that resistance could be prevented by simultaneous administration of two drugs since the product of the likelihood of resistance emerging to each would be greater than the size of any possible infecting inoculum, a thesis best justified by the success of multidrug treatment of tuberculosis. An increased mutation rate eventually exerts a fitness cost, but limited rate increases have been found in organisms with resistance attributable to an altered target

(quinolone resistance from *gyrA* mutations) [62] or modified enzyme (expanded-spectrum β-lactam resistance due to extended-spectrum β-lactamases) [63].

Antibiotic resistance has come to be accepted as an inevitable consequence of antibiotic use. The ubiquity of the phenomenon has been amply illustrated with emerging resistance to antiviral, antifungal, and anti-parasitic agents as well. On the positive side understanding the mechanisms of antibiotic resistance has often provided important insights into how antibiotics work. Knowledge about R-factors has unfortunately not made a direct attack on the genetic basis of resistance possible, but insight into resistance mechanisms has guided the development of expanded-spectrum β-lactams (cefepime, cefotaxime, ceftazidime, ceftriaxone, aztreonam, and others), aminoglycosides (amikacin, dibekacin, arbekacin, plazomicin, and others), and tetracyclines (tigecycline) as well as currently available β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) and others undergoing evaluation (avibactam). A number of enigmas remain. Some organisms, such as *S. aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa,* seem particularly adept at acquiring resistance while others are puzzlingly reluctant with certain drugs. *Treponema pallidum* and *Streptococcus pyogenes*, for example, remain fully susceptible to penicillin G despite decades of exposure to the drug while other organisms have become progressively more resistant. The tempo at which resistance develops is also remarkably variable (Table 1.1). Resistance may appear soon after a drug is introduced or only after many years. Methicillin-resistant *S. aureus* were isolated in the United Kingdom within a few years of the drug being introduced [64, 65], but 20 years elapsed before pneumococci with reduced susceptibility to penicillin were isolated and another 20 years before resistance was recognized as a worldwide problem [66]. Vancomycin resistance took even longer to appear [67]. The equilibrium level at which resistance becomes stabilized is also curiously variable. β-Lactamase production has reached 10–30 % in the gonococcus, 15–35 % in *H. influenzae*, 30–40 % in *E. coli,* 75 % in *Moraxella catarrhalis*, and 90 % in *S. aureus*, but what determines these levels is poorly understood. Once it has been acquired, however, resistance is slow to decline [68] and there are few examples of reduced antibiotic use associated with diminished resistance [69] so that prevention of resistance by prudent antibiotic use remains the keystone to control. Appropriate use applies as well to nonhuman applications with restraining antibiotics in animal feed a prominent example.

Table 1.1 Timetable of antibiotic discovery and resistance

Antibiotic	Discovered or reported	Clinical use	Resistance identified	Organism
Sulfonamide	1935	1936	1939	*S. pneumoniae*
Penicillin G	1928	1941	1942	*S. aureus*
	1940 (purified)		1965	*S. pneumoniae*
Methicillin	1960	1960	1961	*S. aureus*
Oxyimino-β-lactams	1978	1981	1983	*K. pneumoniae*
				E. coli
Streptomycin	1944	1946	1946	*E. coli*
Tetracycline	1948	1952	1959	*S. dysenteriae*
Erythromycin	1952	1955	1957	*S. aureus*
Vancomycin	1956	1958	1987	*E. faecium*
Gentamicin	1963	1967	1970	*K. pneumoniae*
				P. aeruginosa

References

1. Ehrlich P. Ueber moderne chemotherapie. Leipzig: Akademische Verlagsgesellschaft m.b.H.; 1909. p. 167–202.
2. Neuschlosz S. Untersuchungen über die gewöhnung an gifte. Pflüger's Arch Physiol. 1919;176:223–35.
3. Nikaido H. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol. 1996;178:5853–9.
4. Hadley P. Microbic dissociation: the instability of bacterial species with special reference to active dissociation and transmissible autolysis. J Infect Dis. 1927;40:1–312.
5. Hinshelwood CN. The chemical kinetics of the bacterial cell. Oxford: The Clarendon Press; 1946.
6. Lederberg J, Lederberg EM. Replica plating and indirect selection of bacterial mutants. J Bacteriol. 1952;63:399–406.
7. Hotchkiss RD. Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. Symp Quant Biol. 1951;16:457–61.
8. Cairns J, Foster PL. Adaptive reversion of a frameshift mutation in *Escherichia coli*. Genetics. 1991;128:695–701.
9. Rosenberg SM. Evolving responsively: adaptive mutation. Nat Rev Genet. 2001;2:504–15.
10. Riesenfeld C, Everett M, Piddock LJ, Hall BG. Adaptive mutations produce resistance to ciprofloxacin. Antimicrob Agents Chemother. 1997;41:2059–60.
11. Xiong Y-Q, Bayer A, Potel G. Adaptive resistance to antibiotics.p. In: Hughes D, Andersson D, editors. Antibiotic development and resistance. London and New York: Taylor and Francis; 2001. p. 53–63.
12. Epidemiological Unit Number 22. Sulfadiazine resistant strains of beta hemolytic streptococci. J Am Med Assoc 1945;129:921–7.
13. Damrosch DS. Chemoprophylaxis and sulfonamide resistant streptococci. J Am Med Assoc. 1946;130:124–8.
14. Goodale WT, Schwab L. Factors in the resistance of gonorrhea to sulfonamides. J Clin Invest. 1944;23:217–23.
15. Feldman HA. Sulfonamide-resistant meningococci. Annu Rev Med. 1967;18:495–506.
16. Schmith K, Reymann FE. Experimental and clinical investigations on sensitivity of gonococci to sulfapyridine. Nord Med Tid. 1940;8:2493–9.
17. Wentworth FH, Wentworth B. Development of sulfadiazine resistance during outbreak of shigellosis due to *Shigella sonnei* form I. J Dis Child. 1957;93:551–4.
18. Frisch AW, Price AE, Myers GB. Development of sulfadiazine resistance, transmission by cross infection and persistence in carriers. Ann Intern Med. 1943;18:271–8.
19. Ross RW. Acquired tolerance of pneumococcus to M.& B. 693. Lancet. 1939;233:1207–8.

20. Woods DD. The relation of para-aminobenzoic acid to the mechanism of action of sulphanilamide. Br J Exp Pathol. 1940;21:74–90.
21. MacLeod CM. The inhibition of the bacteriostatic acion of sulfonamide drugs by substances of animal and bacterial origin. J Exp Med. 1940;72:217–32.
22. Landy M, Larkum NW, Oswald EJ, Streightoff F. Increased synthesis of p-aminobenzoic acid associated with the development of sulfonamide resistance in Staphylococcus aureus. Science. 1943;97:265–7.
23. Wise Jr EM, Abou-Donia MM. Sulfonamide resistance mechanism in *Escherichia coli*: R plasmids can determine sulfonamide-resistant dihydropteroate synthases. Proc Natl Acad Sci U S A. 1975;72:2621–5.
24. Huovinen P, Sundström L, Swedberg G, Sköld O. Trimethoprim and sulfonamide resistance. Antimicrob Agents Chemother. 1995;39:279–89.
25. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. Nature. 1940;146:837.
26. Rammelkamp CH, Maxon T. Resistance of *Staphylococcus aureus* to the action of penicillin. Proc Soc Exp Biol Med. 1942;51:386–9.
27. Kirby WMM. Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. Science. 1944;99:452–3.
28. Barber M, Rozwadowska-Dowzenko M. Infection by penicillin-resistant staphylococci. Lancet. 1948;2:641–4.
29. Rolinson GN. Forty years of ß-lactam research. J Antimicrob Chemother. 1998;41:589–603.
30. Brown DF, Reynolds PE. Intrinsic resistance to ß-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. 1980;122:275–80.
31. Hartman BJ, Tomasz A. Altered penicillin binding proteins in methicillin-resistant strains of *Staphylococcus aureus*. Antimicrob Agents Chemother. 1981;19:726–35.
32. Medeiros AA. Evolution and dissemination of ß-lactamases accelerated by generations of ß-lactam antibiotics. Clin Infect Dis. 1997;24 Suppl 1:S19–45.
33. Finland M. Emergence of antibiotic-resistant bacteria. N Engl J Med. 1955;253:909–22.
34. Anand N, Davis BD. Damage by streptomycin to the cell membrane of *Escherichia coli*. Nature. 1960;185:22–3.
35. Spotts CR, Stanier RY. Mechanism of streptomycin action on bacteria: a unitary hypothesis. Nature. 1961;192:633–7.
36. Flaks JG, Cox EC, White JR. Inhibition of polypeptide synthesis by streptomycin. Biochem Biophys Res Commun. 1962;7:385–9.
37. Davies J, Gilbert W, Gorini L. Streptomycin, suppression, and the code. Proc Natl Acad Sci U S A. 1964;51:883–90.
38. Weisblum B, Davies J. Antibiotic inhibitors of the bacterial ribosome. Bacteriol Rev. 1968;32:493–528.
39. Yamada T, Tipper D, Davies J. Enzymatic inactivation of streptomycin by R factor-resistant *Escherichia coli*. Nature. 1968;219:288–91.
40. Watanabe T. Infective heredity of multiple drug resistance in bacteria. Bacteriol Rev. 1963;27:87–115.
41. Watanabe T. Infectious drug resistance in enteric bacteria. N Engl J Med. 1966;275:888–94.
42. Datta N, Hedges RW. Compatibility groups among *fi-* R factors. Nature. 1971;234:222–3.
43. Meyers JA, Sanchez D, Elwell LP, Falkow S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol. 1976;127:1529–37.
44. Hedges RW, Jacob AE. Transposition of ampicillin resistance from RP4 to other replicons. Mol Gen Genet. 1974;132:31–40.
45. Stokes HW, Hall RM. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol Microbiol. 1989;3:1669–83.
46. Toleman MA, Bennett PM, Walsh TR. IS*CR* elements: novel gene-capturing systems of the 21st century? Microbiol Mol Biol Rev. 2006;70:296–316.
47. Datta N, Kontomichalou P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature. 1965;208:239–41.
48. Elwell LP, De Graaff J, Seibert D, Falkow S. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type b. Infect Immun. 1975;12:404–10.
49. Elwell LP, Roberts M, Mayer LW, Falkow S. Plasmid-mediated beta-lactamase production in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 1977;11:528–33.
50. Richmond MH, Sykes RB. The ß-lactamases of gram-negative bacteria and their possible physiological role. Adv Microb Physiol. 1973;9:31–88.
51. Matthew A, Harris AM, Marshall MJ, Ross GW. The use of analytical isoelectric focusing for detection and identification of ß-lactamases. J Gen Microbiol. 1975;88:169–78.
52. Sanders CC, Sanders Jr WE. Emergence of resistance to cefamandole: possible role of cefoxitin-inducible beta-lactamases. Antimicrob Agents Chemother. 1979;15:792–7.
53. Jacoby GA, Munoz-Price LS. The new ß-lactamases. N Engl J Med. 2005;352:380–91.
54. Hughes VM, Datta N. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature. 1983;302:725–6.
55. Benveniste R, Davies J. Aminoglycoside antibiotic-inactivating enzymes in Actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc Natl Acad Sci U S A. 1973;70:2276–80.
56. Marshall CG, Broadhead G, Leskiw BK, Wright GD. D-Ala-D-Ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin-resistance ligases VanA and VanB. Proc Natl Acad Sci U S A. 1997;94:6480–3.
57. D'Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. Antibiotic resistance is ancient. Nature. 2011;477:457–61.
58. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE. 2012;7, e34953.
59. Dowson CG, Hutchison A, Brannigan JA, George RC, Hansman D, Liñares J, Tomasz A, Smith JM, Spratt BG. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of Streptococcus pneumoniae. Proc Natl Acad Sci U S A. 1989;86:8842–6.
60. Spratt BG, Zhang QY, Jones DM, Hutchison A, Brannigan JA, Dowson CG. Recruitment of a penicillin-binding protein gene from *Neisseria flavescens* during the emergence of penicillin resistance in *Neisseria meningitidis*. Proc Natl Acad Sci U S A. 1989;86:8988–92.
61. Stanhope MJ, Walsh SL, Becker JA, Italia MJ, Ingraham KA, Gwynn MN, Mathie T, Poupard JA, Miller LA, Brown JR, Amrine-Madsen H. Molecular evolution perspectives on intraspecific lateral DNA transfer of topoisomerase and gyrase loci in *Streptococcus pneumoniae*, with implications for fluoroquinolone resistance development and spread. Antimicrob Agents Chemother. 2005;49:4315–26.
62. Komp Lindgren P, Karlsson A, Hughes D. Mutation rate and evolution of fluoroquinolone resistance in *Escherichia coli* isolates from patients with urinary tract infections. Antimicrob Agents Chemother. 2003;47:3222–32.
63. Baquero MR, Galán JC, del Carmen Turrientes M, Cantón R, Coque TM, Martínez JL, Baquero F. Increased mutation frequencies in *Escherichia coli* isolates harboring extended-spectrum ß-lactamases. Antimicrob Agents Chemother. 2005;49:4754–6.
64. Jevons MP. "Celbenin"-resistant staphylococci. Br Med J. 1961;1:124–5.

65. Barber M. Methicillin-resistant staphylococci. J Clin Pathol. 1961;14:385–93.
66. Klugman KP. Pneumococcal resistance to antibiotics. Clin Microbiol Rev. 1990;3:171–96.
67. Leclerq R, Derlot E, Duval J, Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med. 1988;319:157–61.
68. Enne VI, Livermore DM, Stephens P, Hall LM. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet. 2001;357:1325–8.
69. Seppälä H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. N Engl J Med. 1997;337:441–6.

Evolutionary Biology of Drug Resistance

2

Fernando Baquero and Rafael Cantón

1 Introduction

It is widely upheld that evolution is the result of two essential forces: variability (chance) and selection (necessity). This assumption is confirmed by a number of simple phenomena in antibiotic resistance. Variability is created by random mutation (also recombination), and some of these variants (for instance, those with a mutation in the antibiotic target) become resistant. These variants are selected by antibiotic use and consequently they increase the frequency of resistance. If we increase variability (as in a hyper-mutable strain) or the intensity of selection (antibiotic hyper-consumption), the result is more resistance. This is true, but not the whole truth. Most determinants of antibiotic resistance are not based on simple mutations, but rather on sophisticated systems frequently involving several genes and sequences; moreover, resistance mutations are seldom transmitted by lateral gene transfer. The acquisition of any type of resistance produces a change. In biology, any change is not only an opportunity, but is also a risk for evolution. Bacterial organisms are highly integrated functional structures, exquisitely tuned by evolutionary forces to fit with their environments. Beyond the threshold of the normal compliance of these functions, changes are expected to disturb the equilibrium. Therefore, the acquisition of resistance is not sufficient to survive; evolution should also shape and refine the way of managing resistance determinants. Under the perspective of systems biology, this biological dilemma is presented as "evolvability versus robustness", where only robust systems (able to tolerate a wide range of external changes) survive, but in the long term they should reorganize their compositional network so that they can address new and unexpected external changes. In fact, we can expect a constant cycle between robustness and evolvability in antibiotic resistance, which is manifested by changes in the frequency of some particular resistant clones.

Indeed, the field of research in drug resistance is becoming more and more complex, and constitutes a growing discipline. More than 40 years ago, Yves A. Chabbert (a brilliant pioneer in research about resistance) and one of us (F.B.) asked the pharmacologist John Kosmidis to coin the right Greek expression to describe "the science of studying resistance", and he immediately produced the word "antochology" (from Αντοχυ, resistance). To our knowledge, it was not used before the publication of the first edition of this book in 2009. In this chapter, we will examine the concept of resistance genes, the effectors of antibiotic resistance, and two essential processes that shape microbial evolution of drug resistance. First, **variability**, the *substrate of evolution*, the process providing material in evolutionary processes. Second, **selection**, the *mechanism of evolution* [1], the process by which evolution is able to adapt genetic innovation to environmental needs in the bacterial world. These evolutionary processes are embedded in a complex hierarchical network of interactions involving population dynamics of the biological elements involved in resistance, from particular genetic sequences, to genes, operons, mobile genetic elements, clonal variants, species, consortia of microorganisms, microbiotas, hosts and their communities, and the environment.

2 Resistance Genes, the Effectors of Antibiotic Resistance

Resistance genes are those that produce a protective or adaptive effect in a microorganism in response to the deleterious input following exposure to anthropogenic antimicrobial

F. Baquero, M.D., Ph.D. (✉)
Biology and Evolution of Microorganisms, Ramón y Cajal Institute for Health Research (IRYCIS), CIBERESP, Ramón y Cajal University Hospital, Madrid, Spain
e-mail: baquero@bitmailer.net

R. Cantón, Ph.D.
Department of Microbiology, Ramón y Cajal University Hospital, University Madrid, Madrid, Spain

Department of Microbiology, Faculty of Pharmacy Complutense, University Madrid, Madrid, Spain

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_2

agents. Note that implicitly this definition contains the concept that, in a strict sense, antibiotic resistance is resistance to antibiotic therapy, that is, resistance as a threat for public health and consequently for the patient and for human population. It is true that there are differences in antibiotic susceptibility among different bacterial organisms, but certainly "bacteria were not born susceptible"; by reasons totally unrelated with antibiotic exposure, many bacterial organisms are unsusceptible or poorly susceptible to some antimicrobial agents. For instance, *Escherichia coli* is "resistant" to macrolides, only because the structure (lipopolysaccharides-based) and function (physiological pumps, such as AcrAB) of the *E. coli* outer membrane do not allow these drugs to reach in sufficient quantity at the otherwise "susceptible" ribosomal targets. Obviously the genes encoding for the outer membrane cannot be considered antibiotic "resistance genes", and "resistance" can be considered here as a "false phenotype". However, if genes involved in lipopolysaccharide or AcrAB pumps are functionally eliminated, *E. coli* become more susceptible to macrolides, but that does not make them "resistance genes". In fact bacterial cells of all species contain a large number of genes (may reach 1 % of the genome) whose knock-out (or eventually mutations) or hyper-expression results in a decrease in susceptibility to antimicrobial agents. These genes constitute the "intrinsic resistome" for a given bacterial species [2]. The "natural resistance" or "intrinsic resistance" of particular species to certain antibiotics depends on these genes, which are normally part of the bacterial chromosome "core" genome, involved in the physiological functions of the cell.

Metagenomic studies have identified many of these genes as "resistance genes", and are inappropriately included as such in databases. As frequently new "resistance genes" are defined by homology with existing genes, the noise in databases may increase exponentially. Most of the mistakes in such attribution are related with three groups of genes: (1) genes belonging to the intrinsic resistome, (2) genes encoding antibiotic targets harbouring particular mutations, and (3) genes with insufficient degrees of genetic identity with resistance genes of clinical importance.

However, we cannot fully exclude that some of these genes could act as "true" resistance genes when they enter in another (susceptible) organism exposed to antibiotics. In their original host, these genes perform physiological functions, and are generally inserted in a functional network. Out of the original host, decontextualized genes might be selected as true resistance genes. The first condition for this is that these genes could be captured by mobile genetic elements (MGEs). Second, the bacteria harbouring resistance genes in MGEs should have sufficient genetic and ecologic connectivity with bacteria able to produce infections in humans. Third, that these genes encode for resistance to relevant antibiotics used in the therapy of infections, more so if these antibiotics were not known to be detoxified by other mechanisms. Considering these main factors, the different resistance genes that might be found in metagenomic resistomes can be classified into different levels of risk for health [3, 4].

3 Variability: The Substrate of Evolution of Drug Resistance

3.1 The Complexity of Antibiotic Action and the Variety of Resistance Phenotypes

The classic dominance of either mechanistic or clinical thought in microbiology has oversimplified the image of the possible harmful consequences of exposure to industrially produced antibiotics in the microbial world. From this point of view, antibiotics are considered as *anti-biotics*, anti-living compounds found or designed to either stop the growth or kill bacterial organisms. Their main molecular targets have been identified. Nevertheless, recent studies on sub-inhibitory effects of antibiotics demonstrate that the effects of antibiotic exposure in bacteria are much larger, and therefore the adaptive and evolutionary consequences of their action are also much more complex. First, at the cellular level, the effect of antibiotic exposure is not confined to the inhibition of a single lethal target and may cause secondary effects on bacterial metabolism. Second, at the population level, the effect of antibiotic exposure is not confined to the local extinction of a harmful bacterial organism. Antibiotics exert actions on the individual cells at concentrations far lower than those needed to inhibit growth or kill bacteria.

Recent studies of gene expression suggest that a number of cellular functions (some of them increasing fitness) are modified when bacteria are exposed to sub-inhibitory concentrations of antibiotics [5, 6]. Sub-inhibitory concentrations of aminoglycoside antibiotics induce biofilm formation in *Pseudomonas aeruginosa* and *E. coli.* In *P. aeruginosa*, the aminoglycoside response regulator gene (*arr*) is essential for this induction and has contributed to biofilm-specific aminoglycoside resistance [7]. These results support the notion that antibiotics in nature are not only bacterial weapons for fighting competitors, but they are also signalling molecules that may regulate the homeostasis of microbial communities. Competition, in microbial communities, is seldom a permanent effect; competitors might just be sufficiently aggressive to control the size of their populations, in order to avoid dominance of a single genotype. Diversity, rather than dominance of a particular group, is the hallmark of evolutionary success. Indeed the major aim of evolution is to survive, to persist in time; finally, the gain in space or in cell numbers only serves to assure persistence in time [8]. This view about an ecological role of antibiotics, serving as both weapons and signals (the classic armament-ornament duality) should immediately influence our view about the evolution of resistance traits [5]. If antibiotics act as weapons in nature, antibiotic resistance develops not only to prevent

Table 2.1 Levels of specificity in antibiotic resistance

• Target mutation or alternative target production
• Inducible enzyme protecting target
• Constitutive enzyme protecting target
• Inducible enzyme detoxifying the antibiotic
• Constitutive enzyme detoxifying the antibiotic
• Rewiring of physiological systems altered by antibiotic exposure
• Mutation in specific mechanism for antibiotic uptake
• Inducible efflux system
• Constitutive efflux system
• Alterations in general mechanisms of antibiotics uptake
• Nonspecific envelope permeability alterations
• Global stress adaptive responses
• Phenotypic tolerance related with cell cycle
• Environment-dependent resistance

suicide in the producer organisms, but also to protect the diversity of the coexisting microbial communities. If in natural environments the weapons are intended to be just sublethal, just to modulate the growth rate or to alter the gene expression profile of microbes sharing the same habitat, resistance traits are modifiers or back-modulators of these effects. Indeed we should be open to consider that the emergence and evolution of resistance not only applies for high-level, clinically relevant resistance, but also for resistance protecting the modulation of microbial interactions. If these interactions are important to maintain the bacterial lifestyle, resistance will develop even at very low "signalling" concentrations. In short, there are a multiplicity of effects of antibiotics in bacteria; consequently, there are many levels on which antibiotic resistance is exerted, from very specific to very general ones (Table 2.1).

3.1.1 Adaptation Without Change: Redundancy and Degeneracy of Bacterial Systems

Even though antibiotics might exert a number of effects on the bacterial cell even at low antibiotic concentrations, a number of cells within a population will be essentially unaffected and could restore the original population (see also "phenotypic tolerance" in the next Sect. 3.1.2). At biological system level, this is an example of environmental *canalization* defined as the property of a biological system to maintain the normal standard phenotype despite environmental perturbations. This *robustness* or inertia to perturbation depends in part on the redundancy and degeneracy of the biological system. *Redundancy* means that multiple identical units perform the same or very similar functions inside the system. For instance, by assuring high reproductive rates, which results in high cell densities, the negative effects of variation on the entire population is diluted. Indeed small populations have a high risk of extinction by deleterious variation. Interestingly, bacteria tend to increase their replication rate at concentrations of growth-inhibiting substances that are only slightly lower than those that prevent multiplication, but the adaptive impact of this phenomenon has as yet been scarcely explored.

If a number of individuals are lost after a challenge, many other almost-identical individuals are available to replace them, thus repairing the system. Note that the reconstruction of the population depends on a relatively low number of individuals, and therefore the new population will be purged to some degree of its original genetic diversity (periodic selection). At higher complexity levels, degenerate individuals may also compensate for losses in units within a system. *Degeneracy* means that structurally different units can perform the same or very similar functions in the system. Probably clonal diversification can be viewed as a way of increasing degeneracy within bacterial species. In short, redundancy and degeneracy tend to prevent antibiotic-mediated disordering events in high-level complexity bacterial systems, and lead to highly optimized tolerance. In the bacterial world, as redundant individuals are disposable they may be imported by other similar systems under danger of disorder. Hence, we can add *connectivity*—the ability of elements and systems to interact—as a means for increasing such tolerance.

3.1.2 Phenotypic Tolerance

Non-inherited antibiotic resistance (non-susceptibility) illustrates the flexibility of bacterial populations to adapt to antibiotic challenges. As stated in the previous paragraph, fully susceptible bacteria from the genetic point of view (that is, lacking specific mechanisms of resistance) might exhibit phenotypic tolerance to antibiotics, that is, they are able to persist at concentrations in which the majority of the population is dying. Cells regrown from these refractory bacteria remain as susceptible to the antibiotic as the original population [9]. Although canalization, redundancy, and degeneracy probably contribute to this phenomenon, it is the changes in the physiological state of bacterial organisms along the cell cycle that are probably critical. In practical terms, the main trait of the phenotype is slow growth. Experiments have shown that when growing bacteria are exposed to bactericidal concentrations of antibiotics, the sensitivity of the bacteria to the antibiotic commonly decreases with time and substantial fractions of the bacteria survive, without developing any inheritable genetic change [10]. Interestingly, these tolerant subpopulations generated by exposure to one concentration of an antibiotic are also tolerant to higher concentrations of the same antibiotic and can be tolerant to other types of antibiotics. It is possible that in any bacterial population, a certain spontaneous switch might occur between normal and persister cells, and it has been proposed that the frequency of such a switch might be responsive to environmental changes [11]. Such switching is probably stochastic, and depends on the random induction of persister cells through the activation of the alarmone (p)ppGpp resulting in increasing function of mRNA endonucleases [12]. In fact, we could designate as "persistence" the result of such a

switch, and phenotypic tolerance or indifference to drugs as the physiological status of any cell to become refractory to drugs. However, in our opinion such distinctions are not always clear. Mathematical modelling and computer simulations suggests that phenotypic tolerance or persistence might extend the need of antibiotic therapy, cause treatment failure of eradication, and promote the generation and ascent of inherited, specific resistance to antibiotics [13].

3.2 The Source of Antibiotic-Resistance Genes

Genes currently involved in antibiotic-resistance may have evolved for purposes other than antibiotic resistance (Table 2.2). From this point of view, resistance should be considered as a chance product, determined by the interaction of an antibiotic and a particular genotype. This is not incompatible with the idea of a gradual modification of some genes of pre-existing cellular machinery to finally "convert" into resistance genes. Some genes which may be neutral or almost neutral in the prevailing non-antibiotic environment may possess a latent potential for selection that can only be expressed under the appropriate conditions of antibiotic selection. In this case we are probably facing a *pre-adaptation* [14, 15], in the sense of assumption of a new function without interference with the original function via a small number of mutations, or gene combinations. In a later paragraph we will see in details the possible origin of enzymes hydrolyzing beta-lactam antibiotics (beta-lactamases) as an alteration of the tridimensional structure of the active site of cell wall biosynthetic enzymes (transglycosylases-transpeptidases). In other cases, the mere amplification of genes with small activity for the purposes of resistance may also result in a resistant phenotype [16]. Finally, we can have an *exaptation* [17] if the genetic conditions which exist for a function are equally well adapted to serve for antibiotic resistance.

A reservoir of "unknown" resistance genes in the intestinal microbiome has been suggested [18] even though a number of these genes have not been functionally confirmed (might have structural resemblance with resistance genes, but the resistance function was not proven). Cryptic beta-lactamase-mediated resistance to carbapenems is present in intestinal *Bacteroides* or in *Listeria* [19–21]. Metallo-beta-lactamases (MBLs) can be found in the genomes of 12 different Rhizobiales [18]. Fifty-seven open reading frames were classified as potential MBLs. Four of them were functionally analysed and one was demonstrated to be a functional MBL. Broad-spectrum chromosomally mediated beta-lactamases are usually found in Gram-negative organisms. Quinolone-resistance *qnr* genes, now plasmid-mediated, were originated in the chromosome of aquatic bacteria, such as *Shewanella algae* [22, 23]. Cryptic tetracycline-resistance determinants are present in the chromosomes of susceptible *Bacillus*, *Bacteroides*, or *E. coli* strains as well as aminoglycoside modifying enzymes in some Enterobacteriaceae species and *P. aeruginosa* . Resistance mediated by drug-efflux pumps constitutes an excellent example of exaptation. For instance, a blast search for proteins similar to the macrolide-resistance Mef protein of *Streptococcus* reveals hundreds of hits of similar sequences encompassing all microorganisms, including *Neisseria, Bacteroides, Legionella, Enterococcus, Desulfitobacterium, Lactococcus, Lactobacillus, Ralstonia, Bacillus, Geobacter, Thermologa,* or *Streptomyces*. More recently, the possibility that genetic variants of the aminoglycoside-inactivating enzyme *aac*(6′)-*Ib* gene might reduce the susceptibility to quinolones was reported [22]. A number of these enzymes are normal chromosomal genes in a number of species, such as members of Enterococci, where they can contribute to so-called *natural resistance* to aminoglycosides and quinolones. Clinical resistance to aminoglycosides is also due to target modification by A1408 16SrRNA methyltransferases, which have been found in environmental Actinobacteria and Firmicutes [24].

The evolution of vancomycin-resistance multigene determinants is particularly intriguing. They are found in a limited number of complex operon-clusters. However these clusters are composed of genes from different sources, and almost certainly originated from a genus other than *Enterococcus*, such a *Bacillus* and *Paenibacillus* for *vanA, Clostridium, Atopobium, or Eggerthella* for *vanB,* that is, environmental aerobic or strict anaerobic bacteria from the bowel flora. The classic "**eye evolution problem**" applies here. It is difficult to conceive how such a complicated mechanism of defence against glycopeptidic antibiotics might have evolved, as apparently all its intrincate functions are required for the vancomycin-resistance phenotype. In the case of the many different elements that are needed to "construct" an eye, a principal component should emerge first (in the eye, the starting point is the existence of light-sensitive cells). Some small degree of glycopeptide resistance must have evolved first (probably mediated by D-Ala:D-lac ligases) and this must have been selected and eventually refined by further evolutionary steps, that certainly include the modular recruitment of genes with functions primarily unrelated with antibiotic resistance, as two-component stimulus–response coupling (sensing-transcription) mechanisms. Without this inducible mechanism there is in fact a drastic reduction in the levels of resistance to beta-lactam antibiotics and vancomycin [25]. It is likely that unsuccessful combinations have been produced along time, and probably a number of different "solutions" have arisen. Indeed photoreceptors or eyes have also independently evolved more than 40 times in the animal kingdom. This example illustrates how nature evolves in many parallel ways, and the same occurs for drug resistance. The high diversity in determinants of resistance strongly suggests that many of them have evolved to the current function from "pre-resistance" molecules originated

Table 2.2 Examples of resistance mechanisms in clinical strains that evolved from natural functions in non-clinical organisms

Antibimicrobial group	Mechanisms	Related natural protein	Natural reservoirs
Aminoglycosides	Acetylation	Histone-acetylases	*Streptomyces*
	Phosphorylation	Protein kinases	*Actinobacteria, Firmicutes*
	16S rRNA methyltransferases	The same	
Tetracyclines	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	*Streptomyces*
Chloramphenicol	Acetylation	Acetylases	*Streptomyces*
	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	
Macrolides	Target site modification	rRNA methylases	*Streptomyces*
ß-lactams (methicillin)	PBP2a	Homologous PBP2a	*Staphylococcus sciuri*
ß-lactams (cefotaxime)	CTX-M-3 beta-lactamase	Homologous beta-lactamases	*Kluyvera ascorbata*
ß-lactams (carbapenems)	OXA-48 like beta-lactamase	Homologous beta-lactamases	*Shewanella xiamenensis*
Glycopeptides (vancomycin)	Target site modification: D-ala-D-ala replacement (Van operon)	Van operon homologous genes	*Paenibacillus, Streptomyces, Amycolatopsis*
Fluoroquinolones	Topoisomerase protection	Qnr like protein	*Shewanella algae*
	Topoisomerase protection	QnrS like protein	*Vibrio splendidus*
	Efflux	QepA protein	*Streptomyces*

from different evolutionary lineages. Indeed we know about dozens of aminoglycoside-modifying enzymes, thousands of beta-lactamases, many of them redundantly inactivating the same antibiotic substrates.

This panorama helps to visualize the almost unlimited number and variety of potential antibiotic-resistance determinants in the microbial world. Because most bacterial pathogens enter periodically or are hosted in the environment, research on antibiotic resistance should be placed in the field of environmental microbiology [26, 27]. Many of the ancestor or current genes involved in actual or potential mechanisms of resistance are located in environmental bacteria. In a particular location, the ensemble of all these resistance genes constitutes the local **resistome** [28, 29]. The size of the environmental resistome can be determined by metagenomic technology dissecting local microbiomes, using gene-capture platforms particularly sensitive for the detection of resistance genes along with recent bioinformatic approaches for data mining and metagenomics.

Antibiotic-producing microorganisms might still be considered as a suitable source of highly efficient resistance determinants. It can be presumed that both antibiotic biosynthetic pathways and the mechanisms of resistance avoiding self-damage may be the result of a co-evolutionary process. In fact, resistance can be viewed as a pre-condition for significant antibiotic production. The benefit associated with antibiotic production (probably preventing habitat invasion by sensitive competitors) [30] probably also selected the producer strains harbouring the more efficient resistance strategies. As previously stated before, these resistance mechanisms may in their turn have originated in housekeeping genes (for instance, sugar kinases or acetyl-transferases for aminoglycoside resistance) [31, 32] (Table 2.1).

At closer evolutionary times, it is undeniable that most of the current mechanisms of antibiotic resistance might be derived from commensal organisms of the normal microbiota of human and animals, after older exchanges with environmental organisms. Because of that, research on antibiotic resistance forms part of the "One Health" approach, encompassing humans, animals, and the environment [33].

3.2.1 Origin of Drug Resistance: The Case of Beta-Lactamases

The origin and function of beta-lactamases in nature are still a matter of debate. Current knowledge upholds that PBPs and beta-lactamases are related to each other from a structural and an evolutionary point of view and that these proteins might have common ancestors in primitive antibiotic producer bacteria [34]. Certainly, at their turn, both beta-lactamases and PBPs should derive from ancient carboxypeptidases. It has been traditionally postulated that antibiotic-producing bacteria need to produce their own antidote to avoid committing suicide and that beta-lactam and beta-lactamase production in these organisms could be co-regulated. The filamentous soil bacteria such as *Streptomyces, Nocardia*, and *Actinomadura* produce, among others, beta-lactam antibiotics and beta-lactamases and soil fungi such as *Penicillium* are also able to produce beta-lactam antibiotics. Some of the genes participating in the biosynthesis of beta-lactams, such as *cef* or *pcb* gene variants, share similar sequences in different species of antibiotic producers, including *Cephalosporium*, *Streptomyces,* and *Penicillium*. Amino acid sequence alignment and bioinformatic analysis led to the proposal that all these genes have evolved from an ancestral gene cluster that was later mobilized from ancient bacteria to pathogenic organisms. Horizontal gene transfer must have taken place in the soil about 370 million years ago and multiple gene transfer events

occurred from bacteria to bacteria or bacteria to fungi [35]. Beta-lactam gene clusters participating in antibiotic biosynthesis also often include genes for beta-lactamases and PBPs. The beta-lactamase gene products have been shown to participate in part in the regulation of the production of these antibiotics such as cephamycins in *Nocardia lactamdurans* or cephalosporin C in *Streptomyces clavuligerus*. The latter also produces a potent inhibitor of class A beta-lactamase, probably to protect itself from formed antibiotics.

Beta-lactamases and PBPs also share issues other than potential common ancestors, gene sequences, or potential involvement in antibiotic biosynthesis regulation. Both of them have functions in relation to cell wall and peptidoglycan, which are more evident in the case of PBPs. These proteins are responsible for assembly, maintenance, and regulation of peptidoglycan structure. They are mainly anchored in the bacterial inner membrane, with their active site in the periplasmic space in Gram negatives and the corresponding space in Gram positives. In parallel, most of the beta-lactamases are secreted to the periplasmic space in the Gram negatives or evade the peptidoglycan barrier in the Gram-positive organisms. All PBP classes, with the exception of one which appears to be Zn^{2+} dependent, and beta-lactamase classes are serine active site proteins (see below). Peptidoglycan degrading products can regulate the production of beta-lactamases in certain Gram-negative bacteria due to the action of PBPs or beta-lactam antibiotics. In contrast, natural chromosomal beta-lactamases in these organisms have been shown to participate in the regulation of precursors of peptidoglycan.

Amino acid sequence analysis of PBPs and beta-lactamases argue in favour of a common origin of these proteins. Both proteins are members of a single superfamily of active-serine enzymes that are distinct from the classical serine proteases. The amino acid alignments of the main PBPs and different beta-lactamases reveal the presence of conserved boxes with strict identities or homologous amino acids. Moreover, site-directed mutagenesis in the residues essential for the catalytic activity of PBP in *E. coli* and the counterpart residues in class A beta-lactamases has shown similar features in these positions. In essence, the same structural motifs that bind penicillin in PBPs can be used to hydrolyze beta-lactams for beta-lactamases [36].

Structural evidence also supports the proposal that beta-lactamases descend from the PBP cell wall biosynthesis enzymes [37]. PBPs are ancient proteins as bacteria came into existence approximately 3.8 billion years ago, but the development of beta-lactamases is a relatively recent event, which must have taken place after the evolution of the first biosynthetic pathway in beta-lactam-producing organisms. It has been argued that this process has been reproduced several times to generate the different class A, C, and D beta-lactamases. Beta-lactamases have had to undergo structural alterations to become effective as antibiotic resistance enzymes, avoiding the interaction with the peptidoglycan or peptidoglycan precursors, which are the substrates for PBPs. This has been disclosed in X-ray interaction models with cephalosporin derivatives and AmpC beta-lactamase variants from *E. coli*. These models revealed not only three dimensional structural similarities but also that the surface for interaction with the strand of peptidoglycan that acylates the active site, which is present in PBPs, is absent in the beta-lactamase active site. The possible mutational pathways of evolution from PBPs to beta-lactamases have been investigated [38], but certainly this process might have evolved separately, by mutation and/or recombination, on many occasions.

Alternative hypotheses of the origin and function of beta-lactamases have also been postulated. Antibiotics are known to be secondary metabolite compounds that are normally released in the early stationary growth phase. For this reason, it has been hypothesized that beta-lactamases may also play a role as "peptidases", in catalysing the hydrolysis of the beta-lactam nucleus to reutilize carbon and nitrogen as an energy source in adverse conditions and they may act as nutrients for potential growing bacteria [39]. Some environmental organisms, including some *Burkholderia cepacia* genomovars and *Pseudomonas fluorescens,* have been shown to grow in the presence of penicillin as a sole carbon and nitrogen source and to stimulate the synthesis of beta-lactamase under this condition. From an evolutionary point of view, the beta-lactamase-producing bacteria have had advantages over non-beta-lactamase-producing organisms, particularly in soil communities. The former have been able not only to avoid the action of natural beta-lactam products secreted by these antibiotic producers but also to simultaneously use beta-lactams as nutrients.

3.3 Global Stress Regulation and Antibiotic Resistance

In most cases, antibiotic resistance requires time to be expressed in a particular bacterial cell. The best example is when this expression occurs as a consequence of antibiotic exposure (antibiotic-mediated induction). Only bacteria able to survive during the time required for full induction of resistance mechanisms will be able to resist antibiotic effects and consequently be selected. This "need-to-resist-to-become-resistant" paradox deserves some explanation. Antibiotic action, even at sub-inhibitory conditions, results in alterations of the bacterial physiological network. Physiological networking and signalling mechanisms increase (amplify) any cell disturbance, just as a cob-web increases small oscillations, and immediately provoke non-specific mechanisms of global adaptation. Phenotypic tolerance or formation of "persister cells" might be among this type of responses (see above), with mechanisms involving

the alarmone (p)ppGpp being involved in cell survival, and consequently in antibiotic resistance [40]. Other mechanisms might involve sigma factors, key-components of the translation cell machinery that are responsive to different types of stress [41, 42]. Sigma-S defective strains are more susceptible to antimicrobial agents [43]. Sigma-regulons are induced by beta-lactam agents, fosfomycin, teicoplanin, rifampicin, or polymyxins [44–46]. Probably heat-shock proteins also contribute to nonspecific antibiotic defence [47]. Of course that means that the excitement of global stress responses by factors other than antibiotics might non-specifically reduce the antibiotic potency. SOS adaptive response might also be unspecifically triggered by antibiotics. For instance, beta-lactam-mediated PBP-3 inhibition results in the induction of the SOS machinery in *E. coli* through the DpiBA two-component signal transduction system [48, 49]. Among the immediate consequences of such an early antibiotic sublethal effect is that bacteria might reduce their growth rate, eventually entering in some degree of phenotypic tolerance to drugs, and also that some other adaptive responses are triggered [49].

3.4 Genetic Variation: Mutation

3.4.1 Mutation Frequency and Mutation Rate

In the case of antibiotic resistance, the mutation "rate" is frequently and inappropriately defined as the in vitro frequency at which detectable mutants arise in a bacterial population in the presence of a given antibiotic concentration. Such a determination is widely considered an important task for the prognosis of the emergence of antibiotic-resistant bacteria. In the scientific jargon regarding antibiotics, a "mutation rate" is frequently presented in a characteristically naive way that can sometimes be understood as an intrinsic property of a new antimicrobial drug in its interaction with the target bacteria, with a "low mutation rate" that is considered an advantage over competitors. "This drug induces (?) a low mutation rate" is a familiar but completely mistaken expression. Note that in these types of tests we are recording the number of mutant cells and not the number of mutation events. In fact, we are recording only the selectively favourable mutations for the bacteria that lead to a visible antibiotic resistance phenotype, and therefore we are determining "mutation frequencies" and not "mutation rates". From the pioneering works of Luria and Delbrück, it became clear that evaluation of mutation rates is not easy. The methods for distinguishing the value of the observed frequency of mutants from the real mutation rate are not easy to apply, and fluctuation tests for analysis of the presence of populations of pre-existing mutants in the tested populations should be applied here. In the case of antibiotic resistance, the problem is complicated by the fact that the phenotype does not always reflect the same genotypes in all selected mutants, as mutations in different genes can produce similar antibiotic resistance phenotypes. For example, when a quinolone resistance mutation rate is determined, this rate is really the result of the combination of the mutation rates of the genes that encode the synthesis of GyrA, GyrB, ParA, ParC, and several different multidrug resistance (MDR) systems, and eventually other inactivating and target-protection mechanisms. In this respect, the calculated "phenotypic" mutation frequency is the result of several different "genotypic" mutation events.

The most important part of the adaptive possibilities of bacterial populations to environmental challenges, including adaptation to the anthropogenic antibiotic exposure, results from the huge quantity of bacterial individual cells. Simple calculations can provide an intuitive image of the mutation frequency in bacterial populations. *E. coli* genome has typically a size of 5,000,000 base pairs (5×10^6 bp), corresponding approximately to 5000 genes. The mutation rate of *E. coli* is 1×10^{-3} per genome (cell) per generation [50]. Divided by the number of genes, $0.001/5000 = 0.0000002 = 2 \times 10^{-7}$ p er gene and (cell) generation. Considering a cell density of 10^9 cells/ml in the colon, and a volume of 1000 ml in this part of the colonized intestine, we have 10^{12} *E. coli* cells in a single host (for instance, a particular patient) meaning that each day, supposing that *E. coli* divides only once/day in the colon, we have 200,000 mutations per gene/day for the entire *E. coli* population established in a single host. Of course resistance genes, or pre-resistance genes, will also evolve at this rate. Many *E. coli* clones are living in our intestine for years [51], so that the number of generations might be huge, and so the cumulative number of possible mutations offered to natural selection. How might bacteria tolerate such mutational load? Certainly due to purifying or stabilizing selection, that is, the alleles produced by most mutations are selectively removed if deleterious.

3.4.2 Hyper-mutation

The above calculations were based on huge bacterial populations in a shared environment (as *E. coli* in a "common" intestinal space in our example). However, many bacterial populations can be disaggregated, occupying small and eventually non-connected niches, with lower bacterial local densities in these compartments. Under immune response or antibiotic therapy, bacterial populations can also be reduced in size, and that applies in nature to all kinds of stressful conditions and bottlenecks. In environments where bacteria reach high population sizes, the normal mutation rates are more than enough to provide a sufficient wealth of mutational variation. However, when confined to low population sizes in compartmentalized habitats, variants with increased mutation rates (mutators) tend to be selected since they have an increased probability of forming beneficial mutations. Hyper-mutation is frequently due to the impairment of the

mismatch repair system, and more particularly involves alterations in *mutS* gene, but also in *mutL*, or *mutH*. Note that in an asexually reproducing organism, a mutator allele (for instance, the *mutS* allele that hyper-generates mutation) and the beneficial mutations are physically and genetically associated in the same chromosome. As a result the mutator allele will hitch-hike to increased frequency in the population together with the beneficial mutation.

One exemplary case is the selection of hyper-mutator populations in highly compartmentalized, chronic infections under frequent antibiotic exposure. This is the case of bronchopulmonar colonization in cystic fibrosis patients or those with bronchiectasis [52]. Determination of spontaneous mutation rates in *P. aeruginosa* isolates from cystic fibrosis patients revealed that 36 % of the patients were colonized by a hypermutable (mutator, mostly *mutS* deficient) strain (exceeding by 10–1000× the normal mutation frequency, 10^{-8}) that persisted for years in most patients. Mutator strains were not found in a control group of non-cystic fibrosis patients acutely infected with *P. aeruginosa*. This investigation also revealed a link between high mutation rates in vivo and high rates of antibiotic resistance [53]. An analogous rise in the proportion of hyper-mutable strains in cystic fibrosis patients has been documented for other organisms, including *Streptococcus*, *Haemophilus*, *Staphylococcus*, or *Stenotrophomonas*, and for analogous clinical conditions, as chronic obstructive pulmonary disease [54–56].

About 1 % of the *E. coli* strains have at least 100× the modal mutation frequency of 10^{-8} (strong mutators) and a very high proportion of strains, between 11 and 38 % in the different series, had frequencies exceeding by 4–40 times this modal value (weak mutators) [57] (Fig. 2.1). These proportions are obviously far higher than could be expected by random mutation of the genes that stringently maintain the normal mutation frequency. Moreover, increased mutation frequency may result in a loss of fitness for the bacterial population in the gut [58] as random deleterious mutations are much more frequent than the advantageous ones. Therefore the abundance of strains with increased frequency of mutation ought to be maintained by positive selection for the hyper-mutable organisms [59]. Without positive selection, the hypothesis is that these mutator populations would be extinct because of their unbearable mutational load (burden). However, we have shown in long-term evolution experiments that hyper-mutators might find mechanisms to reduce their rates of mutation, even if they cannot reacquire the repair function (for instance, the wild-type MutS gene) by horizontal gene transfer. These mechanisms involve protecting the cell against increased endogenous oxidative radicals involved in DNA damage, and thus in genome mutation [60].

The problem of combining the generation of variation required for adaptive needs and the required integrity of the bacterial functions might also be solved by strategies of low-level mutation, and "transient hyper-mutation". Possibly the fitness cost in terms of deleterious mutations is lower in a weak mutator and this allows their rising to higher frequencies in the population, and there might be a "reserve of low level mutators" in many bacterial populations, coexisting with the normo-mutable population. Indeed mutators are fixed in competition with non-mutators when they reach a frequency equal or higher than the product of their population size and mutation rate [61]. In populations of sufficient size, advantageous mutations tend to appear in weak mutators, and the selective process will therefore enrich low mutating organisms. The adaptive success of weak mutators may indeed prevent further fixation of strong mutators [61]. The "transient hypermutation" strategy will be treated in a paragraph below.

Striking differences have been found in the frequency of hyper-mutable *E. coli* strains depending on the origin; faecal samples of healthy volunteers, urinary tract infections, or bloodstream infections. *E. coli* strains from blood cultures are typically isolated from hospitalized patients and are therefore expected to have had a longer exposure to different hosts and antibiotic challenges. For instance, the frequency

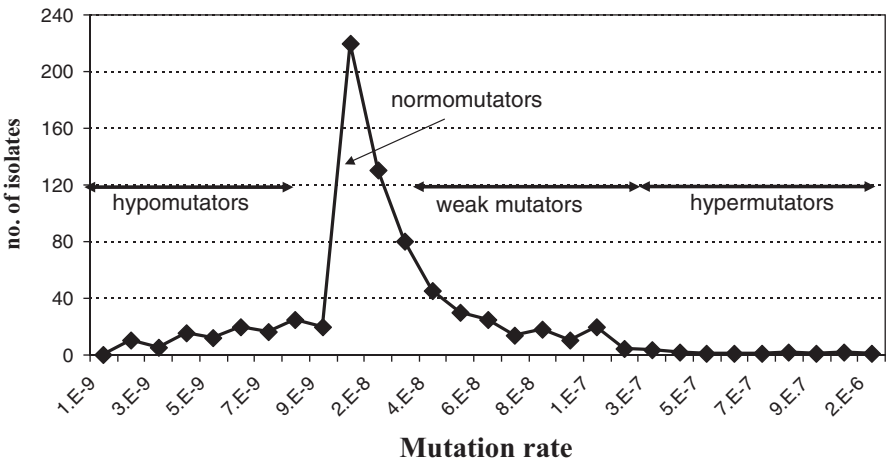


Fig. 2.1 Distribution of mutation frequencies for rifampicin-resistance in a large international series of *Escherichia coli* isolates recovered from patients and healthy volunteers. Hypermutators only account for 1 % of the strains, but weak-mutators are frequently found in clinical strains, but rare among healthy volunteers [37]

of hyper-mutable *E. coli* strains is higher among *E. coli* strains producing extended-spectrum beta-lactamases [62]. In general, adaptation to complex environments, including pathogenic ones, and the facilitation of between-hosts spread, leads to a certain microevolutionary "clonalization" (predominance of a particular clonal variant in a particular environment), which is facilitated by hypermutation [63]. In summary, mutation rates show a certain degree of polymorphism, and differences between isolates might reflect the degree of unexpected variation of the environment in which they are located [53, 64–67].

3.4.3 Antibiotics Inducing Mutations: Transient Mutation

A number of antibiotics induce adaptive responses to their own action, frequently—but not exclusively—by induction of the SOS repair system. SOS induction might be mediated by the SOS repair systems, not only those acting on DNA, but also on cell wall, as previously stated. One of the non-SOS effects (LexA/RecA independent)related to the PBP3-inhibition cell-wall damage response is the induction of *dinB* transcription, resulting in the synthesis of an error-prone DNA polymerase IV [68]. The consequence of this is an increase in the number of transcriptional mistakes, which might result in the emergence of adaptive mutations producing resistance to the challenging agents [67, 69]. Antibiotics that produce mistranslation, as aminoglycosides, induce translational stress-induced mutagenesis (non-inheritable!) [70]. Many antibiotics induce the SOS repair system, resulting in mutational increases, not only DNA-damaging agents, such as fluoroquinolones [71], but also beta-lactam agents [72]. The reason for mutational increase is the SOS-mediated induction of alternative error-prone DNA polymerases PolII, PolIV, and PolV.

3.5 Genetic Variation: Gene Recombination, Gene Amplification

Gene recombination might act as a restorative process which opposes gene mutation. Indeed a mutated gene, leading to a deleterious phenotype, might be replaced by homologous recombination with the wild gene if it is accessible in the same chromosome, or in other replicons of the same or different organism. For instance, if a mutated gene leading to antibiotic resistance is associated with a high biological cost in the absence of antibiotics, reducing fitness of the resistant organism, the mutated gene could be replaced by the wild-type gene, restoring both fitness and antibiotic susceptibility. This phenomenon might explain the partial penetration of some resistance traits in bacterial populations.

On the contrary, gene recombination might assure spread of mutations associated with antibiotic-resistance phenotypes. This might occur inside the same bacterial cell (intragenomic recombination) or between cells; in the last case, horizontal genetic transfer is required. Intragenomic recombination facilitates spread of homologous repeated genetic sequences. Gene conversion assures non-reciprocal transfer of information between homologous sequences inside the same genome. This might lead to minimizing the costs associated with the acquisition of a particular mutation (replacing the mutated sequence), or, on the contrary, to maximizing the benefits of mutations that confer a weak advantage when present as a single member (spreading copies of the mutated sequence) [73]. For instance, single-mutated rRNAs easily produce antibiotic resistance to aminoglycosides (and probably this is the case for other antibiotics) when the rest of the copies of rRNA sequences remain unchanged: the advantageous mutation spread by gene conversion [74].

Recombination in fact provides an extremely frequent mechanism for bacterial adaptation, being reversible in many cases. **Gene duplication-amplification** processes (either RecA-dependent or RecA-independent) are highly relevant in the adaptation to antibiotic exposure because they generate extensive and reversible genetic variation on which adaptive evolution can act [75–77].

For instance, sulfonamide, trimethoprim, or beta-lactams resistance (including resistance to beta-lactam plus beta-lactamase inhibitors) occur by increased gene dosage through amplification of antibiotic hydrolytic enzymes, target enzymes, or efflux pumps [78]. These cells now are now selectable by low antibiotic concentrations, increase in number and therefore also increase the probability for new adaptive mutations occurring in one of the amplified genes, eventually leading to higher levels of resistance. Once that occurs, low-level resistance by amplification-only is no longer efficiently selected. Moreover, gene amplification is inherently instable, and also might produce fitness costs, as each additional kilobase pairs of DNA reduces fitness by approximately 0.15% [79] so that the amplification will return to the original single gene-status. No signal will remain of this transient event in the genome sequence, and that is the reason why this evolutionary mechanism remains underdetected.

The possibility of gene recombination between bacterial organisms is highly dependent on the availability of horizontal gene-transfer mechanisms and the acceptance by the recipient cell of the foreign DNA. For instance, DNA uptake in *Neisseria meningitidis* or *Haemophilus influenzae* is highly sequence-specific. Transformation with *Streptococcus pneumoniae* DNA is exceptional outside this genus. In these very human-adapted organisms, intrageneric transfer facilitates the required variability in the surface proteins needed for colonization of mucosal surfaces in the human host, but the same strategy has been applied for optimizing mechanisms of antibiotic resistance. A variety of mosaic (hybrid)

genes, encoding antibiotic-resistant variants of the target-proteins for beta-lactam antibiotics, have appeared in those organisms which are under antibiotic pressure. In aminoglycosides and in tetracyclines, mosaic hybrid genes are also frequent [80, 81].

3.6 Genetic Variation: Modularization

Modularization is a process by which variability is produced as a consequence of the building-up of different combinations among modular genetic elements, creating alternative genetic orders. Genomes of bacterial communities, species, and plasmids, and transposons, and integrons, frequently harbour or are constituted by modular genetic units. Genetic modules are any kind of repeated, conserved cohesive genetic entities that are loosely coupled [41, 82]. In fact there is a modular organization of nature, in which modules from different (non-genealogical) origins interact and integrate at different hierarchical levels [83]. Common or highly related genetic sequences (from small to very large ones) encoding resistance traits or associated with resistance genes have been found among different bacterial organisms, frequently belonging to different species and phylogenetic groups. The commonality of these sequences can be explained by a common phylogeny, by convergent evolution, or, probably more frequently, by lateral transmission of modular units, in a kind of reticulate evolutionary process. Incremental modularization, the addition of new "resistance" modules to a particular region might occur because there is a "module-recruiting" module (for instance, a recombinase), or by duplication of a pre-existing module, or by insertion of an incoming module. As the incoming modules or multi-modular structures frequently provide new interactive sequences, module accretion increases the local possibilities of recruitment of new modules. As this process of modularization occurs at particular genetic regions, these tend to become highly recombinogenic and module-promiscuous (high-plasticity zones). The cumulative collection of antibiotic resistance traits within particular multi-modular structures (integrons, transposons, plasmids) results from this type of nested evolution. The assemblage of modular components occurs by transposition, homologous recombination, and illegitimate recombinational events. Insertion sequences (ISs) are frequently involved in modularization. For instance, IS*26* mediates mobilization of bla_{SHV} genes encoding extended-spectrum beta-lactamases (ESBLs). The success of a plasmid containing one given $bla_{CTX\text{-}M}$ gene, as is the case of $bla_{CTX\text{-}M\text{-}15}$, also assures the spread of several IS*26* copies which might be involved in further modularization processes leading to multi-resistance [84].

The most beautiful example of the capturing efficiency of IS modules is the ability of the IS*Ecp1*B element to capture a wild beta-lactamase CTX-M-2 gene from the environmental organism *Kluyvera ascorbata* and mobilizing it into *E. coli*, that has now become resistant to third generation cephalosporins [85]. This recruiting module is involved in the expression and mobilization of many ESBLs [86]. Interestingly, the capturing ability of the IS*Ecp1*B module is dependent on a malfunctioning of this insertion sequence for excising itself in a precise way, and so integrating in the excising module sequences adjacent to the point of insertion. It has indeed been proposed that "imprecision" favours DNA arrangements and modularization. Another highly efficient IS module capturing and transposing not only ESBLs, but also metallo-beta-lactamases or co-trimoxazol, aminoglycoside, chloramphenicol and even fluoroquinolone resistance and even large chromosomal modules (genomic islands) are IS*CR*-type modules [87]. IS*CR*, IS with CR (common region), is a designation that implicitly reflects the modular structure of the module itself. A final example is IS*1999*, which when inserted upstream of a novel antibiotic resistance gene mediating very-broad spectrum beta-lactam resistance promotes its mobilization [88]. In principle, most modules involved in adaptive functions, including antibiotic resistance of every kind (from detoxifying enzymes to porin genes) might be recruited and translocated by IS modules. Other elements involved in module mobilization are DNA transposons and retrotransposons (that move by means of an RNA intermediate).

Modularization might act at the genome level as mutation acts at gene sequence level. Just as in the case of mutations, we should admit stochasticity as the major source of different modular combinations. We can expect that probably most of the combinations do not provide any fitness benefit, or might even reduce fitness of some module-associated functions. Nevertheless, some models suggest that even in the absence of any selective advantage, genotypic modularity might increase through the formation of new sub-functions under near-neutral processes [89]. Certainly it might be well conceived that some of these combinations could provide some direct adaptive benefits to the host cell, such as antibiotic resistance. Probably, successful combinations tend to perpetuate the connection among particular series of modules that act more and more now as a single complex module. For this reason there is a synthetic dimension of modularity, during which evolution tends to a number of genetic and biological orders, in a "doll-inside-doll" model. Note that modularity implies that bacterial entities are not formed or maintained as strict hierarchies, either from the top down (from ecosystem, communities, species, phylogenetic sub-specific groups, clones, genomes, long or short genetic sequences), or bottom-up (from short genetic sequences to ecosystem).

Indeed we know that not every bacterial phylogenetic group within a given bacterial species is represented in different ecosystems; no single clone is equally distributed among different hosts; every plasmid is not present at equal frequency among different bacterial species or sub-specific

groups. We also know that every type of mobile element is not equally distributed in any bacterial clone within a species, nor transposon is inserted with similar frequency in each type of plasmid, nor any kind of integron in any transposon, nor any antibiotic-resistance gene in any integron. These disequilibria are probably the result of cumulated selective events, exerted simultaneously at different hierarchical levels [90].

3.7 Horizontal Genetic Transfer and Bacterial Variation

The history of most commonly identified antibiotic resistance genes follows its original capture by different genetic units and further mobilization of novel "operational units" containing such antibiotic resistance genes into composite platforms (often comprising integrons, transposable elements, and/or insertion sequences) within plasmids that facilitate multiple DNA rearrangements among disparate genetic entities. Evolution based on gene recombination and modularization is greatly facilitated by horizontal (or lateral) genetic transfer. In particular, many drug resistance determinants spread between bacterial cells and species using plasmids, conjugative transposons, and probably phages. The evolution of resistance on these elements occurs in a modular fashion by sequential assemblage of resistance genes in specific sequences which are frequently mediated by specialized genetic elements such as integrons and transposable elements.

3.7.1 Plasmids and Drug Resistance Evolution

A plasmid is a double stranded, circular, or linear DNA molecule capable of autonomous replication. Plasmids frequently encode maintenance systems to assure copy-number and self-perpetuation in clonal bacterial populations. A plasmid may encode for a long-life cell-killing substance that is detoxified by a short-life plasmid product. If the plasmid is lost, the bacterial host is killed. To a certain extent, the same strategy has been applied to antibiotic (or heavy metals) resistance; only the clones harbouring plasmid-determined resistance will survive in an antibiotic-polluted environment. Therefore, plasmids use selective forces for their own maintenance and spread, and their spread in bacterial populations may be proportional to the intensity of these forces.

Facing in the 50s an increasingly selective antibiotic environment, historical (pre-antibiotic) plasmids immediately incorporated antibiotic resistance determinants. The study of pre-antibiotic collections of plasmids strongly suggests that the appearance of resistance genes in plasmids has only occurred during the last five decades. Indeed the diversity of the main plasmid families remains relatively limited, illustrating the success in continuous adaptation and spread of old plasmids thanks to antibiotic-mediated selection.

An example is the recent dissemination of old plasmids due to the incorporation to their genetic sequence of genes encoding for ESBLs. Promiscuous FII chimeric plasmids, widely spread among Enterobacteriaceae already before antibiotic discovery, are responsible for the current pandemic spread of $bla_{\text{CTX-M-15}}$, or bla_{TEM}, among other antibiotic resistance genes [91–94]. These plasmids harbour operational genetic platforms containing gene capture units as IS*ECp1*, IS*26,* or IS*CR*s, thus recruiting diverse antibiotic resistance genes [93, 95].

In many cases, the final success of resistant clones depends on the sequential acquisition of adaptive features unrelated to antibiotic resistance, facilitating in some of them to spread between hosts and/or environments (epidemicity) [96–100]. Moreover, the recent outburst of the OXA-48 enzyme, a widely distributed carbapenemase, has been related with the insertion of Tn1999 into the *tir* gene, encoding a transfer inhibition protein. This results in a high transfer frequency of plasmid harbouring $bla_{\text{OXA-48}}$ that might explain the successful dissemination of this enzyme [101]. All these observations indicate that the total plasmid frequency in bacterial populations might be increasing as a result not only of the more and more extensive anthropogenic release of selective agents, such as antimicrobial agents, but also to the advantage that they provide in circulating among microbiomes of multiple hosts, or in mediating resistance to other environmental organic chemicals or heavy metals [102]. This plasmid increase might have consequences on the full evolutionary machinery of bacterial populations, enlarging the number and variety of genetic interactions. In self-transmissible plasmids, there is always a possibility of entering (particularly under stress) into a new host resistant to the new drug, which may harbour another plasmid determining resistance to this drug. Plasmids from natural populations of *E. coli* frequently show a mosaic modular structure. Apparently plasmid–plasmid interaction and coevolution of recombinants (modular exchanges) depends on the possibility of plasmid coexistence. Plasmid promiscuity is limited by the phenomenon of plasmid incompatibility (two plasmids in the same cell might compete for the replication site, so that only one will be maintained), which depends on the amino acid sequence of the replication initiator proteins (RIP). However, a plasmid might enter into a cell where an incompatible plasmid is located, and still, before segregation, will have time to exchange modular traits with the resident one; on the other hand, many plasmids collect more than one Rep protein to be able to be replicated even if one of its Rep proteins is shared with the resident "incompatible" plasmid. No wonder that a multiple antibiotic environment has led the plasmid evolution towards the acquisition of multiple antibiotic resistance determinants in a single replicon unit, and even in the same gene cluster.

The possibility of a progressive increase in plasmid frequency and diversity (within classic plasmid backbones) in relation with an escalation of stressful and selective forces in nature, including antibiotic exposure, could be theoretically limited by plasmid incompatibility (inability of two related plasmids with common replication controls to be stably propagated in the same cell line), and progressive capture of plasmid genes by chromosomal sequences which make the cost of plasmid maintenance unnecessary. Recent advancements in the methods to determine plasmid relatedness, by restriction fragment pattern analysis, by classification into incompatibility groups (Inc), by PCR-based replicon (rep) typing (PBRT) [103], or more significantly by relaxase-typing by PLANC-Net methodology [104], or plasmid reconstruction in metagenomic analysis [105] have permitted the analysis of large series of resistance plasmids. These studies suggest that the limitation of plasmid incompatibility might be eventually surpassed by the evolution of multi-replicon plasmids or by plasmid co-integration.

An important point that is worth being investigated in more depth is the basis for specific stable maintenance of given plasmids in particular hosts. The development of solid systems for phylogenetic classification of sub-specific groups of bacteria are revealing that particular types of plasmids which eventually harbour particular types of resistance determinants are preferentially present in particular lineages. These bacterial lineages are acquiring the ever-lasting advantage of hosting evolutionary-active, plastic (modular) plasmids. The maintenance of a given type of plasmid in a given host depends on the "plasmid ecology" within the cell (host-plasmid mutual dependence, restriction-modification systems, presence of other plasmids), the reduction in the costs of maintenance, the rate of intra-populational transfer, and the frequency of selection for plasmid-encoded traits. The concept of specific stable maintenance means that, despite the potential transferability of plasmids to different hosts, some of them will be privileged in hosting particular plasmids, and these lineages or clones should have an increased evolvability in terms of developing antibiotic resistance.

But also mobile genetic elements might be shared by "genetic exchange communities", as "common adaptive goods" for ecologically integrated groups of (generally related) organisms [106, 107]. In such a way, antibiotic resistance tends to assure not only the survival of a particular lineage, but also of clouds of lineages, or even consortia of ecologically and functionally interconnected bacterial communities.

3.7.2 Transposable Elements

It is mainly transposable elements that have produced genetic transference of resistance in *S. aureus* and other gram-positive organisms. Class I transposons are able to mobilise themselves among different DNA sequences due to the presence of IS flanking their structure [108]. Different examples of Class I integrons are those involved in the transference of aminoglycoside resistance genes such as streptomycin, kanamycin or bleomycin (Tn5), chloranphenicol (Tn9) and tetracycline (Tn10). Tn4001, which is associated with IS256, is one of the most successfully disseminated transposons among gram-positive organisms. This element harboured the *aac6′-aph2″* gene which encodes a bifunctional enzyme able to inactivate most of the aminoglycoside antibiotics [109].

Class II transposons are widely disseminated among both gram-negative and gram-positive bacteria. They have a complex structure, which allows their mobilization from the bacterial chromosome to plasmids present in the bacteria. They have a genetic structure flanked by inverted repeat sequences which also include sequences with functional activity (transposase and resolvases) that facilitate their recombination and integration within the chromosome or a plasmid sequence. Some of these class II transposons contain resistance genes such as Tn*3* which harbour $bla_{\text{TEM-1}}$ gene or Tn*21* and their derivatives containing mercury or cadmium resistance genes, which may act as cofactors in the selection process [110, 111]. Another example of class II transposons are Tn*916*-Tn*1545* harbouring a tetracycline resistance gene in Enterobacteriaceae or Tn*1456* encoding glycopeptide resistance in enterococci. Moreover, some transposons are able to be transferred with a circular structure similar to that of plasmids (conjugative transposons). Some examples include tetracycline resistance (*tetM*) in *S. pneumoniae* or enterococci.

Transposons are important in the dissemination and maintenance of resistance genes and resistant bacteria. A transposon can be inserted inside another transposon and may contain more than one resistance determinant or even integron structure [109]. These latter elements are able to capture resistance genes (cassettes) due to the recognition of homologous sequences (integrase) and facilitate their expression [111, 112]. In general, bacteria harbouring integrons are more resistant to antimicrobials than those lacking these structures as an integron may present more than one resistance cassette. It is important to note that integrons can be mobilized by transposable elements which are also located in plasmids. This structure can be considered as an example of the "doll-inside-doll" model which undoubtedly gives advantages for the selection of resistant bacteria.

Most of the integrons have been described in organisms of high sanitary importance such as *Salmonella* Typhimurium, ESBL-producing *K. pneumoniae,* or *E. coli*. Within the integrons, class I integrons (according to the type of the integrase) have been successfully disseminated probably due to their integration in transposable elements and plasmids. The best example is that of integrons associated with the IS*CR1* structure (or ORF513) that are commonly associated with certain ESBL genes (*blaCTX-M*), carbapenemase genes, the *qnrA* gene which produces quinolone resistance, or ammonium quaternary compound resistance [84, 113].

3.7.3 Phages

The association of antibiotic resistance with bacterial phages has been overlooked for decades. We should remember that bacteriophages are probably the most abundant organism on Earth. Their ability to insert in bacterial genomes, to excise from them eventually carrying host DNA sequences, and to transfer to other bacterial cells, makes them potential vectors for disseminating antibiotic resistance. A number of examples of antibiotic resistance genes spreading by generalized or specialized phage transduction are available for *E. coli*, *P. aeruginosa, Staphylococcus epidermidis*, *S. aureus*, and *Actinobacillus. Burkholderia cepacia* transduce the resistance determinants to cotrimoxazol, trimethoprim and erythromycin to *Shigella flexneri*. A multiresistance gene cluster (*tetG, floR*, bla_{PSE1}) has been transduced from *Salmonella enterica* serovar Typhimurium DT104 to other serovars of *S. enterica*. A wide variety of β-lactamases (bla_{OXA-2}, bla_{PSE-1}, bla_{PSE-4}, or *bla*P) from *Proteus* have been found associated with bacteriophages isolated from sewage samples. The study of the genetic environment surrounding $bla_{CTX-M-10}$ β-lactamase gene has revealed the presence of upstream sequences with homology to conserved phage tail proteins [114]. It is not known whether these genes are part of a functional phage carrying $bla_{CTX-M-10}$ gene or only a reminiscent of an ancestral transduction event.

Abundant phage particles have been found in the supernatant of *Streptococcus pyogenes* harbouring the proton-dependent macrolide efflux system encoded by *mef(*A) gene, and these phage preparations have conferred macrolide resistance to a macrolide-susceptible strain [115]. High throughput sequencing has revealed phylogenetically diverse macrolide-resistant *S. pyogenes* strains carrying *mef*(A) inserted in different prophage or prophage-like elements, as Tn*1207.3,* alone or in combination with *tet(*O) gene. *Bacillus anthracis* carries a very diverse array of phages; among them are γ phages which contain a gene conferring resistance to fosfomycin. Bacteriophages isolated from food might in fact contribute to the propagation of antibiotic resistance [116].

3.8 Genetic Variation: Clonalization

Bacterial populations inside species are frequently subdivided in clones, particular lineages or units of descent that probably reflect different evolutionary histories. Multilocus sequence typing has pointed out that most isolates in a clonal population belong to one of a limited number of genotypic clusters (clonal complexes) that are thought to emerge from the rise in frequency and subsequent radial diversification of clonal founders [117, 118]. Rise in frequency is in most cases the consequence of selective events favouring the outburst of particular clones and clonal complexes in particular environmental circumstances. Each clone will correspond to a fitness peak, to an "ecotype" [119]. This means that the clonal structure of a bacterial population might reflect the changing variety of environments (including environmental gradients) to which the *ensemble* of the species is regularly exposed, and small changes among clones favours microevolution [117]. Therefore, we can conceive a bacterial species as a macro-structure composed of a number of clones and clonal complexes that might or might not be present in a particular location. In this sense, clones might behave as adaptive modules of a hierarchical superior entity, a "regional community structure", able to provide alternative stable states [120]. Mobile elements containing antibiotic resistance genes, such as plasmids, might circulate more effectively in such a genetically highly homogeneous multi-clonal structure, leading to typical complex endemic antibiotic-resistance situations [121] also termed resistance "allodemics" (see Sect. 4.3.1), and Fig. 2.2 [122–124].

3.9 Generation of Variation in Response to Antibiotic Stress

We have shown in Sect. 3.4.3 the influence of antibiotics on the mutation rate. Indeed that is a particular case of adaptive response to stress. Mutational events (base substitutions, frameshifts, excisions, insertions, transpositions) are increased by orders of magnitude under stress [125–127]. Probably, bacterial cells under extreme antibiotic-provoked stress (with membrane or cell wall damage, or compromised protein synthesis, or altered DNA supercoiling) could increase the rate of mutation, which would result in this type of adaptive response. Mutation rates can transiently increase depending on conditions of bacterial growth like starvation and environmental situations that cause bacterial stress, including induction of the SOS response. The SOS cascade can be induced by numerous antibiotics, presumably because these antibiotics cause the production of ssDNA [128]. DNA topoisomerase subunit A inhibitors, such as ciprofloxacin and other quinolones have a strong inducer SOS response [71, 129], however the subunit B inhibitors as novobiocin are not inducers [130]. On the other hand, antibiotics are also enhancing gene spread among bacterial populations: macrolides, tetracyclines, and beta-lactam agents facilitate intracellular and intercellular gene transfer. Conjugational transfer of the antibiotic-resistant transposon Tn*916* containing a tetracycline-resistance determinant, increases more than 1000-fold in the presence of tetracycline [131]. Most prophages are SOS-inducible so that SOS-inducing agents will dramatically increase the spread of prophages. This might significantly influence the spread of antibiotic-resistance genes [132], as it does for virulence factors. Indeed antibiotics might contribute to the spread of resistance genes modifying virulence and host-to-host frequency of transfer.

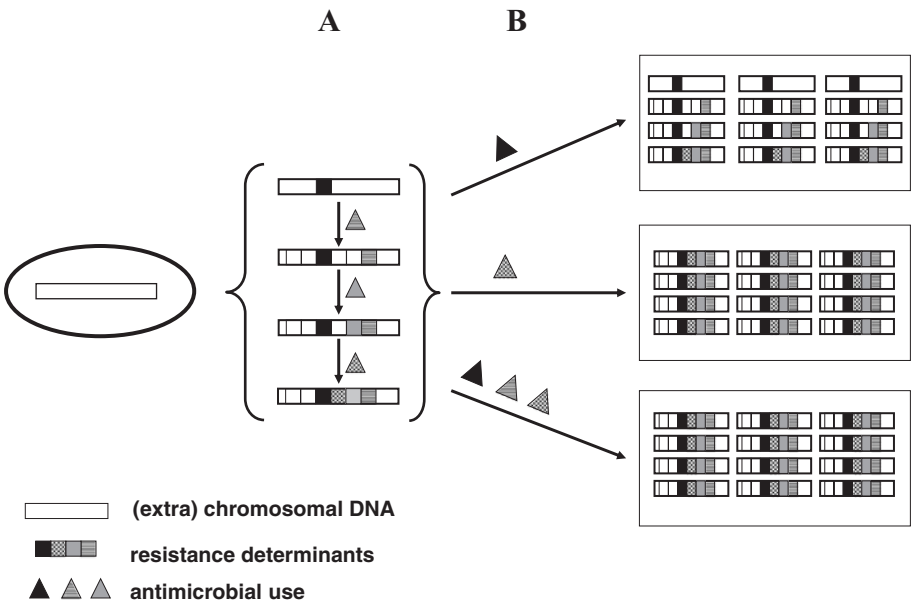


Fig. 2.2 Emergence of multi-resistance by sequential acquisition of antimicrobial resistance determinants (mutation or gene transfer) and selection of resistant bacteria under different antimicrobial selective pressures. (**a**) The sequential exposure to different antimicrobials may accumulate resistance determinants in bacteria. (**b**) The use of different antimicrobials may select resistant bacteria with different patterns of resistance determinants; note that eventually exposure to a single antibiotic produces the same selective effect for multidrug resistance that exposure to different drugs

For instance, the prophage-encoded shiga-toxin gene is SOS-induced and treatment of the hemolytic-uremic syndrome with SOS-inducers, such as fluoroquinolones, worsens the syndrome, amplifying the population of phages encoding shiga toxin [133]. Goerke et al. have demonstrated the increase of the expression of virulence factors and titres of particle phages in *Staphylococcus aureus* strains carrying ϕ13 lysogen, after being exposed to concentrations of ciprofloxacin near the threshold of growth inhibition [134, 135]. Other antibiotics such as trimethoprim have also been reported to cause phage induction [135]. In summary, antibiotic pressure in the environment may well contribute simultaneously to the increase in mutant-resistant phenotypes, to the selection of the fittest among them, and to the dispersal of resistance genes, which is expected to result in an acceleration in the rate of microbial evolution.

3.10 Phenotypic Variation and Genetic Variation: The Baldwin Effect

As stated in an earlier section (3.1) there is a certain degree of plasticity in bacterial cells and populations that are able to tolerate a determined concentration of antibiotics without requiring any inheritable genetic change. Regulatory factors influencing DNA supercoiling, catabolic repression or growth-phase specific regulators, translational modifications, and/or induction or stress responses might provide this flexibility. In a certain sense, the mechanisms of resistance that are induced by the presence of antibiotic agents also provide adaptive phenotypic variation, as is the case of AmpC related chromosomal beta-lactamases in *Enterobacter* or *P. aeruginosa* [136]. A classic important and still unanswered question in evolution is whether survival provided by phenotypic variation influences the emergence of specific inheritable genetic changes [137]. Apparently, phenotypic variation should limit the selective power of antibiotics for heritable changes, slowing evolution. Nevertheless, plasticity might help crossing adaptive valleys in a fitness landscape. For instance, antibiotic selection will favour the cells in the plastic population that are the most effective in resisting antibiotic action. Low-level antibiotic-resistance mutations arising in this population will probably be more effective than in the cells with lower expression of plasticity, and might be hooked by selection. Cells that are super-inducible for resistance might be prone to evolve to constitutive production of the mechanism. Indeed, a stress-inducible phenotype could be selectively enriched to the extent where it is stably (constitutively) expressed in the absence of stress [138].

4 Selection: The Mechanism of Evolution of Drug Resistance

The common wisdom supports that the emergence of drug resistance is a direct consequence of the selective events imposed by the use of antibiotics in clinical infections. That is probably true in terms of clinically relevant antibiotic resistance, involving a relatively high number of strains with high levels of resistance. In reality, the mere discovery of an antibiotic effect frequently reveals the presence of resistance to this antibiotic, and in many occasions the description of relevant mechanisms of resistance precedes the launching of the drug for clinical use (Table 2.3). Resistance is always there.

Table 2.3 Chronological introduction of different antimicrobial agents in therapeutics and emergence of resistance mechanisms

Antimicrobial agent	Discovery (introduction)	Resistance 1st reported	Mechanisms of resistance	Organisms
Penicillin G	1940 (1943)	1940	Penicillinase	*Staphylococcus aureus*
Streptomycin	1944 (1947)	1947	S12 ribosomal mutations	*M. tuberculosis*
Tetracycline	1948 (1952)	1952	Efflux	*Shigella dysenteriae*
Erythromycin	1952 (1955)	1956	23S rRNA methylation	*Staphylococcus aureus*
Vancomycin	1956 (1972)	1988	D-Ala-D-Ala replacement	*Enterococus faecalis*
		2004	D-Ala-D-Ala replacement	*Staphylococcus aureus*
Methicillin	1959(1961)	1961	MecA (PBP2a)	*Staphylococcus aureus*
Gentamicin	1963 (1967)	1969	Modifying enzymes	*Staphylococcus aureus*
Nalidixic acid	1962 (1964)	1966	Topoisomerase mutations	*Escherichia coli*
Cefotaxime	1975 (1981)	1981	AmpC ß-lactamases ESBLs	Enterobacteriaceae
		1983		Enterobacteriaceae
Imipenem	1976 (1987)	1986	Acquired carbapenemases	*Pseudomonas aeruginosa*
				Serratia marcescens
Linezolid	1979 (2000)	1999	23S RNA mutations	*Staphylococcus aureus*
				Enterococcus faecalis
Daptomycin	1980 (2004)	2005	Cell wall thickening	*Staphylococcus aureus*
				Enterococcus faecalis
Ceftaroline	2003[a] (2010)	2012	PBP modifications	*Staphylococcus aureus*

[a]Year of first publication

4.1 Selection by Low Antibiotic Concentrations

Antibiotic resistance is frequently recognized by clinicians as a therapeutic problem only after an extremely prolonged period of "subclinical resistance". During this cryptic period, a huge number of selective and evolutionary events take place among the originally susceptible bacterial populations challenged by continuous, intermittent or fluctuating antibiotic pressure, in the same or in different hosts. Bacterial spontaneous variability, perhaps increased after antibiotic-mediated mass extinction events, offers the selective process an important number of mutants, some of them exhibiting very low levels of antibiotic resistance. In most cases, these mutants remain indistinguishable from the fully "susceptible" strains applying the current standard susceptibility testing procedures that (implicitly) assume their selectability, considering that the peak antibiotic concentration in serum by far exceeds the concentration needed to inhibit the variant. Nevertheless, retrospective genetic and populational analysis of recently emerging resistant bacterial organisms, such as beta-lactam-resistant *S. pneumoniae* or *Enterobacteriaceae* harbouring ESBLs or carbapenemases, strongly suggests that low-level resistant variants have indeed been selected during treatments, and that they have evolved, after new cycles of mutation and selection, to high-level resistant organisms.

The discussions on the evolution of antibiotic resistance in microorganisms have been greatly dominated by some a priori beliefs. The first of them probably originated from human chemotherapy: to be considered "resistant" to an antibiotic, a given microorganism should express a relevant increase in the minimal inhibitory concentration (MIC) to this drug. In this view, "minor" increases are meaningless, since the patient can still be successfully treated with antibiotic concentrations exceeding this MIC value. A derivative belief is that: "only significant antibiotic concentrations apply in selection of resistance". Therefore, as antibiotics are mostly excreted in very small amounts by natural microorganisms in the environment, the origin of resistance as a result of these small selective forces (outside of the producing organism) tends to be disregarded. A third belief, closely related to the first, is that "resistance genes" are only those related to "significant" high-level resistance. Under natural circumstances, the preservation of susceptible bacteria may depend on the fact that the selective effect could be preferentially exerted in a given spatial compartment, in a "small niche" according to Smith and Hoekstra [139]. We propose that this compartment, responsible for this type of "confined selection", could be considered as the space or niche in which a precise concentration of antibiotic provides a punctuate selection of a particular resistant bacterial variant. The antibiotic concentration exerting such an effect is here designated as the "selective antibiotic concentration".

4.2 Concentration-Specific Selection: The Selective Window

Any antibiotic concentration can potentially select a resistant variant if it is able to inhibit growth of the susceptible population but not that of the variant harbouring the resistance mechanism. In other words, a selective antibiotic concentration is

that which exceeds the minimal inhibitory concentration (under the local conditions) of the most susceptible population, but not that of the variant population (even if it is very close). If MICs of both susceptible and variant populations are surpassed, then no selection of the variant is expected to occur, and the same applies when the antibiotic concentration is below the local MICs of both populations. Therefore, the selection of a particular variant may happen only in a very narrow range of drug concentrations [140].

Among the more efficient new TEM-beta-lactamase variants that have evolved to hydrolyze cefotaxime are those which differ from the earlier molecules by several amino acids. Assuming the known mutation rates in *E. coli* (see above) it is unlikely that two or more point mutations would appear simultaneously in a beta-lactamase gene. Therefore, if the TEM-1 beta-lactamase is the ancestor of these multiple multiplied variants, it is most likely that the variants arose by a process of sequential point mutation and selection of singly mutated intermediates. For such a scenario to be plausible, each mutation would need to confer a selective advantage over the ancestral strain. In many cases, strains with monomutated TEM-1 enzymes (such as TEM-12, resulting from a single substitution of arginine for serine at position 164) exhibit only a very small increase in resistance to cefotaxime. Typically, TEM-1-producing *E. coli* is inhibited by 0.008 μg/ml, and TEM-12-producing *E. coli* is inhibited by 0.015 μg/ml. Both in vitro and in vivo experiments have demonstrated that despite such a small phenotypic difference, TEM-12-containing strains are efficiently selected by cefotaxime exposure, thereby providing the genetic background for double-mutated, more efficient enzymes for example TEM-10 [141]. Such selection only occurs in particular antibiotic concentrations that define a "selective window for selection" [6].

4.3 Antibiotic Gradients in Antibiotic Selection

Sublethal antibiotic concentrations are able to efficiently select for antibiotic resistance [142, 143]. At any dosage, antibiotics used in chemotherapy create a high diversity of concentration gradients, which inevitably include sublethal (but selective) antibiotic concentrations. These gradients are due to pharmacokinetic factors, such as the different diffusion rates into various tissues, or variation in the elimination rate from different body compartments. The direct effect of microbes of the normal or pathogenic flora, that possess antibiotic-inactivating enzymes, also contributes to the gradient formation. Bacterial populations in the human body probably face a wide range of antibiotic concentrations after each administration of the drug. Since the spontaneous genetic variability of microbial populations also provides a wide range of potentially selectable variant subpopulations, it is appropriate to determine which antibiotic concentration is able to select one or other of these particular subpopulations.

Theoretically, each particular variant population showing a definite MIC will have the possibility of being selectively enriched by a particular antibiotic concentration. This conclusion appears obvious. Surprisingly, the theoretical and practical consequences of such a conclusion remain to be explored in the aim of a better understanding of the evolution of antibiotic-resistant bacterial populations. Bacterial populations show impressive natural genetic polymorphism. For many antibiotics, spontaneous gene variation frequently results in a multiplicity of low-level mechanisms of resistance and the emergence of more specific high-level mechanisms are less frequent (except for a limited number of antibiotics, or by uptake of exogenous highly specialized genes). In the real world, antibiotic concentrations challenging bacteria are mostly located in the low-level margin; those populations showing small increases in MIC would be expected to be preferentially selected by these antibiotics. We emphasize once more the importance of the selection of low-level resistant bacterial mutants to explain the spread of high-level resistance. First of all, several consecutive rounds of selection at the selective antibiotic concentration will produce a progressive enrichment of the low-level variant, and this occurs during most multi-dose treatments. Once a critical number is reached, new variants may arise which can then be selected in the following selective antibiotic concentration, so increasing the antibiotic resistance level. On the other hand, low-level resistant variants can arrive at a position permitting the incorporation of foreign resistance genes in an antibiotic-rich medium. In conclusion, these studies of population selective amplification suggest that at the different points of a concentration gradient, selective forces may be acting with different selective specificity. To a certain extent, the continuous variation of antibiotic concentrations may resemble a tuning device which selects a determined radio frequency emission. Under or over such a frequency (the antibiotic selective concentration), the emission (the particular variant) is lost (selection does not take place). The saddle between the concentrations inhibiting the susceptible and resistant populations is the frequency signal recognized by the selective antibiotic concentration.

A more practical conclusion has been developed in this field when Drlica and collaborators proposed to use antibiotics at dosages that should surpass the "mutant prevention concentration" to avoid the selection of resistance mutants [144].

4.4 Fluctuating Antibiotic Environments

Fluctuating antibiotic environments may facilitate the possibility of evolution of a resistant organism towards higher adaptive peaks than fixed environments. Despite the large

number of in vitro mutations that increase resistance to extended-spectrum cephalosporins in TEM-type beta-lactamases, only a small number occur in naturally occurring enzymes. In nature, and particularly in the hospital setting bacteria that contain beta-lactamases encounter simultaneous or consecutive selective pressure with different beta-lactam molecules. All variants obtained by submitting an *E. coli* strain that contains a $bla_{\text{TEM-1}}$ gene to fluctuating in vitro challenge with both ceftazidime and amoxicillin contain only mutations previously detected in naturally occurring beta-lactamases. Nevertheless, some variants obtained by ceftazidime challenge alone contained mutations never detected in naturally occurring TEM beta-lactamases. A number of modulating mutations might arise that are neutral by themselves but in addition to others might equilibrate the antibiotic substrate preference in fluctuating antibiotic environments [141]. Indeed it can be suggested that extended-spectrum TEM variants in hospital isolates result from fluctuating selective pressure with several beta-lactams rather than selection with a single antibiotic.

4.5 Selection Towards Multi-Resistance: Genetic Capitalism

The concept of genetic capitalism has been applied to MDR pathogens [90]. It refers to further adaptive possibilities of organisms to accumulate resistance mechanisms, either via mutational or gene acquisition events. This reflects a kind of genetic capitalism—the rich tend to become richer. In the last years different examples illustrate this concept such as methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, ESBL-producing Enterobacteriaceae, or carbapenemase-producing Enterobacteriaceae. Genetic capitalism has determined not only the increase in prevalence of MDR resistance pathogens but also the spread and maintenance of resistance genes among clinical isolates, those belonging to the microbiota and in the environment [145]. Obviously, in environments where exposure to different selective agents (antimicrobial drugs) is frequent, the organism harbouring more resistant traits should have higher possibilities of being selected (multi-lateral selection), and a single antibiotic might select multi-resistant strains. This process is illustrated in Fig. 2.3. Moreover, the acquisition of resistance genes, or even virulence traits, may increase clonal fitness and may facilitate the uptake of more and more adaptive advantages. Examples of dispersion of specific genes among bacterial isolates from different compartments are those conferring resistance to tetracyclines (*tet*), macrolides (*erm*), beta-lactamases (*bla*), aminoglycosides (*aac, aad, aph*), sulfonamides (*sul*), trimethoprim (*dfr*), and more recently colistin (*mcr*). In certain cases, the persistence of resistance genes such as those affecting sulfonamides and streptomycin cannot be explained by the current antibiotic selection pressure, as these antibiotics are scarcely used. However, the concomitant presence of other resistance genes may drive this selection process and explains this paradox. Moreover, the genetic support of resistance genes, including integrons, transposons or plasmids, also facilitates their persistence without selective force [146].

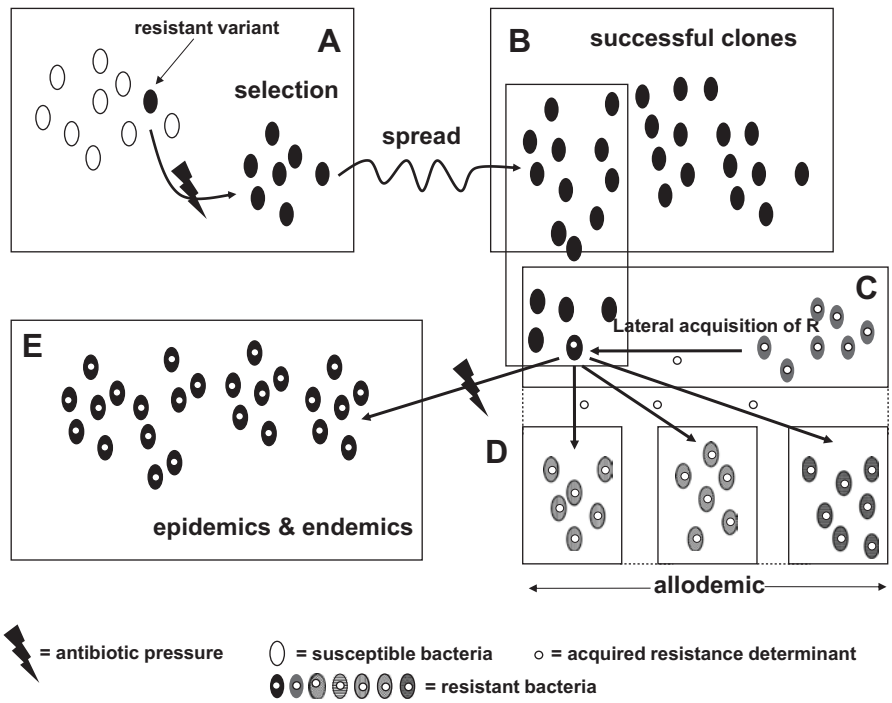


Fig. 2.3 Epidemiological scenarios for the selection and spread of antimicrobial-resistant bacteria: (**a**) the use of an antimicrobial agent may select resistant bacterial variants within a susceptible population; (**b**) selection might contribute to the dominance (success) of the resistant clones, favouring spread in different compartments; (**c**) because of the dominance, successful spreading clones are prone to contact with resistant organisms and to acquire resistance genes by lateral transfer processes; (**d**) at their turn, these resistant clones might act as donors of resistance to other clones depicting an allodemic (or polyclonal) resistance situation); (**e**) resistant clones with acquired resistance genes may become dominant in particular environments depicting epidemic or endemic situations

5 Evolution of Drug-Resistance: Future Prospects

5.1 Units of Variation and Units of Selection

What is selected when we speak about selection of antibiotic resistance? Evolution acts on variation of individual entities. Of course, an individual is not only a single cell, individual animal or plant. In general, an individual can be defined as any simple or complex structure with the potential to maintain, replicate, or reconstruct its self-identity, and also able to escape or at least postpone death, a destructuring or disordering process. Organisms are units of selection, evolutionary units, in a sense "evolutionary individuals", defined as any entity that, independently from the number of elements that enters into its composition or from its hierarchical level of complexity, is selected and evolves as a unit [147]. Because interactions lead to order, individuals should interact with one another. With this perspective, we imagine different kinds of individuals, including "primary order", or elementary individuals, but also secondary, tertiary, and still-higher orders, in which those simpler groupings form more complex assemblies. At any level of the hierarchy variation might occur, and, in a sense the individuals are also units of variation. The modern hierarchical theory of evolution suggests that all types of individuals, at several different levels of integration, independent objects of selective forces, offering a new perspective, one that may be considered as ultra- or hyper-Darwinism. In classic Darwinism, the ordering finger of evolution operates within the selfish organism and, in the later Dawkinian sense, the selfish gene. Ultra-Darwinism serves as a reminder that evolution may occur not only at the level of individual organisms and species, as conceived by Darwin it, but also at the sub- and supraorganismal levels.

Suborganismal evolution may involve molecules such as peptides and proteins. Thus, relatively simple forces, such as chemical stability in a certain environment or modular structures within a particular protein conformation, may exert selective pressures within the "protein universe". Suborganismal evolution may also involve genes; operons; stable chromosomal fragments; mobile genetic elements such as plasmids, transposons, integrons, and insertion sequences; and "nuons". This term, coined in 1992 by Brosius and Gould [148], encompasses any nucleic acids that could act as an elementary unit of selection. Thus, nuons might include genes, gene fusions, gene modules encoding protein catalytic domains, intergenic regions, introns, exons, promoters, enhancers, slippage regions, terminators, pseudogenes, microsatellites, and long or short interspersed elements. Organismal evolution is exerted on units of selection that are typically microbial clones or cell lineages with particular genomic contents, including also demes or local populations. Supra-organismal evolution is exerted on microbial species, with species considered here as a biological individual with a birth, a transformation and possible death; on clades which are monophyletic groups of species; on communities of microbial species, which include microbiomes, possessing metagenomes; and also on stable associations of microbiomes with particular hosts or host communities (metabiota). We frequently use the term "system" to describe the structure of individuals of higher complexity.

Because of that, the analysis of antibiotic resistance requires the study of the multi-level population biology of antibiotic resistance. Antibiotics have natural functions, mostly involving cell-to-cell signalling networks. The anthropogenic production of antibiotics, and its release in the microbiosphere results in a disturbance of these networks, antibiotic resistance tending to preserve its integrity. The cost of such adaptation is the emergence and dissemination of antibiotic resistance genes, and of all genetic and cellular vehicles in which these genes are located. Selection of the combinations of the different evolutionary units (genes, integrons, transposons, plasmids, cells, communities and microbiomes, hosts) is highly asymmetrical. Each unit of selection is a self-interested entity, exploiting the higher hierarchical unit for its own benefit, but in doing so the higher hierarchical unit might acquire critical traits for its spread because of the exploitation of the lower hierarchical unit. This interactive trade-off shapes the population biology of antibiotic resistance, a composed-complex array of the independent "population biologies". Antibiotics modify the abundance and the interactive field of each of these units. Antibiotics increase the number and evolvability of "clinical" antibiotic resistance genes, but probably also many other genes with different primary functions but with a resistance phenotype present in the environmental resistome. Antibiotics influence the abundance, modularity, and spread of integrons, transposons, and plasmids, mostly acting on structures present before the antibiotic era. Antibiotics enrich particular bacterial lineages and clones and contribute to local clonalization processes. Antibiotics amplify particular genetic exchange communities sharing antibiotic resistance genes and platforms within microbiomes. In particular human or animal hosts, the microbiomic composition might facilitate the interactions between evolutionary units involved in antibiotic resistance. The understanding of antibiotic resistance implies expanding our knowledge on multi-level population biology of bacteria [149].

5.2 The Limits of Drug-Resistance Evolution

5.2.1 Saturation Constraints, Short-Sighted Evolution

There are potential bottle necks for the evolution of antimicrobial resistance. For instance, genetic variation inside the modified target, determining more and more effective antibiotic resistance levels, may arrive to exhaustion. As the efficiency

of the mechanism of resistance improves incrementally, the selective advantage of each increment will diminish, until a saturation point is reached at which increments in functional efficiency result in negligible improvements in fitness [150]. Typically this may occur in enzyme kinetics (for instance, hydrolyzing ability of a beta-lactamase for a given beta-lactam antibiotic). When this stage is reached, random changes in amino acid sequence are more often expected to impair enzyme performance than improve it. In the case that the modified antibiotic target retains some vital functions in the bacterial cell, the mutational modifications required to reach very high-level antibiotic resistance may reach a lethal situation. This can be considered as a case of "short-sighted evolution".

5.2.2 Minimizing the Costs of Evolvability

In a well-adapted organism, any change including acquisition of drug-resistance, has a biological risk. Hence bacterial organisms have developed mechanisms to reduce variation to the lower possible level compatible with evolvability, evolutionary innovation, and ability to adapt. The most obvious way to reduce the necessary costs associated with variation is by reducing genetic variation itself, even at the expense of decreasing variability. The most basic mechanism reducing genetic variation is the degeneracy of the genetic code as a number of nucleotide changes are not reflected in changes in amino acid sequence (synonymous nucleotide substitutions). Variation is also reduced by assuring a high-fidelity transcriptional process during DNA replication, or by using highly effective mechanisms of repair of transcriptional mistakes, including increased homologous recombination or daughter strand gap repair. Interestingly, a number of bacteria might have evolved effective mechanisms to reduce the mutation frequency below the average (hypomutation). Mechanisms for stress reduction should also reduce evolvability; indeed the full adaptation of an organism to a very specific niche reduces stress, but stress is maximized when this well-adapted strain is obliged to leave its normal environment. A number of antibiotic resistance mechanisms involved in detoxification of a drug or its expulsion decrease antibiotic-mediated stress and probably reduce variation and evolvability [151].

As stated above, the biological risks associated with the acquisition of drug-resistance might be diminished by management of sequences determining such resistance in modules (relatively "external" to the basic cell machinery) and particularly modules contained in module-carrying elements (as plasmids).

5.2.3 Cost of Antibiotic Resistance

As said before, gene mutants that have been selected for novel resistance phenotypes may have maladaptive pleiotropic effects [152]. This means that acquisition of resistance may de-adapt the resistant organism to its environment thus reducing its competitiveness. Under antibiotic pressure, the competitor organisms may be incapable of taking advantage of this, and therefore the resistant bacteria genotypes have a chance to compensate maladaptation by selection of modifiers [153, 154]. This process of adaptation to its own resistance determinants may completely eliminate the biological cost of resistance. The costs associated with the acquisition of non-advantageous changes might be compensated by the acquisition of new changes. Intragenic or extragenic changes (including, for instance, restorative mutations, gene silencing, or excision) might compensate the cost in a particular environment, but this compensation might even increase the cost in other circumstances. Gene duplication might compensate for decreases in function of a mutated gene and this compensatory effect alone might have important evolutionary consequences. Interestingly, compensatory changes in the bacterial genome may be fixed for reasons other than antibiotic resistance, thus perpetuating the resistance characters in particular genotypes, even in the absence of antibiotic selection. Indeed chromosomal compensatory mutations may eventually increase the bacterial fitness, even if the antibiotic-resistant determinant is lost. At the same time, these organisms may be in the optimal situation of being able "without cost" to lose the mechanism if necessary. Frequently, resistant genes are located in large plasmids, but plasmid carriage usually reduces the competitive fitness of bacteria in the absence of selection for plasmid-encoded functions. It could be expected that plasmid-mediated antibiotic resistance may not be able to persist in bacterial populations in the case of discontinuation of antibiotic use. Interestingly, the cost of plasmid carriage may be compensated in some cases by the mechanisms of resistance encoded, even in the absence of selection. For instance, a tetracycline-efflux pump (determining resistance to this antibiotic) may be used for exporting toxic metabolites from the cell [154]. The in-practice non-functional bleomycin-resistance gene in plasmids harbouring the transposon Tn*5* may confer improved survival and growth advantage [155].

5.3 Epidemiology and Evolution of Antibiotic Resistance

Bacterial selection may result from the acquisition of resistance to environmental changes that are deleterious for competing populations as happens after exposure to antibiotics. Apparently, resistance does not add new capabilities to the survivor: it just compensates (equilibrates) the reduction in reproductive output imposed by the antibiotic. Consequently, immediate intuition associates selection of antibiotic-resistant microbes with the classic expression "*survival* of the fittest". Note that resistant organisms are only "the fittest" in the presence of antibiotics. Certainly natural selection also acts on positive differences when the acquisition of

a novel trait is able to increase the ability of the bacterial organism to exploit a given environment thus provoking a selective difference with the competitors. It is frequently unrecognized that antibiotic resistance provides this type of selective advantage, which is not only a compensation for a loss but *at the same time* is also the gain of a new possibility of habitat exploitation. Frequently, antibiotic-producing microorganisms simultaneously produce antibiotic-resistance mechanisms [31, 156]. It may be that the objective (benefit) of antibiotic production is to obtain an *exclusive* environment where only the producer is able to survive, because of resistance. As a consequence, all the resources of the environment can be exploited exclusively by the producing strain. In other words, in the presence of the antibiotic, antibiotic resistance is a colonization factor to gain *exclusivity* for resources. Etymologically, exclusive means "closed for the others". It may be well conceived that in a world in which antibiotics have become frequent components from microbial environments (in particular in humans and animals), the acquisition of antibiotic resistance is evolving not only a protective mechanism but also a factor assuring *exclusivity* for the resistant populations in antibiotic-containing areas. The increase in the absolute number of antibiotic-resistant organisms is the proof of the benefits of this strategy.

5.3.1 Resistance, Epidemics, Endemics, and Allodemics

Antibiotic resistance is expected to have a minor biological or clinical effect in the absence of effective spread of resistant organisms. As stated in the last paragraph antibiotic resistance might help a given organism to spread, particularly in environments assuring frequent exposure to these drugs. Eventually hyper-mutable organisms might be better suited for host colonization, host-to-host transmission, survival in inert environments, and also for developing antibiotic resistance, either by mutation or by homeologous recombination with exogenous genes. On the other hand, pathogenic and epidemigenic organisms are probably more frequently exposed to antibiotic therapy. Therefore, a certain convergence between virulence, epidemigenicity and resistance could be expected to occur [65]. Interestingly, antibiotic-resistant clones frequently coincide with "successful clones" well adapted for colonization or spread *before* acquiring antibiotic resistance. This convergent process of selection, leading to the dissemination of antibiotic resistance determinants in different bacterial populations is illustrated in Fig. 2.3. Examples of this can be found in beta-lactam-resistant *S. pneumoniae, E. faecalis* and *S. aureus* or in glycopeptide-resistant *E. faecium* [157–161].

However, consistent with the concept of the multiplicity of units of selection stated before (paragraph 5.1.), a particular epidemigenic "resistant clone" does not constitute the only selectable unit of antibiotic resistance. The wide application of molecular techniques, such as restriction pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) to the definition of bacterial clones have offered a totally new view of several 'epidemic' phenomena. A surprising diversity of clones was found when the clones responsible for the progressive and steep increase of enterobacterial strains harbouring ESBLs in a single hospital were studied. For instance, *K. pneumoniae* strains harbouring $bla_{\text{CTX-M-10}}$ or $bla_{\text{OXA-48}}$ belonged to more than thirteen different clones! Therefore the case was "epidemics of $bla_{\text{CTX-M-10}}$ or $bla_{\text{OXA-48}}$ resistance" and but not "epidemics" in the classic sense. The term "**allodemics**' (from Greek *allos*, other, different; and *demos*, people), in the sense of "something is being produced in the community by different causal agents" has been proposed to describe this pattern (Fig. 2.3) [122]. Note that the infection (or in our case the frequency of antibiotic resistance) may cluster but not necessarily its causative organism. In other words, the phenotype may cluster, but not the genotype. Indeed the concept of allodemics emphasizes the importance of the asymmetry between phenotype and genotype in natural selection. Its practical consequences are quite obvious. In documented allodemic situations, interventions should be focused more to the environmental causes of the problem than to the classical approaches including clone-directed measures to limit host-to-host spread, or search-and-destroy strategies. For instance, in our particular case, a reduction in the intensity of use of antibiotics potentially able to *select for* ESBLs or carbapenemases could be an appropriate environmental intervention for controlling allodemic situations.

5.3.2 Resistance as a Colonization Factor

In the absence of antibiotics, resistance does not generally add new basic capabilities to the physiology of the bacterial cell and often produces reduction in fitness. In other words, resistance does not "improve" the cell machinery but only just compensates (equilibrates) the reduction in reproductive output imposed by the antibiotic. From this point of view, can antibiotic resistance be considered a factor triggering important changes in long-term bacterial evolution?

Certainly, natural selection also acts on positive differences when the acquisition of a novel trait is able to increase the ability of the bacterial organism to exploit a given environment thus provoking a selective difference with the competitors. It is often unrecognized that antibiotic resistance provides this type of selective advantage and being not only a compensation for a loss, but *at the same time* the gain of a new possibility of habitat exploitation. Antibiotic-producing microorganisms produce antibiotic-resistance mechanisms simultaneously [31, 156]. When this occurs it may be that the biological benefit of antibiotic production is to obtain an exclusive environment, in which only the producer is able to survive because of resistance. The same might be true if a bacterial organism resistant to antibiotic A were able to

induce antibiotic A production in another antibiotic-producing organism such as another bacteria, fungus, plant or animal. Antibiotic release will eliminate competitors. In a certain sense, antibiotic-resistant bacteria have taken ecological advantage of human production and release of a number of antibiotics. The increase in the absolute number of antibiotic-resistant organisms is the proof of the benefits of such an evolutionary trend.

5.3.3 Biogeography and Local Biology of Antibiotic Resistance

Biogeography of resistance is the study of the distribution of diversity of resistance over space and time [162]. In the words of Brendan Bohannan, "space is the next frontier in biology". The world is a spatially structured place, with localized dispersal, localized interactions and localized selective events. In environments under high intensity of selective forces (for instance, in the hospital, because of pathogenesis, host-to-host spread, and local usage of antiseptics and antimicrobial agents), the local tool-kit of evolutionary active elements should be very large. Locally successful sub-specific groups, clones, plasmids, transposons, integrons or antibiotic resistance genes (see Sect. 5.1 about individuals and units of selection) will be cumulatively selected, and possibilities of interaction (accessibility-connectivity) will necessarily increase. Consequently in these environments we can expect acceleration in the evolution (construction-selection) of complex structures eventually involved in antibiotic resistance. Organisms that are ecologically and/or phylogenetically distant, present in a low density or submitted to environmental isolation might have reduced possibilities for genetic exchange and evolvability. The term "exchange community" has been proposed to identify the biological systems able to exchange genes [163]. It is possible that genetic exchange might occasionally occur among organisms sharing similar lifestyles across a wide phylogenetic range, as such "ecologically close" ensembles of organisms tend to conserve equivalent regulatory networks [164]. The presence of the same antibiotic resistance genes in ecologically connected bacterial genera indicates a complex history of genetic interactions in which antibiotic resistance genes have parasitized the natural circuits of adaptive gene flow. Note that "genetic exchange communities" are necessarily local ones [165]. Different environments with different cumulative histories of antibiotic use and local epidemics/endemics may harbour different ensembles of evolutionary pieces. Therefore the emergence and development of new antibiotic-resistance patterns is probably of biogeographical dimension [166]. Of course "global spreading clones" disseminate a number of the genetic elements involved in antibiotic resistance but once in touch with local biological ensembles, a local phylogeographic diversification tends to take place [167].

5.3.4 Antibiotics as Ecosystem-Damaging Agents: The Role of Resistance

Simply put, antibiotic agents are chaos-promoting factors for microbial ecosystems because these agents provoke functional disorders and death in many kinds of bacteria. The use (particularly the abuse) of such agents leads to collapse in the diversity of these microorganisms along with entire ranges of individuals. It can be stated that Nature will be always able to recover some degree of biological equilibrium. We should be aware that the extensive use and release of drugs may be provoking the emergence of new biological orders. It is difficult to predict whether these new orders will be better for the whole system or will lead to new adaptive difficulties. The short-term relief that we derive from using antibiotics may be followed by longer term difficulties that are the hallmark of any evolutionary trend.

Supracritical release of antimicrobial agents should disturb microbial populations, affecting many different types of individuals (units of selection) within those populations. Among individuals at the supracellular level, for instance, within intestinal bacterial communities or the soil microbiota at a particular site, the functional loss of bacteria within a particular system can be repaired by residual "redundant" populations that survive such a challenge, by degenerate populations of other bacteria fulfilling a similar function, by imported populations migrating from a connected system or eventually by the emergence of novel variant organisms. At the level of the individual organism—for instance, a single bacterial cell—redundant or degenerate genes can repair or otherwise overcome the damage that follows an antibiotic challenge. This reordering may depend on replacing those functions that the antibiotic inhibited, by importing foreign genes that can deactivate the antibiotic or by mutation- or recombination-dependent innovation that leads to antibiotic resistance. Because of the hypothesis of multiple-units of selection affected by antibiotics, these drugs might have a second-order evolutionary impact on suborganismal individuals—for instance, on plasmids, integrons, operons, genes, insertion sequences, and proteins. Critically, antibiotics or any other agent or circumstance promoting disorder may expand across the whole hierarchy of evolutionary individuals. For instance, local disordering events may select different types of bacterial clones in a particular environment, such as that within a specific hospital. Genes or proteins carried by these clones may be enriched. The amplifying selective process increases the possibilities of interaction among certain clones, genetic elements, and other molecules. The best combinations for local survival increase in number which facilitates further adaptive possibilities and reflects a kind of genetic capitalism—the rich tend to become richer. From this perspective, antibiotic resistance might constitute an ecological risk and at the same time—deactivating the effect of antimicrobial drugs—a factor of ecological protection.

5.3.5 Evolutionary Ecology and Spread of Antibiotic Resistance: The Four P's

All evolutionary ecology of antibiotic resistance, involving its spread and diversification can be summarized in the consideration of the four P's: (1) **P**enetration in microbial ecosystems, including microbiotas, of highly effective pathogenic clones and plasmids, (2) **P**romiscuity of genetic traits involved in antibiotic resistance by lateral gene transfer, (3) **P**lasticity of genetic vehicles and platforms, taking advantage of highly recombinogenic sequences, and (4) **P**ersistence and maintenance of different pathogenic multidrug-resistant high-risk clones and globally distributed plasmids [168]. These four P's determine the **three main processes** shaping the natural history of antibiotic resistance, involving the emergence, invasion, and occupation by antibiotic-resistant genes of significant environments for human health. The process of emergence in complex bacterial populations is a high-frequency, continuous swarming of ephemeral combinatory genetic and epigenetic explorations inside cells and among cells, populations and communities, expanding in different environments (migration), creating the stochastic variation required for evolutionary progress. Invasion refers to the process by which antibiotic resistance significantly increases in frequency in a given (invaded) environment, led by external invaders local multiplication and spread, or by endogenous conversion. Conversion occurs because of the spread of antibiotic resistance genes from an exogenous-resistant clone into an established (endogenous) bacterial clone(s) colonizing the environment; and/or because of dissemination of particular resistant genetic variants that emerged within an endogenous clonal population. Occupation of a given environment by a resistant variant means a permanent establishment of this organism in this environment, even in the absence of antibiotic selection. Specific interventions on emergence influence invasion, those acting on invasion also influence occupation and interventions on occupation determine emergence. Such interventions should be simultaneously applied, as they are not simple solutions to the complex problem of antibiotic resistant.

How has antibiotic resistance reached a planetary spread among all kind of environments? We cannot discard that other bacterial adaptive traits may also be spreading with high efficiency among bacterial populations, and that antibiotic resistance provides easy-to-detect phenotypes of obvious importance in public health. In any case, it seems reasonable to think that humans have–through the excessive use of antibiotics, biocides, and industrial pollution-accelerated the building-up and selection of genes and genetic platforms involved in antibiotic resistance. General factors derived from societal changes in human populations and the environment, including changes in land use (intensified human encroachment on natural environments, and globalization of planet biology—including human population growth; livestock and production methods; international travel or long distant trade of humans, animals, and vegetables, breakdown in public health infrastructure, and eventually geo-anthropological changes (such as global warming), microbial adaptation to drug or vaccine use or to new host species, might have also contributed to the global invasion by antibiotic-resistant bacteria. The rising consciousness about this multi-causal complexity requires a novel reconsideration of the priorities among possible interventions aiming to fight antibiotic resistance, including the possibility of influencing with complex treatments, restoration strategies, and may be with "drugs for the environment" the ecology and evolution of antibiotic-resistant bacteria [169, 170]. We should take seriously the possibilities of reducing antibiotic selection in the environment, as in fact in an environment polluted by low concentrations of antibiotics and potential human-pathogenic bacterial organisms serves as "training field" for the emergence and evolution of novel resistance traits.

5.3.6 Might Evolution of Antibiotic Resistance be Predicted?

The ultimate reason for any human scientific knowledge is the optimization or improvement of our current and future interactions with our environment. The reason for research in antibiotic resistance is, obviously, the possibility of disarming bacteria of their ability to counteract antibiotics. In a broader perspective, as was stated in the last paragraph, the aim is the preservation of a healthy microbial ecosystem surrounding humans. These objectives require mastering the evolutionary trajectories resulting in antibiotic resistance. Is that a feasible task? According to conventional scientific knowledge, evolution is essentially based on random processes which are exposed to an extremely large number of unexpected influences and is therefore essentially unpredictable. However, we generally act against this intuition with, for instance, hygienic procedures and implementation of antibiotic policies to prevent the development of antibiotic resistance are common practices in modern medicine. Indeed research in microbiological sciences applied to public health is currently based on the implicit belief that microbial variation and infectious diseases are predictable and therefore might (and should) be controlled before causing problems to mankind. If we are constantly seeking huge amounts of genomic and proteomic data from microbes, if we are building-up complex phylogenies, structural and mathematical models and developing advanced procedures based on systems biology to understand interactions between elements, it is only because we do not discard the possibility of preventing the emergence and dissemination of antibiotic-resistant microbial pathogens. Preventing this emergence and dissemination implies mastering the evolutionary trajectories of microbial pathogens, something that as previously stated goes against our conventional view of the process of evolution.

The main problem is the multi-causal origin of the spread of antibiotic resistance. To describe to a certain extent the main processes involved, a number of composite parameters should be analysed [171]. The main ones are certainly the following ones: (1) *contact rates*; this set of parameters refers to the probability that two particular elements involved in antibiotic resistance could be in close contact during a sufficient period of time, enabling potential interactions; for instance, susceptible and resistant cells, or plasmids carrying or not carrying particular genes; (2) *transfer rates*; this set of parameters refers to the probability that one of these elements moves into another element of the same or different hierarchical level; as a plasmid into a cell, or a gene into a plasmid; (3) *integration rates*; this set of parameters refers to the probability that one transferred unit could be stably maintained in coexistence with another element or assembled with it; (4) *replication* rates; this set of parameters refers to the probability that a particular element involved in antibiotic resistance will increase in copy number at a certain speed and reach certain final densities; (5) *diversification* rates; this set of parameters refers to the probability that a particular element (a clone, a plasmid, a gene) produces genetic variants at certain rates, and variants of these variants; and (6) *selection* rates; this final set of parameters refers to the probability that a particular element involved in antibiotic resistance might be replicating differentially than other units of the same hierarchical level as the result of the carriage of genes providing higher fitness.

The parametric space resulting from the above set of six rates measuring interactions of relevance in antibiotic resistance is certainly modified (even determined) by another group of parameters, the ecological parameters. These are environmental parameters whose changes might influence the above-mentioned rates. Among these parameters we can mention: density of colonized and colonizable hosts; population sizes of bacteria per host during colonization and infection; susceptibility to colonization of hosts, including age, nutrition, illness-facilitated colonization; frequency of between-host interactions (such as animal–human interaction); host natural and acquired immune response to colonizing organisms; ecological parameters of colonizable areas, including interaction with local microbiota and frequency and type of antibiotic-resistant commensals; migration and dispersal of colonized hosts; antibiotic exposure; overall density of antibiotic use, type of antibiotics and mode of action, dosage and duration of therapy, adherence to therapy, selective concentrations, antibiotic combinations; mode of transmission of resistant organisms; transmission rates between hosts (antibiotic treated and untreated, infected, and uninfected); time of contact between hosts; exposure to biocides; hygiene, infection control, sanitation; food, drinking-water and water body contamination, and host exposure; and environmental contamination by resistant organisms in soil, including sewage and water bodies.

Of course to obtain data to define this parametric field is an extremely complex task, certainly to be completed this century. We can of course with the powerful available bioinformatic technologies start to dissect this complexity. In the case of modular structures associated with resistance, the predictive process should be based on research about the "grammar of affinities" between modular elements. Techniques of comparative genomics have been used to infer functional associations between proteins based on common phylogenetic distributions, conserved gene neighbourhood, or gene fusions. The use of scoring-schemes in the building-up of networks describing possible associations between modules facilitates the prediction of novel functions [172, 173]. Similar types of methods could be developed to predict functional associations between modules involved in the emergence, expression, mobilization, or evolution of antibiotic resistance. A concern of these studies is their unaffordable complexity. Nevertheless, as in the case of mutation, genetic architectures based on modules might have an affordable complexity as they show reuse of alignments or circuit patterns which allow construction of complex adaptive systems by using common series of modules [174, 175]. From the perspective of a modular "genome system architecture" [176] it is possible to find in different organisms, plasmids, transposons, integrons or protein sequences such as recombinases, identical modules combined in different ways. The study of the corresponding linkage patterns has become critical for understand the evolution of evolvability [177]. Indeed MDR is the result of combinatorial genetic evolution [178, 179]. If it were possible to make comprehensive catalogues of modular functional units, combination of these modules in local alignments could be predicted that might fulfil the expected bacterial adaptation [180]. The building-up of comprehensive interconnected databases where modules could be stored in function of their combinations has been proposed [181]. These combinations probably depend on particular codes by which particular units are accepted (integrated) within others after introgressive events. For instance, (1) codes determining the compatibility of a acquired resistance gene with the functional structure of a cell or of mobile genetic elements; in fact the new character should be compatible with the metabolon, or selfish (a kind of individual) metabolism [182]; (2) codes determining the compatibility of resistance and virulence plasmids or ICEs with particular bacterial species and clones; (3) codes determining the compatibility of a particular bacterial resistant or virulent clone with specific microbiotic ensembles in particular hosts, including different animals. Unfortunately, we know very little about these codes, but such a knowledge is certainly needed for public health, to establish desirable surveillance and control measures assuring healthy relations between humans and animals, and the microbiosphere [183]. Bioinformatics (network genomics and proteomics) using

approaches like combinatorics, fuzzy logic models and principles learned from linguistics and semiotics may be able in the future to accomplish the task of finding a grammar of modular affinities [90, 176, 184] to approach one of the major objectives of all biological sciences: to be able to predict ("the topology of the possible" [185]) evolutionary trajectories of living beings. System biology [186, 187] and *ad-hoc* computational methods will take advantage of these data to establish predictions for antibiotic resistance [188].

Acknowledgements To the group of people who daily shares our interest in evolutionary biology of antibiotic resistance, and in particular to José-Luis Martínez, Teresa Coque, Rosa del Campo, Juan-Carlos Galán, María-Isabel Morosini, and Patricia Ruiz-Garbajosa, some of whom have provided data for the present review. We acknowledge also to EU Commission and Innovative Medicine Initiative (IMI) for funding projects on this field (COBRA-LSHM-CT-2003-503335; DRESP-LSHM-CT-2005-018705, EAR-LSHM-CT-2005-518152 ACE-LSHM-CT-2006-037410,TROCAR-LSHM-CT-2008-223031,R-GNOSIS-FP7-HEALTH-F32011-282512, EVOTAR-FP7-HEALTH-2011-282004, MON4STRAT-FP7-HEALTH-2013-INNOVATION-1-602906-2, IMI-6-ENABLE-2014-115583-2,IMI-9-COMBATE-CARE-115620-2,IMI-11-iABC-115721).

References

1. Bell G. Selection, the mechanism of evolution. New York: Chapman & Hall; 1997.
2. Olivares J, Bernardini A, Garcia-Leon G, Corona FB, Sanchez M, Martinez JL. The intrinsic resistome of bacterial pathogens. Front Microbiol. 2013;4:103.
3. Martínez JL, Coque TM, Baquero F. What is a resistance gene? Ranking risk in resistomes. Nat Rev Microbiol. 2015;13:116–23.
4. Galán JC, González-Candelas F, Rolain JM, Cantón R. Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the β-lactamases world. Front Microbiol. 2013;4:9.
5. Linares JF, Gustafsson I, Baquero F, Martinez JL. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci U S A. 2006;103:19484–9.
6. Cantón R, Morosini MI. Emergence and spread of antibiotic resistance following exposure to antibiotics. FEMS Microbiol Rev. 2011;35:977–91.
7. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. 2005;436:1171–5.
8. Baquero F. Evolution and the nature of time. Int Microbiol. 2005;8:81–91.
9. Morosini MI, Cantón R. Tolerance and heteroresistance in Gram-positive microorganisms. Med Clin (Barc). 2010;135 Suppl 3:16–22.
10. Wiuff C, Zappala RM, Regoes RR, Garner KN, Baquero F, Levin BR. Phenotypic tolerance: antibiotic enrichment of non-inherited resistance in bacterial populations. Antimicrob Agents Chemother. 2005;49:1483–94.
11. Kussell E, Kishony R, Balaban NQ, Leibler S. Bacterial persistence: a model of survival in changing environments. Genetics. 2005;169:1807–14.
12. Germain E, Roghanian M, Gerdes K, Maisonneuve E. Stochastic induction of persister cells by HipA through (p)ppGpp-mediated activation of mRNA endonucleases. Proc Natl Acad Sci U S A. 2015;112:5171–6.
13. Levin BR, Rozen DE. Non-inherited antibiotic resistance. Nat Rev Microbiol. 2006;4:556–62.
14. Gould SJ, Vrba S. Exaptation—a missing term in the science of form. Paleobiology. 1982;8:4–15.
15. Gould SJ, Lloyd EA. Individuality and adaptation across levels of selection: how shall we name and generalize the unit of Darwinism? Proc Natl Acad Sci U S A. 1999;96:11904–9.
16. Torres C, Perlin MH, Baquero F, Lerner DL, Lerner SA. High-level amikacin resistance in *Pseudomonas aeruginosa* associated with a 3′-phosphotransferase with high affinity for amikacin. Int J Antimicrob Agents. 2000;15:257–63.
17. Gould SJ. In: Gould SJ, editor. The structure of evolutionary theory. Cambridge, MA/London: The Belknap Press of Harvard University Press; 2002.
18. Sommer MO, Dantas G, Church GM. Functional characterization of the antibiotic resistance reservoir in the human microflora. Science. 2009;325:1128–31.
19. Charpentier E, Courvalin P. Antibiotic resistance in *Listeria* spp. Antimicrob Agents Chemother. 1999;43:2103–8.
20. Edwards R, Read PN. Expression of the carbapenemase gene (cfiA) in *Bacteroides fragilis*. J Antimicrob Chemother. 2000;46:1009–12.
21. Gudeta DD, Bortolaia V, Amos G, Wellington EM, Brandt KK, Poirel L, Nielsen JB, Westh H, Guardabassi L. The soil microbiota harbors a diversity of carbapenem-hydrolyzing ß-Lactamases of potential clinical relevance. Antimicrob Agents Chemother. 2015. pii: AAC.01924-15 [Epub ahead of print].
22. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12:83–8.
23. Hernández A, Sánchez MB, Martínez JL. Quinolone resistance: much more than predicted. Front Microbiol. 2011;2:22.
24. Witek MA, Conn GL. Expansion of the aminoglycoside-resistance 16S rRNA (m(1)A1408) methyltransferase family: expression and functional characterization of four hypothetical enzymes of diverse bacterial origin. Biochim Biophys Acta. 2014;1844:1648–55.
25. Sieradzki K, Tomasz A. A highly vancomycin-resistant laboratory mutant of *Staphylococcus aureus*. FEMS Microbiol Lett. 1996;142:161–6.
26. Martinez JL. General principles of antibiotic resistance in bacteria. Tackling antibiotic resistance: the environmental framework. Drug Discov Today Technol. 2014;11:33–9.
27. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons MN, Kreuzinger N, Huovinen P, Stefani S, Schwartz T, Kisand V, Baquero F, Martinez JL. Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol. 2015;13:310–7.
28. Cantón R. Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin Microbiol Infect. 2009;15 Suppl 1:20–5.
29. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. Science. 2006;311:374–7.
30. Wiener P, Tuljapurkar S. Migration in variable environments: exploring life-history evolution using structured population models. J Theor Biol. 1994;166:75–90.
31. Davies J. Inactivation of antibiotics and the dissemination of resistance genes. Science. 1994;264:375–82.
32. Finley RL, Collignon P, Larsson DG, McEwen SA, Li XZ, Gaze WH, Reid-Smith R, Timinouni M, Graham DW, Topp E. The scourge of antibiotic resistance: the important role of the environment. Clin Infect Dis. 2013;57:704–10.
33. Travis DA, Sriramarao P, Cardona C, Steer CJ, Kennedy S, Sreevatsan S, Murtaugh MP. One medicine one science: a framework for exploring challenges at the intersection of animals, humans, and the environment. Ann N Y Acad Sci. 2014;1334:26–44.

34. Massova I, Mobashery S. Structural and mechanistic aspects of evolution of beta-lactamases and penicillin-binding proteins. Curr Pharm Des. 1999;5:929–37.
35. Aharonowitz Y, Cohen G, Martín JF. Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. Annu Rev Microbiol. 1992;46:461–95.
36. Massova I, Mobashery S. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agents Chemother. 1998;42:1–17.
37. Kelly JA, Dideberg O, Charlier P, Wery JP, Libert M, Moews PC, Knox JR, Duez C, Fraipont C, Joris B, et al. On the origin of bacterial resistance to penicillin: comparison of a beta-lactamase and a penicillin target. Science. 1986;231:1429–31.
38. Gherman BF, Goldberg SD, Cornish VW, Friesner RA. Mixed quantum mechanical/molecular mechanical (QM/MM) study of the deacylation reaction in a penicillin binding protein (PBP) versus in a class C beta-lactamase. J Am Chem Soc. 2004;126: 7652–64.
39. Medeiros AA. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin Infect Dis. 1997;24:S19–45.
40. Martínez JL, Rojo F. Metabolic regulation of antibiotic resistance. FEMS Microbiol Rev. 2011;35:768–89.
41. Wolf DM, Arkin AP. Motifs, modules and games in bacteria. Curr Opin Microbiol. 2003;6:125–34.
42. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001;9:34–9.
43. Greenway DL, England RR. The intrinsic resistance of *Escherichia coli* to various antimicrobial agents requires *ppGpp* and sigma *s*. Lett Appl Microbiol. 1999;29:323–6.
44. Cao M, Wang T, Ye R, Helmann JD. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* sigma (W) and sigma (M) regulons. Mol Microbiol. 2002;45:1267–76.
45. Bandow JE, Brotz H, Hecker M. *Bacillus subtilis* tolerance of moderate concentrations of rifampin involves the sigma(B)-dependent general and multiple stress response. J Bacteriol. 2002;184:459–67.
46. Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol Microbiol. 1999;34:305–16.
47. Powell JK, Young KD. Lysis of *Escherichia coli* by beta-lactams which bind penicillin-binding proteins 1a and 1b: inhibition by heat shock proteins. J Bacteriol. 1991;173:4021–6.
48. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN. SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. Science. 2004;305:1578–9.
49. Blázquez J, Gómez-Gómez JM, Oliver A, Juan C, Kapur V, Martín S. PBP3 inhibition elicits adaptive responses in *Pseudomonas aeruginosa*. Mol Microbiol. 2006;62:84–99.
50. Foster PL, Lee H, Popodi E, Townes JP, Tang H. Determinants of spontaneous mutation in the bacterium *Escherichia coli* as revealed by whole-genome sequencing. Proc Natl Acad Sci U S A. 2015;112:E5990–9.
51. Selander RK, Levin BR. Genetic diversity and structure in *Escherichia coli* populations. Science. 1980;210:545–7.
52. Jorth P, Staudinger BJ, Wu X, Hisert KB, Hayden H, Garudathri J, Harding CL, Radey MC, Rezayat A, Bautista G, Berrington WR, Goddard AF, Zheng C, Angermeyer A, Brittnacher MJ, Kitzman J, Shendure J, Fligner CL, Mittler J, Aitken ML, Manoil C, Bruce JE, Yahr TL, Singh PK. Regional isolation drives bacterial diversification within cystic fibrosis. Lungs Cell Host Microbe. 2015;18:307–19.
53. Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science. 2000;288:1251–4.
54. Román F, Cantón R, Perez-Vazquez M, Baquero F, Campos J. Dynamics of long-term colonization of respiratory tract by Haemophilus influenzae in cystic fibrosis patients shows a marked increase in hypermutable strains. J Clin Microbiol. 2004;42:1450–9.
55. Prunier AL, Malbruny B, Laurans M, Brouard J, Duhamel JF, Leclercq R. High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. J Infect Dis. 2003;187: 1709–16.
56. del Campo R, Morosini MI, de la Pedrosa EG, Fenoll A, Muñoz-Almagro C, Maiz L, Baquero F, Cantón R. Spanish Pneumococcal Infection Study Network. Population structure, antimicrobial resistance, and mutation frequencies of *Streptococcus pneumoniae* isolates from cystic fibrosis patients. J Clin Microbiol. 2005;43:2207–14.
57. Baquero MR, Nilsson AI, del Carmen Turrientes M, Sandvang D, Galán JC, Martinez JL, Frimodt-Moller N, Baquero F, Andersson DI. Polymorphic mutation frequencies in *Escherichia coli*: emergence of weak mutators in clinical isolates. J Bacteriol. 2004;186:5538–42.
58. Giraud A, Matic I, Tenaillon O, Clara A, Radman M, Fons M, Taddei F. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. Science. 2001;291:2606–8.
59. Shaver AC, Dombrowski PG, Sweeney JY, Treis T, Zappala RM, Sniegowski PD. Fitness evolution and the rise of mutator alleles in experimental *Escherichia coli* populations. Genetics. 2002;162:557–66.
60. Turrientes M-C, Baquero F, Levin BR, Martínez J-L, Ripoll A, González-Alba J-M, Tobes R, Manrique M, Baquero MR, Rodríguez-Domínguez MJ, Cantón R, Galán JC. Normal mutation rate variants arise in a mutator (Mut S) Escherichia coli population. PLoS ONE. 2013;8, e72963.
61. Chao L, Cox EC. Competition between high and low mutating strains of *Echerichia coli*. Evolution. 1983;37:125.
62. Baquero MR, Galán JC, del Carmen Turrientes M, Cantón R, Coque TM, Martinez JL, Baquero F. Increased mutation frequencies in *Escherichia coli* isolates harboring extended-spectrum beta-lactamases. Antimicrob Agents Chemother. 2005;49: 4754–6.
63. Saxer G, Krepps MD, Merkley ED, Ansong C, Deatherage Kasier BL, Valovska M-T, Ristic N, Yeh PT, Prakash VP, Leiser OP, Nahleh L, Gibbons HS, Kreuzr HW, Shamoo Y. Mutations in global regulators lead to metabolic selection during adaptation to complex environments. PLoS Genet. 2014;10(12), e1004872.
64. Miller K, O'Neill AJ, Chopra I. Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes. J Antimicrob Chemother. 2002;49: 925–34.
65. Martinez JL, Baquero F. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. Clin Microbiol Rev. 2002;15:647–79.
66. Tanaka MM, Bergstrom CT, Levin BR. The evolution of mutator genes in bacterial populations: the roles of environmental change and timing. Genetics. 2003;164:843–54.
67. Miller K, O'Neill AJ, Chopra I. *Escherichia coli* mutators present an enhanced risk for emergence of antibiotic resistance during urinary tract infections. Antimicrob Agents Chemother. 2004;48:23–9.
68. Pérez-Capilla T, Baquero MR, Gómez-Gómez JM, Ionel A, Martín S, Blázquez J. SOS-independent induction of dinB transcription by beta-lactam-mediated inhibition of cell wall synthesis in *Escherichia coli*. J Bacteriol. 2005;187:1515–8.
69. Baquero F, Blázquez J. Evolution of antibiotic resistance. Trends Ecol Evol. 1997;12:482–7.
70. Balashov S, Humayun MZ. Mistranslation induced by streptomycin provokes a RecABC/RuvABC-dependent mutator phenotype in *Escherichia coli* cells. J Mol Biol. 2002;315:513–27.

71. Phillips I, Culebras E, Moreno F, Baquero F. Induction of the SOS response by new 4-quinolones. J Antimicrob Chemother. 1987;20:631–8.
72. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN. SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. Science. 2004;305:1629–31.
73. Santoyo G, Romero D. Gene conversion and concerted evolution in bacterial genomes. FEMS Microbiol Rev. 2005;29:169–83.
74. Prammananan T, Sander P, Springer B, Bottger EC. RecA-mediated gene conversion and aminoglycoside resistance in strains heterozygous for rRNA. Antimicrob Agents Chemother. 1999;43:447–53.
75. Andersson DI, Jerlström-Hultqvist J, Näsvall J. Evolution of new functions de novo and from preexisting genes. Cold Spring Harb Perspect Biol. 2015;7(6):a017996.
76. Andersson DI, Hughes D. Gene amplification and adaptive evolution in bacteria. Annu Rev Genet. 2009;43:167–95.
77. Andersson DI, Hughes D, Roth JR. The origin of mutants under selection: interactions of mutation, growth, and selection. Ecosal Plus. 2011;4(2).
78. Sandegren L, Andersson DI. Bacterial gene amplification: implications for the evolution of antibiotic resistance. Nat Rev Microbiol. 2009;7:578–88.
79. Adler M, Anjum M, Berg OG, Andersson DI, Sandegren L. High fitness costs and instability of gene duplications reduce rates of evolution of new genes by duplication-divergence mechanisms. Mol Biol Evol. 2014;31:1526–35.
80. Maisnier-Patin S, Roth JR. The origin of mutants under selection: how natural selection mimics mutagenesis (adaptive mutation). Cold Spring Harb Perspect Biol. 2015;7:a018176.
81. Woegerbauer M, Kuffner M, Domingues S, Nielsen KM. Involvement of aph(3′)-IIa in the formation of mosaic aminoglycoside resistance genes in natural environments. Front Microbiol. 2015;6:442.
82. Barile S, Devirgiliism C, Perozzim G. Molecular characterization of a novel mosaic tet(S/M) gene encoding tetracycline resistance in foodborne strains of Streptococcus bovis. Microbiology. 2012;158(Pt 9):2353–62.
83. Pereira-Leal JB, Levy ED, Teichmann SA. The origins and evolution of functional modules: lessons from protein complexes. Philos Trans R Soc Lond B Biol Sci. 2006;361:507–17.
84. Bapteste E, Lopez P, Bouchard F, Baquero F, McInerney JO, Burian RM. Evolutionary analyses of non-genealogical bonds produced by introgressive descent. Proc Natl Acad Sci. 2012;109(45):18266–72.
85. Cantón R, Coque TM. The CTX-M beta-lactamase pandemic. Curr Opin Microbiol. 2006;9:466–75.
86. Lartigue MF, Poirel L, Aubert D, Nordmann P. In vitro analysis of IS*Ecp1B*-mediated mobilization of naturally occurring beta-lactamase gene $bla_{CTX\text{-}M}$ of *Kluyvera ascorbata*. Antimicrob Agents Chemother. 2006;50:1282–6.
87. Poirel L, Decousse JW, Nordmann P. Insertion sequence ISEcp1B is involved in expression and mobilization of a bla(CTX-M) beta-lactamase gene. Antimicrob Agents Chemother. 2003;47:2938–45.
88. Toleman MA, Bennett PM, Walsh TR. IS*CR* elements: novel gene-capturing systems of the 21st century? Microbiol Mol Biol Rev. 2006;70:296–316.
89. Aubert D, Naas T, Heritier C, Poirel L, Nordmann P. Functional characterization of IS*1999*, an IS*4* family element involved in mobilization and expression of beta-lactam resistance genes. J Bacteriol. 2006;188:6506–14.
90. Force A, Cresko WA, Pickett FB, Proulx SR, Amemiya C, Lynch M. The origin of subfunctions and modular gene regulation. Genetics. 2005;170:433–46.
91. Baquero F. From pieces to patterns: evolutionary engineering in bacterial pathogens. Nat Rev Microbiol. 2004;2:510–8.
92. Rodríguez I, Novais Â, Lira F, Valverde A, Curião T, Martínez JL, Baquero F, Cantón R, Coque TM. Antibiotic-resistant Klebsiella pneumoniae and Escherichia coli high-risk clones and an IncFII(k) mosaic plasmid hosting Tn1 (blaTEM-4) in isolates from 1990 to 2004. Antimicrob Agents Chemother. 2015;59:2904–8.
93. Partridge SR, Hall RM. Complex multiple antibiotic and mercury resistance region derived from the r-det of NR1 (R100). Antimicrob Agents Chemother. 2004;48:4250–5.
94. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol Rev. 2011;35:820–55.
95. Partridge SR, Iredell JR. Genetic contexts of blaNDM-1. Antimicrob Agents Chemother. 2012;56:6065–7.
96. Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. FEMS Microbiol Rev. 2011;35:912–35.
97. Jackson RW, Vinatzer B, Arnold DL, Dorus S, Murillo J. The influence of the accessory genome on bacterial pathogen evolution. Mob Genet Elem. 2011;1:55–65.
98. Baquero F, Tobes R. Bloody coli: a gene cocktail in Escherichia coli O104:H4. MBio. 2013;4:e00066–13.
99. Croucher NJ, Coupland PG, Stevenson AE, Callendrello A, Bentley SD, Hanage WP. Diversification of bacterial genome content through distinct mechanisms over different timescales. Nat Commun. 2014;5:5471.
100. Petty NK, Ben Zakour NL, Stanton-Cookm M, Skippington E, Totsika M, Forde BM, Phan MD, Gomes Moriel D, Peters KM, Davies M, Rogers BA, Dougan G, Rodriguez-Baño J, Pascual A, Pitout JD, Upton M, Paterson DL, Walsh TR, Schembri MA, Beatson SA. Global dissemination of a multidrug resistant Escherichia coli clone. Proc Natl Acad Sci U S A. 2014;111:5694–9.
101. Potron A, Poirel L, Nordmann P. Derepressed transfer properties leading to the efficient spread of the plasmid encoding carbapenemase OXA-48. Antimicrob Agents Chemother. 2014;58:467–71.
102. Souza V, Eguiarte LE. Bacteria gone native vs. bacteria gone awry? Plasmidic transfer and bacterial evolution. Proc Natl Acad Sci U S A. 1997;94:5501–3.
103. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 2005;63:219–28.
104. Alvarado A, Garcillán-Barcia MP, de la Cruz F. A degenerate primer MOB typing (DPMT) method to classify gamma-proteobacterial plasmids in clinical and environmental settings. PLoS ONE. 2012;7, e40438.
105. Lanza VF, de Toro M, Garcillán-Barcia MP, Mora A, Blanco J, Coque TM, de la Cruz F. Plasmid flux in *Escherichia coli* ST131 sublineages, analyzed by plasmid constellation network (PLACNET), a new method for plasmid reconstruction from whole genome sequences. PLoS Genet. 2014;10, e1004766.
106. Skippington E, Ragan MA. Lateral genetic transfer and the construction of genetic exchange communities. FEMS Microbiol Rev. 2011;35:707–35.
107. Lanza VF, Tedim AP, Martínez JL, Baquero F, Coque TM. The Plasmidome of Firmicutes: Impact on the emergence and the spread of resistance to antimicrobials. Microbiol Spectr. 2015;3:PLAS-0039-2014.
108. Bennett PM. Genome plasticity: insertion sequence elements, transposons and integrons, and DNA rearrangement. Methods Mol Biol. 2004;266:71–113.
109. Rice LB. Association of different mobile elements to generate novel integrative elements. Cell Mol Life Sci. 2002;59:2023–32.
110. Liebert CA, Hall RM, Summers AO. Transposon Tn21, flagship of the floating genome. Microbiol Mol Biol Rev. 1999;63:507–22.

111. Rowe-Magnus AD, Davies J, Mazel D. Impact of integrons and transposons on the evolution of resistance and virulence. Curr Top Microbiol Immunol. 2002;264:167–88.
112. Fluit AC, Schmitz FJ. Resistance integrons and super-integrons. Clin Microbiol Infect. 2004;10:272–88.
113. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-ß-lactamases: the quiet before the storm? Clin Microbiol Rev. 2005;18:306–25.
114. Oliver A, Coque TM, Alonso D, Valverde A, Baquero F, Cantón R. CTX-M-10 linked to a phage-related element is widely disseminated among Enterobacteriaceae in a Spanish hospital. Antimicrob Agents Chemother. 2005;49:1567–71.
115. Pozzi G, Iannelli F, Oggioni MR, Santagati M, Stefani S. Genetic elements carrying macrolide efflux genes in streptococci. Curr Drug Targets Infect Disord. 2004;4:203–6.
116. Shousha A, Awaiwanont N, Sofka D, Smulders FJ, Paulsen P, Szostak MP, Humphrey T, Hilbert F. Bacteriophages isolated from chicken meat and the horizontal transfer of antimicrobial resistance genes. Appl Environ Microbiol. 2015;81:4600–6.
117. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186:1518–30.
118. Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, Coque TM, Cantón R, Baquero F, Murray BE, del Campo R, Willems RJ. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol. 2006;44:2220–8.
119. Cohen ML. Antimicrobial resistance: prognosis for public health. Trends Microbiol. 1994;2:422–5.
120. Shurin JB, Amarasekare P, Chase JM, Holt RD, Hoopes MF, Leibold MA. Alternative stable states and regional community structure. J Theor Biol. 2004;227:359–68.
121. Jutersek B, Baraniak A, Zohar-Cretnik T, Storman A, Sadowy E, Gniadkowski M. Complex endemic situation regarding extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in a hospital in Slovenia. Microb Drug Resist. 2003;9 Suppl 1:S25–33.
122. Baquero F, Coque TM, Cantón R. Allodemics. Lancet Infect Dis. 2002;2:591–2.
123. Cantón R, Coque TM, Baquero F. Multi-resistant Gram-negative bacilli: from epidemics to endemics. Curr Opin Infect Dis. 2003;16:315–25.
124. Friesen ML, Saxer G, Travisano M, Doebeli M. Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in Escherichia coli. Evolution. 2004;58:245–60.
125. Shapiro JA. Natural genetic engineering in evolution. Genetica. 1992;86:99–111.
126. Mittler JE, Lenski RE. New data on excisions of Mu from *E. coli* MCS2 cast doubt on directed mutation hypothesis. Nature. 1990;344:173–5.
127. Lenski RE, Sniegowski PD. "Adaptive mutation": the debate goes on. Science. 1995;269:285–8.
128. Higashitani N, Higashitani A, Horiuchi K. SOS induction in *Escherichia coli* by single-stranded DNA of mutant filamentous phage: monitoring by cleavage of LexA repressor. J Bacteriol. 1995;177:3610–2.
129. Thomas A, Tocher J, Edwards DI. Electrochemical characteristics of five quinolone drugs and their effect on DNA damage and repair in *Escherichia coli*. J Antimicrob Chemother. 1990;25:733–44.
130. DeMarini DM, Lawrence BK. Prophage induction by DNA topoisomerase II poisons and reactive-oxygen species: role of DNA breaks. Mutat Res. 1992;267:1–17.
131. Beabout K, Hammerstrom TG, Wang TT, Bhatty M, Christie PJ, Saxer G, Shamoo Y. Rampant parasexuality evolves in a hospital pathogen during antibiotic selection. Mol Biol Evol. 2015;32:2585–97.
132. Ubeda C, Maiques E, Knecht E, Lasa I, Novick RP, Penades JR. Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. Mol Microbiol. 2005;56:836–44.
133. Zhang X, McDaniel AD, Wolf LE, Keusch GT, Waldor MK, Acheson DW. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J Infect Dis. 2000;181:664–70.
134. Goerke C, y Papenberg SM, Dasbach S, Dietz K, Ziebach R, Kahl BC, Wolz C. Increased frequency of genomic alterations in *Staphylococcus aureus* during chronic infection is in part due to phage mobilization. J Infect Dis. 2004;189:724–34.
135. Goerke C, Koller J, Wolz C. Ciprofloxacin and trimethoprim cause phage induction and virulence modulation in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2006;50:171–7.
136. Vakulenko SB, Golemi D, Geryk B, Suvorov M, Knox JR, Mobashery S, Lerner SA. Mutational replacement of Leu-293 in the class C *Enterobacter cloacae* P99 beta-lactamase confers increased MIC of cefepime. Antimicrob Agents Chemother. 2002;46:1966–70.
137. Baldwin JM. A new factor in evolution. Am Naturalist. 1896;30:441–51.
138. Aertsen A, Michiels CW. Diversify or die: generation of diversity in response to stress. Crit Rev Microbiol. 2005;31:69–78.
139. Smith JM, Hoekstra R. Polymorphism in a varied environment: how robust are the models? Genet Res. 1980;35:45–57.
140. Baquero F, Negri MC. Selective compartments for resistant microorganisms in antibiotic gradients. Bioessays. 1997;19:731–6.
141. Negri MC, Lipsitch M, Blázquez J, Levin BR, Baquero F. Concentration-dependent selection of small phenotypic differences in TEM-beta-lactamase-mediated antibiotic resistance. Antimicrob Agents Chemother. 2000;44:2485–91.
142. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol. 2014;12:465–78.
143. Hughes D, Andersson DI. Selection of resistance at lethal and non-lethal antibiotic concentrations. Curr Opin Microbiol. 2012;15:555–60.
144. Drlica K. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother. 2003;52:11–7.
145. Alekshun MN, Levy SB. Commensals upon us. Biochem Pharmacol. 2006;71:893–900.
146. Enne VI, Livermore DM, Stephens P, Hall LM. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet. 2001;357:1325–8.
147. Baquero F. Genetic hyper-codes and multidimensional Darwinism: replication modes and codes in evolutionary individuals of the bacterial world. In: Trueba G, editor. Why does evolution matter? . Newcastle upon Tyne, UK: Cambridge Scholars Publishing; 2014. p. 165–80.
148. Brosius J, Gould SJ. On genonomenclature: a comprehensive (and respectful) taxonomy for pseudogenes and other "junk DNA". Proc Natl Acad Sci U S A. 1992;89:10706–10.
149. Baquero F, Tedim AP, Coque TM. Antibiotic resistance shaping multi-level population biology of bacteria. Front Microbiol. 2013;6:4:15.
150. Stebbins GL, Hartl DL. Comparative evolution: latent potentials for anagenetic advance. Proc Natl Acad Sci U S A. 1998;85:5141–5.
151. Piddock LJ. Multidrug-resistance efflux pumps—not just for resistance. Nat Rev Microbiol. 2006;4:629–36.
152. Andersson DI. The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol. 2006;9:461–5.

153. Levin BR, Lipsitch M, Perrot V, Schrag S, Antia R, Simonsen L, Walker NM, Stewart FM. The population genetics of antibiotic resistance. Clin Infect Dis. 1997;24 Suppl 1:S9–16.
154. Lenski RE, Souza V, Duong LP, Phan QG, Nguyen TN, Bertrand KP. Epistatic effects of promoter and repressor functions of the Tn10 tetracycline-resistance operon of the fitness of *Escherichia coli*. Mol Ecol. 1994;3:127–35.
155. Blot M, Hauer B, Monnet G. The Tn*5* bleomycin resistance gene confers improved survival and growth advantage on *Escherichia coli*. Mol Gen Genet. 1994;242:595–601.
156. Davies J. Another look at antibiotic resistance. J Gen Microbiol. 1992;138:1553–9.
157. Coque TM, Willems RJ, Fortún J, Top J, Diz S, Loza E, Cantón R, Baquero F. Population structure of *Enterococcus faecium* causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance? Antimicrob Agents Chemother. 2005;49:2693–700.
158. Willems RJ, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJ. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis. 2005;11:82182–8.
159. del Campo R, Cafini F, Morosini MI, Fenoll A, Linares J, Alou L, Sevillano D, Cantón R, Prieto J, Baquero F, Spanish Pneumococcal Network (G3/103). Combinations of PBPs and MurM protein variants in early and contemporary high-level penicillin-resistant Streptococcus pneumoniae isolates in Spain. J Antimicrob Chemother. 2006;57:983–6.
160. Ruiz-Garbajosa P, Cantón R, Pintado V, Coque TM, Willems R, Baquero F, del Campo R. Genetic and phenotypic differences among *Enterococcus faecalis* clones from intestinal colonisation and invasive disease. Clin Microbiol Infect. 2006;12:1193–8.
161. Gomes AR, Westh H, de Lencastre H. Origins and evolution of methicillin-resistant *Staphylococcus aureus* clonal lineages. Antimicrob Agents Chemother. 2006;50:3237–44.
162. Martiny JB, Bohannan BJ, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M, Krumins JA, Kuske CR, Morin PJ, Naeem S, Ovreas L, Reysenbach AL, Smith VH, Staley JT. Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol. 2006;4:102–12.
163. Jain R, Rivera MC, Moore JE, Lake JA. Horizontal gene transfer accelerates genome innovation and evolution. Mol Biol Evol. 2003;20:1598–602.
164. Madan Babu M, Teichmann SA, Aravind L. Evolutionary dynamics of prokaryotic transcriptional regulatory networks. J Mol Biol. 2006;358:614–33.
165. Lanza VF, Tedimm AP, Martínez JL, Baquero F, Coque TM. The plasmidome of Firmicutes: Impact on the emergence and the spread of resistance to antimicrobials. Microbiol Spectr. 2015;3:PLAS-0039-2014.
166. Stegger M, Wirth T, Andersen PS, Skov RL, De Grassi A, Simões PM, Tristan A, Petersen A, Aziz M, Kiil K, Cirković I, Udo EE, del Campo R, Vuopio-Varkila J, Ahmad N, Tokajian S, Peters G, Schaumburg F, Olsson-Liljequist B, Givskov M, Driebe EE, Vigh HE, Shittu A, Ramdani-Bougessa N, Rasigade JP, Price LB, Vandenesch F, Larsen AR, Laurent F. Origin and evolution of European community-acquired methicillin-resistant *Staphylococcus aureus*. MBio. 2014;5:e01044–14.
167. Xue H, Cordero OX, Camas FM, Trimble W, Meyer F, Guglielmini J, Rocha EP, Polz MF. Eco-evolutionary dynamics of episomes among ecologically cohesive bacterial populations. MBio. 2015;6:e00552–15.
168. Novais A, Viana D, Baquero F, Martínez-Botas J, Cantón R, Coque TM. Contribution of IncFII and broad-host IncA/C and IncN plasmids to the local expansion and diversification of phylogroup B2 Escherichia coli ST131 clones carrying blaCTX-M-15 and qnrS1 genes. Antimicrob Agents Chemother. 2012;56:2763–6.
169. Baquero F, Coque TM, de la Cruz F. Ecology and evolution as targets: the need for novel eco-evo drugs and strategies to fight antibiotic resistance. Antimicrobial Agents Chemother. 2011;55:3649–60.
170. Baquero F, Coque TM, Cantón R. Counteracting antibiotic resistance: breaking barriers among antibacterial strategies. Expert Opin Ther Targets. 2014;18:851–61.
171. Martínez JL, Baquero F. Emergence and spread of antibiotic resistance: setting a parameter space. Upsala J Medical Sci. 2014;119:68–77.
172. von Mering C, Zdobnov EM, Tsoka S, Ciccarelli FD, Pereira-Leal JB, Ouzounis CA, Bork P. Genome evolution reveals biochemical networks and functional modules. Proc Natl Acad Sci U S A. 2003;100:15428–33.
173. Ettema T, van der Oost J, Huynen M. Modularity in the gain and loss of genes: applications for function prediction. Trends Genet. 2001;17:485–7.
174. Lenski RE, Ofria C, Pennock RT, Adami C. The evolutionary origin of complex features. Nature. 2003;423:139–44.
175. Petri R, Schmidt-Dannert C. Dealing with complexity: evolutionary engineering and genome shuffling. Curr Opin Biotechnol. 2004;15:298–304.
176. Shapiro JA. A 21st century view of evolution: genome system architecture, repetitive DNA, and natural genetic engineering. Gene. 2005;345:91–100.
177. Pepper JW. The evolution of evolvability in genetic linkage patterns. Biosystems. 2003;69:115–26.
178. Cantón R, Morosini I, Loza E, Morosini I, Baquero F. Mecanismos de multirresistencia e importancia actual en microorganismos grampositivos y gramnegativos. Enf Infec Microbiol Clin. 2006;5:3–16.
179. Walsh TR. Combinatorial genetic evolution of multiresistance. Curr Opin Microbiol. 2006;9:476–82.
180. Rogozin IB, Makarova KS, Wolf YI, Koonin EV. Computational approaches for the analysis of gene neighbourhoods in prokaryotic genomes. Brief Bioinform. 2004;5:131–49.
181. Toussaint A, Merlin C. Mobile elements as a combination of functional modules. Plasmid. 2002;47:26–35.
182. de Lorenzo V. From the selfish gene to selfish metabolism: revisiting the central dogma. Bioessays. 2014;36:226–35.
183. Baquero F, Lanza VF, Cantón R, Coque TM. Public health evolutionary biology of antimicrobial resistance: priorities for intervention. Evol Appl. 2015;8:223–39.
184. Brent R, Bruck J. Can computers help to understand biology? Nature. 2006;440:416–7.
185. Stadler BM, Stadler PF, Wagner GP, Fontana W. The topology of the possible: formal spaces underlying patterns of evolutionary change. J Theor Biol. 2001;213:241–74.
186. Andrianantoandro E, Basu S, Karig DK, Weiss R. Synthetic biology: new engineering rules for an emerging discipline. Mol Syst Biol. 2006;2:2006–28.
187. Danchin A. The bag or the spindle: the cell factory at the time of system's biology. Microb Cell Fact. 2004;3:13–4.
188. Campos, M., Llorens, C., Sempere, J.M., Futami, R., Rodríguez, I., Carrasco, P., Capilla, R., Latorre, A., Coque, T.M., Moya, A., Baquero, F. A membrane computing simulator of trans-hierarchical antibiotic resistance evolution dynamics in nested ecological compartments (ARES). Biol Direct. 2015;10:41.

Pharmacology of Drug Resistance

3

Elizabeth R. Andrews and Angela D. M. Kashuba

1 Introduction

Pharmacologic parameters influencing antimicrobial resistance can be drug-specific, organism-specific, or host-specific. A basic understanding of antimicrobial pharmacokinetics (PK) and pharmacodynamics (PD) informs how resistance develops, and allows for the selection of optimal dosing strategies. Important pharmacologic parameters influencing antimicrobial resistance can be grouped into three major categories: drug specific, organism specific, and host specific. Since PK–PD relationships are generally developed in preclinical model systems and extrapolated to clinical patients, this chapter reviews the available preclinical and clinical data on antimicrobial PK–PD relationships, and assesses how they can be used to effectively treat infections and epidemiologically conserve active antimicrobials.

2 Drug-Specific Factors

The PK/PD of most antimicrobials have been well characterized at the level of the population. A theoretical concentration versus time graph for a single oral dose of an antibiotic is shown in Fig. 3.1. Knowledge of this profile, together with an understanding of the mechanism of action of the antimicrobial, enables clinicians to make informed decisions about optimal dosing strategies to increase efficacy and decrease the likelihood of resistance. Generally, pharmacokinetic parameters that optimally define antibiotic efficacy (e.g., peak concentration, trough concentration, area under the concentration–time curve (AUC)) are related to the minimum inhibitory concentration (MIC: the lowest concentration of antimicrobial that will inhibit further growth) [1, 2]. Clinically, MIC "breakpoints" that optimize efficacy and minimize the development of drug resistance mutations are widely used. The appropriate selection and maintenance of MIC breakpoints depends on the following data: MIC distributions and wild-type cutoffs, in vitro resistance markers, PK/PD data from animal models and human studies, and outcome data from clinical studies. In vitro PD data are most frequently used in the selection of MIC breakpoints [3]. However, the mutant prevention concentration (MPC) has also been evaluated [4, 5]. First described in 1999 [6], the MPC is defined as the drug concentration required to prevent emergence of all single step mutations in a population of at least 10^{10} bacterial cells [4]. The range between the MIC and the MPC has been called the mutant selection window. Within this window, the antibiotic concentration is sufficient to provide selective pressure on microorganism growth, but insufficient to completely inhibit growth of the microorganism. Although utilized in the research setting, the MPC is infrequently used in the clinical setting in large part due to a high degree of inter-patient variability [4]. Although the following subsections relate to antibiotics, the concepts described in this section also apply to these other antimicrobial agents.

2.1 Concentration-Dependent and Time-Dependent Antibiotic Effects

Antibiotics can be broadly characterized as concentration dependent, time dependent, or both. For concentration-dependent antibiotics, the PK parameter of interest is the maximum concentration achieved in plasma after a dose (Cmax). Therefore, the Cmax:MIC ratio is relevant to evaluating efficacy of these antibiotics [7]. Changes to the dose, rather than the dosing interval, produce changes in Cmax.

E.R. Andrews, Pharm.D.
Clinical Scientist, G1 Therapeutics,
Research Triangle Park, NC, USA

A.D.M. Kashuba, BSc.Phm, Pharm.D. (✉)
Division of Pharmacotherapy and Experimental Therapeutics,
Eshelman School of Pharmacy, University of North Carolina at
Chapel Hill, 1094 Genetic Medicine Building, CB# 7361,
120 Mason Farm Road, Chapel Hill, NC, USA
e-mail: akashuba@unc.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_3

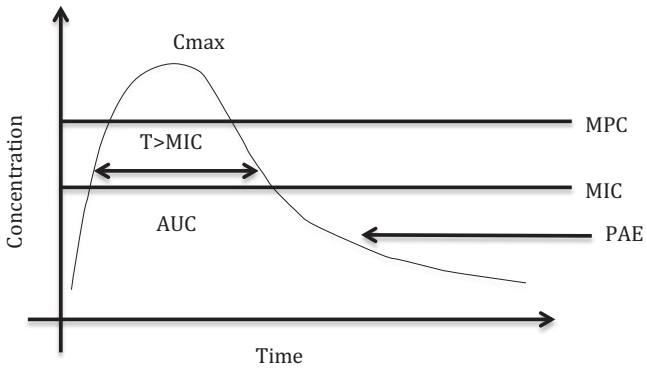


Fig. 3.1 A theoretical concentration versus time graph for a single oral dose of an antibiotic

Examples of concentration-dependent antibiotics include aminoglycosides, daptomycin, and colistin [8]. The concentration-dependent effects can be well described by dose fractionization experiments [9]. For example, Kim et al. designed an in vitro model to investigate the differences between once daily (QD) high dose and three times daily (TID) lower dose gentamicin therapy for the treatment of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) [10]. The model determined that QD dosing achieved approximately a 2.7-fold higher Cmax:MIC than TID dosing. Although both dosing regimens were effective for the first 8 h of therapy, the TID dosing schedule showed regrowth after 24 h in the YSSA1 strain and 8 h in the YSSA2 strain. No regrowth was seen for the duration of the experiment in the QD dosing schedule. Similar results were seen in an in vivo neutropenic murine thigh infection study by Daikos et al. [11]. In this study, mice were inoculated with *Pseudomonas aeruginosa* and treated with either a high initial dose of netilmicin or a lower dose every 2 h for five doses. After 24 h, all of the mice had received the same cumulative dose of netilmicin, and it was demonstrated that mice treated with a high initial dose had 80–90 % fewer surviving CFU [11]. Similar data exist in the literature for daptomycin [12].

The parameter of interest in the evaluation of time-dependent antibiotics is length of time that the plasma concentration is above a certain threshold, typically MIC (time above MIC, or $T>$MIC). To prevent the development of resistance, dosing schedules for these antibiotics aim to maximize the time that the plasma concentration exceeds the MIC. Changes to the $T>$MIC are achieved by changing the dosing interval. Beta lactam antibiotics exhibit time-dependent action [14]: these include the penicillins, cephalosporins, carbapenems, and monobactams. Preclinical data can be used to determine appropriate cutoffs for $T>$MIC [14]. For example, Knudsen et al. evaluated $T>$MIC targets for penicillin in the treatment of *Streptococcus pneumonia* [15]. Time-kill curves were determined in vitro for three bacterial strains as well as a mixed culture at 10^6 and 10^7 CFU/mL at penicillin concentrations of 4 and 16 times the MIC as well as 16 μg/mL. Penicillin was bactericidal to similar extents in individual strains and in the mixed culture. In vivo studies were also conducted, using mouse thigh infection, mouse peritonitis, and rabbit tissue cage models. Using pooled data from the mouse thigh and peritonitis models, a $T>$MIC of ≥40 % was found to be sufficient to eradicate bacteria. Additionally, in all three models, a $T>$MIC of <40 % allowed for selection of resistant bacteria, as demonstrated by regrowth after 12 h in the rabbit model, and changing ratios of susceptible to resistant strains in both mouse models [15]. Similar data are available for ceftizoxime [16].

A growing body of evidence suggests that many antibiotics are neither exclusively time dependent nor concentration dependent. Efficacy of these antibiotics is quantified using the ratio of the area under the concentration–time curve (AUC) to the MIC. The AUC is a parameter that measures total plasma exposure over a dosing interval. Likewise, the AUC/MPC ratio can be evaluated to predict the development of resistance [17]. Examples of these antibiotics include fluoroquinolones, macrolides, tetracyclines, and vancomycin [8].

In 2006, Olofsson et al. published a frequently cited in vitro pharmacokinetic study to demonstrate the relationship between ciprofloxacin (a fluoroquinolone) exposure and the development of resistance. In this study, two strains of *E. coli* were grown in the presence of 4×, 8×, 16×, 32×, and 64× the MIC of ciprofloxacin. The researchers chose to use the MPC to describe the results. They demonstrated that neither maximizing $T>$MPC nor Cmax:MPC were adequate to prevent the development of resistance. Only by optimizing the AUC/MPC to ≥22 could resistance be prevented in 100 % of the strains. For ciprofloxacin, an AUC/MPC ratio ≥22 was the single best predictor in preventing adaptive resistance. The researchers speculate that this is likely achievable for urinary tract infections but, due to high protein binding, may be more difficult to achieve for bloodstream infections [17].

2.2 Post-antibiotic Effect

Post-antibiotic effect (PAE) refers to an antibiotic's ability to continue to inhibit bacterial growth after its serum concentration has dropped below the MIC. The effects of this phenomenon vary widely, and are based both on the infecting organism and the antibiotic [8]. With exceptions, most antibiotics exhibit a PAE with gram-positive infections, and minimal PAE with gram-negative infections [8]. Since the vast majority of PAE data comes from in vitro, *r*ather than in vivo experimentation, the clinical applicability of PAE is still controversial [18].

An example of the clinical application of PAE comes from aminoglycosides. Aminoglycosides exhibit a post-antibiotic effect in the treatment of gram-negative bacilli and *Staphylococcus aureus* [13]. Once daily dosing regimens of aminoglycosides take advantage of the theoretical PAE. By achieving a high Cmax with each dose, the drug concentrations are allowed to fall to low concentrations (less than the MIC) before re-dosing such that PAE could occur before redosing [10]. In the study previously discussed by Kim et al., resistance to gentamicin did not develop during the sub-MIC portion of the dosing interval with daily dosing [10].

2.3 Combination Therapy and PK Drug Interactions

The use of combination therapy is effective in preventing the development of antimicrobial resistance. Combination therapy can be synergistic, additive, or antagonistic. Synergy is defined as an interaction between medications that produces an effect that is greater than the sum of the effects of each of the two medications in isolation. In a research setting, synergy can be more strictly defined as 25 % of the doses of two drugs producing the same antimicrobial effect as a single agent at 100 % of the dose [19]. In evaluating antimicrobial resistance, synergy is important to enhance the PK/PD of an active drug in such a way that the likelihood of developing resistance is significantly decreased [4].

Trimethoprim/sulfamethoxazole (Bactrim®; AR Scientific) is an example of two antimicrobials co-formulated for synergy. Trimethoprim and sulfamethoxazole exert their antibacterial action by acting on successive steps in the folic acid synthesis pathway and interfere with bacterial DNA synthesis [20]. In a 1969 study, Bushby demonstrated that the addition of sulfamethoxazole potentiates the action of trimethoprim in *S. faecalis* and *E. coli*, as demonstrated by an increased percentage of control, as evidenced by a reduced MIC, the magnitude of which is contingent upon the ratio of trimethoprim to sulfamethoxazole, when high inoculums of bacteria were exposed to a combination of trimethoprim and sulfamethoxazole versus trimethoprim alone [21].

Antimicrobials are also used in combination to overcome mechanical mechanisms of resistance, as can be seen in infective endocarditis (IE) [22]. Therapeutic guidelines for IE in patients with prosthetic heart valves recommend the use of rifampin [23] to disrupt biofilms and allow concomitant antibiotics access to the causative bacteria [24]. Similarly, gentamicin can be included in gram-positive IE infections to make bacterial cell walls permeable to intracellularly active agents such as linezolid [25]. Luther et al. recently demonstrated a statistically significant increase in bactericidal activity when gentamicin was added to a daptomycin regimen in vitro ($p = 0.033$) [22].

Another example of a beneficial PK drug interaction is the combination of beta lactam antibiotics with beta lactamase inhibitors. Bacteria producing beta lactamase hydrolyze the beta lactam ring of antibiotics such as amoxicillin and piperacillin, rendering them ineffective. Beta lactamase inhibitors such as clavulanate and tazobactam inhibit antibiotic degradation by beta lactamase by becoming the target for beta lactamase hydrolysis in the place of the active beta lactam [26]. Mentec et al. demonstrated that the addition of tazobactam to piperacillin lowered the MIC of a beta lactamase producing *Klebsiella pneumonia* sufficiently to be considered susceptible [27]. Other beta lactam/beta lactamase inhibitor combinations include amoxicillin/clavulanate (Augmentin®; GlaxoSmithKline), piperacillin/tazobactam (Zosyn®; Pfizer), and ampicillin/sulbactam (Unasyn®; Pfizer).

Drug–drug interactions, by inhibiting a drug-metabolizing enzyme or efflux transporter activity, and thereby increasing drug exposure, can also be used to minimize the risk of resistance development. One example is in the area of HIV treatment. The addition of small doses of the CYP3A and p-glycoprotein inhibitor ritonavir or cobicistat to another active protease inhibitor such as lopinavir, darunavir, atazanavir, saquinavir, or indinavir can increase the latter's Cmax and AUC up to 1622 % [28, 29]. Subtherapeutic concentrations, and the risk of developing drug resistance, are less likely with the addition of these pharmacokinetic enhancers [28].

Conversely, certain detrimental drug interactions have been shown to promote the development of resistance. One example is the combination of rifampin with protease inhibitors. Rifampin is a potent inducer of the drug-metabolizing enzyme CYP3A4 and increases the amount of CYP3A4 in the intestine and liver. This, in turn, increases the rate and extent of metabolism of protease inhibitors. This enhanced metabolism results in a decrease in protease inhibitor AUC and Cmin by up to 87 % [30], thus decreasing efficacy and increasing the likelihood of the development of resistance. Because of this interaction, the HIV treatment guidelines from the Department of Health and Human Services recommend substituting rifabutin for rifampin, which causes less CYP3A induction [31].

3 Organism-Specific Factors

3.1 Resistance at Baseline

The US Centers for Disease Control and Prevention (CDC) estimates that each year, two million illnesses and 23,000 deaths are caused by antibiotic-resistant bacteria nationally [32]. The trend toward increasing MICs represents a threat to the treatment and prevention of bacterial infections. Therefore, the US Department of Health and Human

Services (DHHS) has developed a five-point plan for combating antimicrobial resistance, which includes slowing the emergence of resistant bacteria and preventing their spread, strengthening surveillance efforts to combat resistance, advancing the development and use of diagnostic tests for resistance, accelerating research for the development of new antimicrobials, and improving international collaboration for prevention, surveillance, control and research and development [33]. The report was released in 2014, and most national targets to assess the efficacy of the provisions outlined will be measured in 2020. Some of the 2020 goals include reducing *Clostridium difficile* infections by 50 % and reducing multidrug-resistant *Pseudomonas* infections by 35 %. The plan also states that two New Drug Applications (NDAs) for antibiotics will be submitted to the FDA by 2018 [33].

3.2 Fitness

Fitness is used to describe the pathogenicity of a microorganism. Pathogens that are more fit are more likely to develop acquired resistance to antimicrobials [14]. In the presence of antimicrobials, resistant subpopulations may be unmasked and allowed to proliferate [34]. Mutations that confer antimicrobial resistance often decrease fitness. This loss of fitness still leaves mutated microbes at a distinct evolutionary advantage versus wild type (WT) when exposed to antimicrobials but is considered a significant disadvantage when compared to wild type in the absence of antimicrobials. This has been seen in both bacterial and viral infections. Giraud et al. demonstrated that fluoroquinolone resistance (MIC = 16 μg/mL) increases the generation time of *Salmonella enterica* by twofold [35]. Additionally, the M184I/V mutation that confers resistance to the nucleoside analogue reverse transcriptase inhibitors (NRTIs) lamivudine and emtricitabine, results in decreased replication capacity and fitness compared to WT virus [36].

3.3 Microbial Load

Like fitness, microbial load or inoculum size impacts the development of resistance [14]. In a study using the murine thigh model of *Pseudomonas aeruginosa*, Jumbe and colleagues demonstrated that a larger inoculum size required a larger dose of levofloxacin to adequately eradicate the infection [37]. When the inoculum size was increased by a factor of 10 (from 10^7 to 10^8 CFU/g), approximately 2–6 times the antibiotic exposure (AUC/MIC) was required to achieve the same degree of efficacy. This is due, at least in theory, to the increased probability that a resistant subpopulation will be present in infections of increasing burden. In a clinical setting, treating an infection with a high bacterial load with a standard dose of antimicrobial may select for resistance by providing inadequate exposure [37].

3.4 Acquired Resistance Mutations

In the presence of antimicrobials, microbes will strive to develop specific mutations that enable their continued survival and proliferation. One example of this is the enhanced expression of efflux transporters in bacteria when exposed to intracellularly active antibiotics. This mode of resistance development has been demonstrated for chloramphenicol, tetracyclines, fluoroquinolones, and aminoglycosides, among others [38]. In the case of tetracyclines, the genes associated with efflux are typically located on transposons or plasmids and are inducible by low tetracycline concentrations [39]. These mutations decrease the exposure to the antibiotic (Cmax, AUC) at the drug's site of action, thus rendering the bacteria resistant to the antibiotic in question. Similarly, alterations in a drug's site of action can cause resistance. For example, HIV can develop resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) by altering the allosteric binding site on the reverse transcriptase enzyme where NNRTIs exert their action [40]. These mutations are primarily driven by exposure to the drug in insufficient quantities to completely inhibit viral replication. Once the mutation has developed, the virus is resistant to the drug in question at a concentration that would have otherwise been sufficient [40].

4 Host-Specific Factors

4.1 Penetration

Host-specific factors can have a profound influence on the PK/PD of antimicrobials and the development of resistance. One such factor is the ability of the drug to cross anatomic barriers.

For example, the blood brain barrier (BBB) contains tight junctions and efflux transporters that limit drug exposure. In a systematic review by Stam et al., data from several studies were pooled to reveal differences in the resistance profiles between HIV in the blood and HIV in the cerebral spinal fluid (CSF). Frequently, resistance mutations develop in the blood that are not seen in the CSF. The authors speculate that this is due to poor penetration of antiretrovirals into the CSF. Without achieving an adequate concentration to exert selective pressure in the CSF, resistance mutations are unlikely to develop in HIV sequestered there [41].

4.2 Metabolism

Drug-metabolizing enzymes are under genetic and environmental control. Genetic polymorphisms can increase or decrease drug-metabolizing enzyme activity. In the case of a drug that is administered in its active form, an increase in metabolism would decrease the amount of active drug in the body and could theoretically lead to resistance. For a prodrug, the opposite is true. For example, valacyclovir is an antiviral prodrug that requires enzymatic metabolism to acyclovir by valacyclovirase in order to exert its action on the herpes simplex virus (HSV). Enzymatic polymorphisms that lead to decreased valacyclovirase activity have demonstrated significantly decreased availability of active drug [42]. This decrease in activity has the theoretical potential to increase antimicrobial resistance, although no clinical cases have been noted.

4.3 Altered Physiology

Alterations to the host's physiology, as seen in the cases of sepsis, burns, obesity, and pregnancy, may also influence the PK/PD of antimicrobials. In these cases, the volume of distribution (Vd) is increased, resulting in decreased plasma concentrations [43, 44]. Because decreased plasma concentrations are associated with the development of resistance, it is reasonable to assume that patients with an increased Vd are at an increased risk of developing resistance, and dose increases should be considered.

In the case of burns, it has been demonstrated that PK/PD parameters are often altered and highly variable [45]. These changes include an increased volume of distribution, hypovolemia, hypoalbuminemia, and changes in glomerular filtration rate [45]. It has been recommended that burn patients receive increased doses of aminoglycosides, beta lactams, and vancomycin in order to achieve therapeutic efficacy targets (Cmax/MIC, AUC/MIC, $T>$MIC) and prevent the development of resistance. In this situation, therapeutic drug monitoring has utility [45]. Ventilator-associated pneumonia (VAP) is a significant cause of morbidity and mortality among these patients and the causative pathogens are often nosocomial in nature and potentially highly resistant to antimicrobials [46].

Similar data regarding the Vd and other PK/PD parameter changes exists in the case of sepsis [47], obesity [48], and pregnancy [49], and it is reasonable to suggest that dosing adjustments are also required in these patients to prevent the development of resistance.

4.4 Protein Binding

Some clinicians and researchers also maintain that it is prudent to understand the protein-unbound concentration of antimicrobials, in addition to the total (protein bound + unbound) concentration that is typically measured for therapeutic drug monitoring. Only the protein unbound fraction of drug in plasma or other parts of the body is available to exert antimicrobial activity. This adds an additional level of complexity to PK/PD considerations because protein concentrations are highly variable between patients and may change with altered pathophysiologic states. As early as 1942 [50], it was observed that drug protein binding directly relates to the activity of antimicrobials. It is reasonable to extend this observation to the development of resistance as well [51].

5 Conclusion

The development of antimicrobial resistance is multifactorial. Critical factors include those that are drug specific, organism specific, and host specific. Important factors to consider include the infecting organism, an appropriate dosing regimen per guideline recommendations, and alterations to guideline recommendations based on individual factors. By understanding the complexities of the exposure–response relationship for antimicrobials, clinicians and researchers can make informed choices to minimize the development of antimicrobial resistance. In the clinical setting, it is essential to consider these factors in order to effectively treat the patient's infection and to epidemiologically conserve active antimicrobials. Moving forward, the proper implementation of antimicrobial stewardship programs, as well as the development of novel antimicrobials, will be critical in treating infections appropriately.

References

1. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48 Suppl 1:5–16.
2. Craig WA. Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. Clin Infect Dis. 1998;26(1):1–10, quiz 11-2.
3. Turnidge J, Paterson DL. Setting and revising antibacterial susceptibility breakpoints. Clin Microbiol Rev. 2007;20(3):391–408, table of contents.
4. Roberts JA, Kruger P, Paterson DL, Lipman J. Antibiotic resistance—what's dosing got to do with it? Crit Care Med. 2008;36(8):2433–40. doi:10.1097/CCM.0b013e318180fe62.
5. Zhao X, Drlica K. Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. Clin Infect Dis. 2001;33 Suppl 3:S147–56.

6. Dong Y, Zhao X, Domagala J, Drlica K. Effect of fluoroquinolone concentration on selection of resistant mutants of mycobacterium bovis BCG and staphylococcus aureus. Antimicrob Agents Chemother. 1999;43(7):1756–8.
7. Drusano GL. Role of pharmacokinetics in the outcome of infections. Antimicrob Agents Chemother. 1988;32(3):289–97.
8. Rybak MJ. Pharmacodynamics: relation to antimicrobial resistance. Am J Infect Control. 2006;34:S38–45.
9. Nielsen EI, Cars O, Friberg LE. Pharmacokinetic/pharmacodynamic (PK/PD) indices of antibiotics predicted by a semimechanistic PKPD model: a step toward model-based dose optimization. Antimicrob Agents Chemother. 2011;55(10):4619–30. doi:10.1128/AAC.00182-11.
10. Kim SW, Lee DG, Choi SM, et al. Once-daily gentamicin administration for community-associated methicillin resistant staphylococcus aureus in an in vitro pharmacodynamic model: preliminary reports for the advantages for optimizing pharmacodynamic index. Yonsei Med J. 2010;51(5):722–7. doi:10.3349/ymj.2010.51.5.722.
11. Daikos GL, Lolans VT, Jackson GG. First-exposure adaptive resistance to aminoglycoside antibiotics in vivo with meaning for optimal clinical use. Antimicrob Agents Chemother. 1991;35(1):117–23.
12. Safdar N, Andes D, Craig WA. In vivo pharmacodynamic activity of daptomycin. Antimicrob Agents Chemother. 2004;48(1):63–8.
13. Gonzalez 3rd LS, Spencer JP. Aminoglycosides: a practical review. Am Fam Physician. 1998;58(8):1811–20.
14. Mouton JW, Ambrose PG, Canton R, et al. Conserving antibiotics for the future: new ways to use old and new drugs from a pharmacokinetic and pharmacodynamic perspective. Drug Resist Updat. 2011;14(2):107–17. doi:10.1016/j.drup.2011.02.005.
15. Knudsen JD, Odenholt I, Erlendsdottir H, et al. Selection of resistant streptococcus pneumoniae during penicillin treatment in vitro and in three animal models. Antimicrob Agents Chemother. 2003;47(8):2499–506.
16. Stearne LE, Goessens WH, Mouton JW, Gyssens IC. Effect of dosing and dosing frequency on the efficacy of ceftizoxime and the emergence of ceftizoxime resistance during the early development of murine abscesses caused by bacteroides fragilis and enterobacter cloacae mixed infection. Antimicrob Agents Chemother. 2007;51(10):3605–11. doi: AAC.01486-06 [pii].
17. Olofsson SK, Marcusson LL, Komp Lindgren P, Hughes D, Cars O. Selection of ciprofloxacin resistance in Escherichia coli in an in vitro kinetic model: Relation between drug exposure and mutant prevention concentration. J Antimicrob Chemother. 2006;57(6):1116–21.
18. Craig WA, Vogelman B. The postantibiotic effect. Ann Intern Med. 1987;106(6):900–2.
19. Khalil H, Chen T, Riffon R, Wang R, Wang Z. Synergy between polyethylenimine and different families of antibiotics against a resistant clinical isolate of pseudomonas aeruginosa. Antimicrob Agents Chemother. 2008;52(5):1635–41. doi:10.1128/AAC.01071-07.
20. Vouloumanou EK, Karageorgopoulos DE, Rafailidis PI, Michalopoulos A, Falagas ME. Trimethoprim/sulfametrole: evaluation of the available clinical and pharmacokinetic/pharmacodynamic evidence. Int J Antimicrob Agents. 2011;38(3):197–216. doi:10.1016/j.ijantimicag.2011.04.016.
21. Bushby SR. Combined antibacterial action in vitro of trimethoprim and sulphonamides. The in vitro nature of synergy. Postgrad Med J. 1969;45(Suppl):10–8.
22. Luther MK, Arvanitis M, Mylonakis E, LaPlante KL. Activity of daptomycin or linezolid in combination with rifampin or gentamicin against biofilm-forming enterococcus faecalis or E. faecium in an in vitro pharmacodynamic model using simulated endocardial vegetations and an in vivo survival assay using galleria mellonella larvae. Antimicrob Agents Chemother. 2014;58(8):4612–20. doi:10.1128/AAC.02790-13.
23. Baddour LM, Wilson WR, Bayer AS, et al. Infective endocarditis: diagnosis, antimicrobial therapy, and management of complications: a statement for healthcare professionals from the committee on rheumatic fever, endocarditis, and kawasaki disease, council on cardiovascular disease in the young, and the councils on clinical cardiology, stroke, and cardiovascular surgery and anesthesia, American Heart Association: Endorsed by the infectious diseases Society of America. Circulation. 2005;111(23):e394–434.
24. Zheng Z, Stewart PS. Penetration of rifampin through staphylococcus epidermidis biofilms. Antimicrob Agents Chemother. 2002;46(3):900–3.
25. Le T, Bayer AS. Combination antibiotic therapy for infective endocarditis. Clin Infect Dis. 2003;36(5):615–21.
26. Worthington RJ, Melander C. Overcoming resistance to beta-lactam antibiotics. J Org Chem. 2013;78(9):4207–13. doi:10.1021/jo400236f.
27. Mentec H, Vallois JM, Bure A, Saleh-Mghir A, Jehl F, Carbon C. Piperacillin, tazobactam, and gentamicin alone or combined in an endocarditis model of infection by a TEM-3-producing strain of klebsiella pneumoniae or its susceptible variant. Antimicrob Agents Chemother. 1992;36(9):1883–9.
28. Zeldin RK, Petruschke RA. Pharmacological and therapeutic properties of ritonavir-boosted protease inhibitor therapy in HIV-infected patients. J Antimicrob Chemother. 2004;53(1):4–9. doi:10.1093/jac/dkh029.
29. Tomaru A, Takeda-Morishita M, Banba H, Takayama K. Analysis of the pharmacokinetic boosting effects of ritonavir on oral bioavailability of drugs in mice. Drug Metab Pharmacokinet. 2013;28(2):144–52.
30. Justesen US, Andersen AB, Klitgaard NA, Brosen K, Gerstoft J, Pedersen C. Pharmacokinetic interaction between rifampin and the combination of indinavir and low-dose ritonavir in HIV-infected patients. Clin Infect Dis. 2004;38(3):426–9.
31. Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivisto KT. Pharmacokinetic interactions with rifampicin: clinical relevance. Clin Pharmacokinet. 2003;42(9):819–50.
32. Centers for Disease Control and Prevention. http://www.cdc.gov/drugresistance/threat-report-2013/. Updated 2014. Accessed 17 Nov. 2014.
33. National strategy for combating antibiotic-resistant bacteria [Internet]. Washington DC: The White House; 2014. Available from: http://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf [cited 20 Nov. 2014].
34. Sevillano D, Aguilar L, Alou L, et al. Effects of antimicrobials on the competitive growth of streptococcus pneumoniae: a pharmacodynamic in vitro model approach to selection of resistant populations. J Antimicrob Chemother. 2006;58(4):794–801.
35. Giraud E, Cloeckaert A, Baucheron S, Mouline C, Chaslus-Dancla E. Fitness cost of fluoroquinolone resistance in salmonella enterica serovar typhimurium. J Med Microbiol. 2003;52(Pt 8):697–703.
36. Hu Z, Kuritzkes DR. Interaction of reverse transcriptase (RT) mutations conferring resistance to lamivudine and etravirine: effects on fitness and RT activity of human immunodeficiency virus type 1. J Virol. 2011;85(21):11309–14. doi:10.1128/JVI.05578-11.
37. Jumbe N, Louie A, Leary R, et al. Application of a mathematical model to prevent in vivo amplification of antibiotic-resistant bacterial populations during therapy. J Clin Invest. 2003;112(2):275–85. doi:10.1172/JCI16814.
38. Poole K. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother. 2005;56(1):20–51.
39. Butaye P, Cloeckaert A, Schwarz S. Mobile genes coding for efflux-mediated antimicrobial resistance in gram-positive and gram-negative bacteria. Int J Antimicrob Agents. 2003;22(3):205–10.
40. Menendez-Arias L, Betancor G, Matamoros T. HIV-1 reverse transcriptase connection subdomain mutations involved in resistance to

approved non-nucleoside inhibitors. Antiviral Res. 2011;92(2):139–49. doi:10.1016/j.antiviral.2011.08.020.
41. Stam AJ, Nijhuis M, van den Bergh WM, Wensing AM. Differential genotypic evolution of HIV-1 quasispecies in cerebrospinal fluid and plasma: a systematic review. AIDS Rev. 2013;15(3):152–61.
42. Lai L, Xu Z, Zhou J, Lee KD, Amidon GL. Molecular basis of prodrug activation by human valacyclovirase, an alpha-amino acid ester hydrolase. J Biol Chem. 2008;283(14):9318–27. doi:10.1074/jbc.M709530200.
43. McKinnon PS, Davis SL. Pharmacokinetic and pharmacodynamic issues in the treatment of bacterial infectious diseases. Eur J Clin Microbiol Infect Dis. 2004;23(4):271–88. doi:10.1007/s10096-004-1107-7.
44. Thomas JK, Forrest A, Bhavnani SM, et al. Pharmacodynamic evaluation of factors associated with the development of bacterial resistance in acutely ill patients during therapy. Antimicrob Agents Chemother. 1998;42(3):521–7.
45. Jamal JA, Economou CJ, Lipman J, Roberts JA. Improving antibiotic dosing in special situations in the ICU: burns, renal replacement therapy and extracorporeal membrane oxygenation. Curr Opin Crit Care. 2012;18(5):460–71. doi:10.1097/MCC.0b013e32835685ad.
46. Huzar TF, Cross JM. Ventilator-associated pneumonia in burn patients: a cause or consequence of critical illness? Expert Rev Respir Med. 2011;5(5):663–73. doi:10.1586/ers.11.61.
47. Pea F. Plasma pharmacokinetics of antimicrobial agents in critically ill patients. Curr Clin Pharmacol. 2013;8(1):5–12.
48. Janson B, Thursky K. Dosing of antibiotics in obesity. Curr Opin Infect Dis. 2012;25(6):634–49. doi:10.1097/QCO.0b013e328359a4c1.
49. Hedstrom S, Martens MG. Antibiotics in pregnancy. Clin Obstet Gynecol. 1993;36(4):886–92.
50. Davis BD. Binding of sulfonamides by plasma proteins. Science. 1942;95(2455):78.
51. Merrikin DJ, Briant J, Rolinson GN. Effect of protein binding on antibiotic activity in vivo. J Antimicrob Chemother. 1983;11(3):233–8.

Drug Development for Drug-Resistant Pathogens

4

Jacques Dumas, Michael J. Pucci, and Greg Moeck

1 Introduction

Antibiotic therapy is arguably the most significant achievement of mankind of the twentieth century. Together with vaccines, it has had a tremendous impact in prolonging life. After many successful years following World War II, antibacterial drugs gradually lost their efficacy because of bacterial resistance. Nowadays, certain multidrug-resistant hospital strains cause infections that are almost impossible to treat and lead to mortality rates not dissimilar to those of the pre-antibiotic era. The "antibiotic crisis" has become a matter of priority for governments, regulators, as well as the medical and scientific communities. This chapter provides a brief history of small-molecule antibiotics from the discovery of penicillin to the present day. The second section assesses medical need and key factors contributing to the business landscape. Finally, the last section reviews the current antibacterial clinical pipeline and most recent drug launches.

2 Brief History of Small-Molecule Antibiotics (1928—Now)

2.1 Beginnings of a Medical Revolution

The discovery of penicillin is arguably one of the most important achievements of the twentieth century [1], and by itself a major contributor to the increase in the average human life span that occurred during the same period. In addition to the serendipity of Alexander Fleming's initial observation of penicillin's antibacterial activity on agar plates in 1928 [2], it was most fortunate that the first major antibiotic class discovered were beta-lactams, combining clinical effectiveness with virtually no mammalian toxicity. Stability and production issues delayed widespread use of penicillin until the 1940s, when efforts intensified spurred on by the need engendered by World War II [3]. In order to improve scale-up production, Howard Florey and colleagues moved to the USA and initiated discussions with a number of pharmaceutical companies including Merck, Lederle, Squibb, and Pfizer. Initial supplies were reserved for the military, but by 1945 production became sufficient for civilian use. The clinical antibiotic era had begun. The early experience gained by the pharmaceutical companies in large scale manufacture via fermentation facilitated antibiotic development work that would follow for a number of years. Although Fleming received most of the credit for the discovery of penicillin, Florey's and Chain's contribution led to the first ever clinical trials in 1941, run at the Radcliffe Infirmary in Oxford [4]. Fleming, Florey, and Chain shared the Nobel Prize for Medicine or Physiology in 1945 "for the discovery of penicillin and its curative effect in various infectious diseases" [5]. Around the time that Fleming was discovering penicillin, Gerhard Domagk of the Bayer Division of IG Farben (a consortium of German dye manufacturers) started testing dyes for antibacterial activity against bacteria [6]. In 1932, KI-730 (subsequently named Prontosil) lacked in vitro activity, but was still tested in mice against a *Streptococcus pyogenes* infection where it showed efficacy. The active part of the molecule was later found to be a *p*-aminobenzene sulfonamide [7] leading to the synthesis of a large number of new sulfonamides. Sulfa drugs had a significant impact during World War II, especially in Europe, and, unlike penicillins, were easy to synthesize. Representatives of this antibiotic class are still in use today.

J. Dumas, Ph.D. (✉)
Tetraphase Pharmaceuticals, Watertown, MA, USA
e-mail: jdumas@tphase.com

M.J. Pucci, Ph.D.
Spero Therapeutics, Cambridge, MA, USA

G. Moeck, Ph.D.
Biology, The Medicines Company, Saint-Laurent, QC, Canada

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_4

2.2 Bacteria Fight Back

Since 1945, many generations of novel penicillins demonstrated improved spectrum, pharmacokinetic profiles, and other desirable properties; this class remains a key treatment option today [8]. As early as 1940, Abraham and Chain discovered bacterial enzymes, subsequently designated beta-lactamases, capable of degrading penicillin even before it was mass produced and used clinically [9]. Clinical resistance in staphylococci was reported by 1947 and by 1952 penicillinase-mediated resistance was present in many US hospitals where penicillin was used to treat infections [10, 11]. This rapid onset of antibiotic resistance would continually emerge as more of these drugs were developed and reached the market. For most newly marketed antibiotics, resistance is reported within a few years after introduction. Sometimes a single amino acid substitution greatly diminishes the utility of an antibiotic and requires countermeasures by the pharmaceutical industry to restore effectiveness against the mutant pathogen. The relentless evolution of beta-lactamases by point mutations under the selective pressure of successive introductions of new beta-lactamase resistant penicillins, cephalosporins, carbapenems, and monobactams [12] illustrates this phenomenon. This ongoing toll of antibiotic resistance can be measured in both patient morbidity and mortality as well as in cost of care, and seems destined to plague mankind for the foreseeable future [13, 14].

2.3 Golden Age of Antibiotics

Over the next 50+ years following the discovery of penicillins and sulfa drugs, researchers identified a number of additional important antibiotic classes such as streptomycin (aminoglycosides), tetracycline (tetracyclines), erythromycin (macrolides), vancomycin (glycopeptides), and ciprofloxacin (quinolones). This period was called the "Golden Age of antibiotics" (Fig. 4.1) and can be further divided into the "Golden Age" of discovery of natural antibiotics of clinical significance (1940–1960) and the "Golden Age" of antibiotic medicinal chemistry over the ensuing 50 years [15, 16]. This latter period focused on chemical modification of major antibiotic classes in an attempt to keep ahead of resistant pathogens. Some have recently termed the years between 1962 and 2000 the "innovation gap" as no new major antibiotic classes were discovered during this time [17, 18]. However, the global R&D effort of the latter part of the twentieth century provided a steady flow of treatment options for bacterial infections. Brief descriptions of two additional early antibiotic discoveries are found below.

Perhaps the first successful use of natural product screening was the discovery of streptomycin by Schatz and Waksman as they searched for antibiotics active against *Mycobacterium tuberculosis* [19]. Streptomycin was rapidly isolated, purified, and entered clinical trials for the treatment of tuberculosis in collaboration with Merck. While streptomycin was remarkably successful in these initial trials, resistance developed quickly and ototoxicity was dose-limiting. Continued screening efforts over the next 30 years identified several new and improved aminoglycosides including gentamicin, tobramycin, and spectinomycin [20, 21]. These natural products provided both new clinical therapies and insights into the mechanisms by which bacteria develop resistance. This knowledge was critical to the further semisynthetic expansion of the aminoglycoside class through chemical modification [22, 23]. For example, amikacin, a semisynthetic derivative of kanamycin, showed improved activity against kanamycin-resistant isolates and lower acute toxicity than its parent compound [24]. It was launched in 1976 by Bristol-Myers Squibb as an injection formulation for the treatment of serious infections caused by amikacin-sensitive Gram-negative organisms as well as known or suspected staphylococcal infections.

Apart from Waksman and his group, there were very few experts in antibiotic screening during the early 1940s. Despite

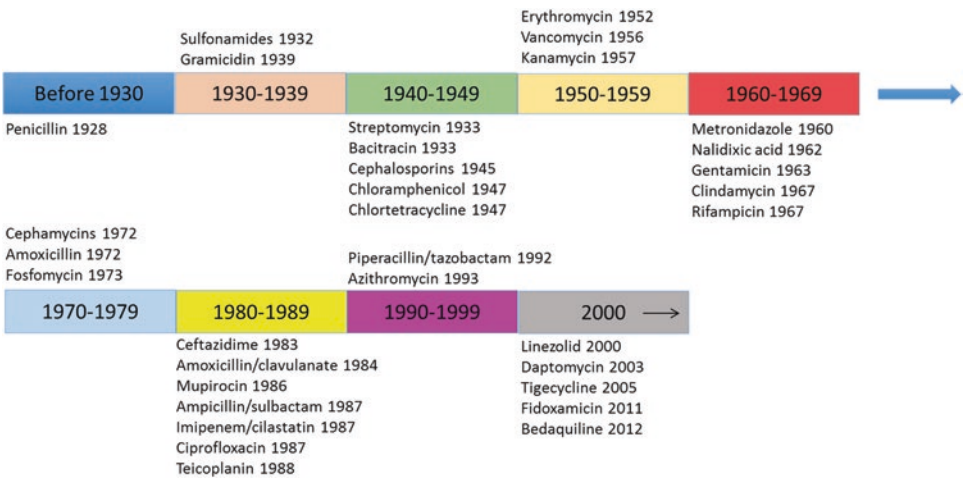


Fig. 4.1 Key milestones in antibiotic development (1928—present)

this dearth of expertise, other important discoveries soon followed. The first tetracycline antibiotic discovered, aureomycin, was isolated in 1945 from a Missouri soil sample at Lederle Laboratories by a team led by Benjamin Duggar—a retired professor from the University of Missouri [25]. This compound inhibited both Gram-positive and Gram-negative bacteria, including strains resistant to sulfa drugs and other antibiotics known at that time. Its antibacterial activity translated in vivo in rodent infection models with little toxicity. The Lederle scientists solved several initial production issues; the compound was obtained in crystalline form in 1947 and subsequently designated as chlortetracycline. Strain and fermentation improvements continued over many years and yields increased sufficiently to meet commercial demands. Second- and third-generation semisynthetic tetracycline analogs resulted in increased potency and efficacy against tetracycline-resistant bacteria as well as with improved pharmacokinetic and chemical properties [26]. The antibacterial activity and broad spectrum of this class will remain important assets for many years to come.

De novo chemistry also provided valuable additions to the natural product antibiotic arsenal. Sterling Winthrop researchers discovered nalidixic acid in 1962 from a by-product in the chemical synthesis of chloroquine. This compound would be the first marketed quinolone antibacterial [27]. It exhibited good Gram-negative activity and is rapidly bactericidal, but lacked Gram-positive activity. Although there was no cross-resistance with other marketed antibiotics, resistant organisms could be readily isolated. Early work on mechanism of action indicated that it was an inhibitor of DNA gyrase, but in 1990 it was found to also inhibit topoisomerase IV, therefore possessing dual target activity [28]. Both target enzymes are involved in bacterial DNA replication and in maintaining the integrity of the DNA helix. The US Food and Drug Administration (FDA) approved nalidixic acid in 1967 for the treatment of urinary tract infections. Subsequent work led to the identification of the first fluoroquinolone, flumequine, in 1977, which, unlike nalidixic acid, now exhibited some Gram-positive activity. Chemistry efforts continued on the fluoroquinolone pharmacophore in order to expand the spectrum, reduce resistance frequencies, and improve pharmacokinetics. Multiple generations of quinolone antibiotics were the result, including ciprofloxacin (approved in 1985), levofloxacin (1996), and moxifloxacin (1999) [29]. Despite growing resistance, quinolones continue to be precious tools in the infectious disease formularies.

2.4 Microbial Genomics: Early Disappointment and Uncertain Future

In 1995, the first complete genome of a bacterium, *Haemophilus influenzae*, was sequenced. This was the beginning of a flood of genomic information that continues to the present day [30]. Many new or improved bacterial genetic methodologies followed in the rush to make new research discoveries [31]. New DNA sequencing technologies greatly reduced the time and expense of sequencing bacterial genomes. The availability of this information profoundly impacted many areas of microbiology including microbial physiology, genetics, and especially studies on gene regulation and systems biology [32]. There have been efforts to define genes that are essential for bacterial growth and survival in the laboratory and in vivo in the host, and to compare these genes across microbial genera [33, 34]. Genomic data would eventually lead to new target ideas for novel antimicrobials as most existing antibiotic classes interacted with a relatively small number of processes in the bacterial cell [35]. There were spectrum implications, too, as one could now survey across a large number of pathogens and identify common key enzymes in essential pathways. There were hopes that the combined technologies of X-ray crystallography, combinatorial chemistry, and high-throughput screening (HTS) would lead to new antibiotics with novel modes of action, and ultimately end the innovation gap. A number of pharmaceutical companies selected targets and employed a broad range of HTS to search for novel inhibitors among chemical libraries. Unfortunately, relatively few inhibitors have yet progressed into clinical development; some observers even declared the entire genomics-based strategy to be a failure [36].

Many potential factors can rationalize why the genomics revolution did not transform antimicrobial drug discovery. The compound libraries of big pharmaceutical companies, although large in size, often did not contain chemical scaffolds that were amenable to antibacterial activity. For example, many of our known antibacterial compounds do not follow "Lipinski's Rules" that are often used to optimize inhibitors in other therapeutic areas and thus constitute the majority of companies' compound libraries [37]. Often, the novel target active site proved unsuitable for "drug-like" inhibitors because it was too exposed to solvent, or too hydrophobic, preventing compound access. Perhaps the major obstacle was the frequent lack of correlation between in vitro target inhibition (screen assays) and whole-cell activity due to membrane permeation or efflux issues, thus making optimization extremely challenging.

High expectations for quick success may have prematurely undermined genomics-based efforts. Also, during the genomics era, several large pharmaceutical companies either eliminated or greatly reduced antibacterial drug discovery for economic reasons (see Sect. 2). One important positive aspect of genomics research was the emergence of assay formats that obtained key information on potential target inhibitors. For example, these technology advances led to the development of sensitive assays allowing scientists to determine whether an inhibitor that demonstrates antibacterial activity acts through its target in the bacterial

cell [38]. This proof-of-mechanism provides confidence that optimization against a given target might lead to parallel improvements in antibacterial activity. The bacterial genomics effort continues in biotechnology companies and those pharmaceutical companies that still have antibacterial R&D units [39]. Hopefully, the scientific community has learned from past failures and soon successful outcomes will emerge.

3 The Current Antibiotic Landscape: Medical Need and R&D Response

The need for new and improved drugs to fight antibiotic resistance remains a major challenge to scientists. In developed countries, medical need can be broadly separated into two separate categories, hospital and community.

3.1 Hospital Setting: No ESKAPE

In the hospitals and nursing homes, the widespread use of antibiotics over the years led to endemic, multidrug-resistant strains causing a large number of deaths worldwide. The now famous "ESKAPE" acronym (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*) was first introduced in 2008 to pinpoint the major causes of hard-to-treat nosocomial infections [40]. While a large, concerted effort to address these pathogens has been underway [41], success remains elusive at this point. Methicillin-resistant *S. aureus* (MRSA) control did benefit from new drugs as well as improved medical practices, yet the management of deep-seated MRSA infections such as osteomyelitis remains challenging [42]. Moreover, the rapid emergence of carbapenemases over the past decade gradually erodes the potency of carbapenems, the last significant line of defense against Gram-negative bacteria. In a striking example of natural response to growth suppression, Gram-negative strains acquired three structural classes of beta-lactamases, such as Ambler Class A carbapenemases [43], Class B metalloenzymes [44], and Class D oxapenemases [45], all sharing the ability to hydrolyze carbapenems. As a result, polymyxins, an older class that had not been used for years because of poor tolerability, have now come back to the forefront of resistance management.

3.2 Community Setting: Often Overlooked Progress of Bacterial Resistance

In the community setting, mortality is much less common, but bacterial resistance leads to unnecessary hospitalizations and healthcare costs in developed countries. Among the many issues reported in the literature, we will outline three specific examples below.

3.2.1 CA-MRSA

According to the Center for Disease Control (www.cdc.gov), community-associated MRSA (CA-MRSA) causes over 15,000 invasive infections per year. These strains, which are often genetically distinct from hospital stains, were rare until the early 1990s [46]. Their prevalence in the community is increasing, affecting patients who have not had any prior contact with the healthcare environment.

3.2.2 Multidrug-Resistant Enterobacteriaceae

Urinary tract infections (UTI) are very common in the community, and until recently, were managed with either beta-lactams, quinolones, or trimethoprim-sulfamethoxazole. Recently, multidrug-resistant strains of enterobacteriaceae harboring extended-spectrum beta-lactamases (ESBL), as well as a variety of mutations rendering them non-susceptible to other drugs, have appeared in the community [47]. The vast majority of these infections are curable, but now require a hospitalization and intravenous antibiotic treatment. This trend is such in some countries that empirical UTI treatment by primary care physicians may soon have to be reevaluated. This outlines an urgent need to introduce new, orally available Gram-negative agents for community use.

3.2.3 Multidrug-Resistant Gonorrhea

Gonorrhea is a sexually transmissible disease caused by *Neisseria gonorrhoea*, a Gram-negative pathogen. Due to its high prevalence and debilitating side effects, virtually every class of antibiotic has been used to cure this infection over the years. Invariably, resistance has emerged, leading to the use of different regimens to control the epidemic. The treatment of choice used to consist of oral drugs, but now requires injectable ceftriaxone, even though ceftriaxone-resistant strains have already been described [48].

3.3 Medical Need Clashes with Economic Realities

Remarkably, while the medical need continued to increase, the number of big pharma players in the antibiotic field diminished greatly [49]. This lack of interest from pharmaceutical companies is due to economics, which have gradually become less favorable. We have attempted to rationalize the causes of this phenomenon, and identified three main drivers below.

3.3.1 The Decline of Large Community Infection Brands

In the past, pharmaceutical companies focused on both oral, well-tolerated drugs for community infections, and high-potency, intravenous drugs to address resistance in the hospital. The community setting often provided the most attractive business proposition. Blockbuster antibacterials such as

amoxicillin/clavulanate [50] or azithromycin [51] not only addressed medical needs in the community, but fueled new advances in the fight against resistance. It is not surprising to see that carbapenems, a major weapon to combat resistance in the hospital, were discovered during the same period [52], despite tremendous synthetic challenges. The new millennium brought a major change to this situation. Because of generic pressure as well as changes in regulatory guidelines, launching new drugs for community indications such as chronic bronchitis, sinusitis, or otitis media is no longer economical; these markets are now largely dominated by generics. With this revolution, the pharmaceutical industry lost a major source of cash flow to fund R&D efforts.

3.3.2 Cost of R&D

The progress in discovery technology since the 1980s did not serve antibiotic research. The difficulty in discovering new antibiotics in the genomics era was already examined in the first section of this chapter [36, 53]. Unfortunately, a number of changes in the regulatory landscape also contributed to higher development costs. During the early 2000s, regulators aimed to increase the quality and power of pivotal trials designed to launch new antibiotics. Inclusion criteria and primary endpoints became more stringent. In addition, trial sizes increased, sometimes dramatically, to satisfy tighter statistical requirements and enhance safety databases. While some of these changes were overdue and made good sense from a medical standpoint, the net effect was to discourage sponsors from starting new trials. For example, hospital-acquired and ventilator-associated bacterial pneumonia (HABP/VABP), for which patient recruitment is particularly cumbersome, became virtually intractable as a launch indication [54, 55].

Given these difficulties, it is not surprising to see that the length of time and risks of antibacterial development have taken a toll on profitability. An independent study conducted by the Eastern Research Group [56], which was commissioned by the US Department of Human and Health Services, built a profit-and-losses model of antibiotic R&D. This work concluded that a HABP/VABP drug developed today would result in negative investment returns for the sponsor, while generating a $1.2B value for society.

3.3.3 Antibiotic Pricing

None of the issues outlined above would be problematic if antibiotic pricing was more favorable. After all, oncology R&D costs also skyrocketed over the past 30 years, but were offset by favorable drug pricing. The antibiotic field, however, brings a unique challenge. The most traditional way to develop antibiotics is to evaluate pharmacokinetics and safety in healthy volunteers, then to compare the candidate drug to a competitor in a non-inferiority trial. In this design, enrolled patients are randomized between the two drugs, while culture and microbiology techniques confirm that both regimens will be capable of curing the infection. This method preserves the safety of the trial, since it would be unethical to assign patients with a life-threatening infection to an ineffective drug. However, it inherently leads to lower pricing: the pivotal trial essentially proves that the new drug is just as good as an older, often generic competitor. In addition, while new life-saving drugs are rapidly adopted, for example, with hepatitis C [57], new antibiotics are often put "in reserve" by doctors in an effort to manage resistance on the long term. This leads to slow sales uptake and lower profitability. In the meantime, the cost of antibiotic failure in the hospital has continued to increase. A 2010 study conducted in the USA [58] estimated that the cost of a hospital-acquired antibiotic-resistant infection results in 29 % higher cost-of-care and 24 % longer hospital stay, compared to the same infection caused by an antibiotic-sensitive strain.

The key to unlock the value of antibiotics is to rapidly identify patients in need of a novel, premium-priced drug, while infections caused by less resistant strains can be managed with cheaper generics. A recent study estimated that a targeted antibiotic capable of treating carbapenemase-resistant *A. baumannii* could easily fetch $10,000 per course, possibly even $30,000 per course, while remaining cost-effective for society [59]. Hence, advances in rapid diagnostic technology might be tightly linked to upward pricing evolution. Fortunately, rapid diagnostics have made great progress over the last decade [60]. It is now technically feasible to identify a given pathogen or mechanism of resistance with fairly good accuracy within a few hours. If these technologies were broadly applied, novel antibiotics could be priced much higher, and used more appropriately to fight resistance. However, adoption and logistics still remain major hurdles. These will have to be overcome before truly targeted, premium-priced antibiotics can replace empirical treatment.

3.4 Government and Regulators Respond to the Challenge

Government and regulators have long been aware of the antibiotic crisis, and engaged in concerted initiatives with the scientific and medical communities to promote antibacterial R&D. This movement has intensified over the past few years and ultimately holds the promise to transform the antibacterial landscape. While it appears unlikely that large, money-making community brands will reappear in the near future, regulatory progress and R&D incentives have already allowed a number of companies to pursue their mission to discover and develop new antibiotics. Among these, we would cite the following as most significant.

3.4.1 The GAIN Act

The GAIN act (Generating Antibiotic Incentives Now) is a US law signed in July 2012 [61], designed to create a financial incentive to companies and accelerate the launch of life-saving drugs. Under this law, companies developing qualified antibiotics gain access to an expedited FDA review, as well as five additional years of data exclusivity in the USA. This initiative was an instant success: as of September 2014, 39 products were granted Qualified Infectious Disease Product (QIDP) status and will therefore benefit from the new legislation. In 2014 alone, four of them have received FDA approval (dalbavancin, tedizolid, oritavancin, and ceftolozane/tazobactam). The original list of 18 bacterial and fungal threats, which included all ESKAPE species, was recently expanded with three additional pathogens in June 2014.

3.4.2 Regulatory Efforts to Expedite the Development of Life-Saving Drugs

On both sides of the Atlantic, the FDA and the European Medicines Authority (EMA) set a goal to introduce new development pathways enabling drugs targeting a single pathogen, rather than a disease (for example, HABP or UTI). Because high-risk pathogens such as *P. aeruginosa* or *A. baumannii* are somewhat infrequent and mostly confined to the hospital, enrolling "all comers" would require thousands of patients in order to find enough occurrences of the right pathogen. Therefore traditional statistical analysis of safety and efficacy in the context of a single pathogen would be highly impractical. A group of key opinion leaders proposed a solution to this conundrum [62] and engaged regulators in a productive dialogue. The ADAPT act [63], presented to the US congress in late 2013, aims to create a new regulatory pathway for pathogen-directed antibiotics under certain label limitations. In addition, the FDA created a special taskforce and issued several draft guidances over the same period [64].

The regulatory progress is not limited to pathogen-directed drugs. For example, regulators agreed to limit the number of pivotal trials to one per indication when a given drug is studied in two indications simultaneously. Moreover, a recent FDA Advisory Committee meeting [65] recommended the approval of ceftazidime/avibactam (see Sect. 3) on the basis of Phase 2 data in two indications.

3.4.3 Public–Private Partnerships

Public private partnerships have become a significant source of funding, both in Europe and the USA, allowing pharmaceutical companies to pursue their discovery programs, especially during discovery, preclinical, and Phase 1 stages, for which attrition is high. Among the numerous institutes and funding agencies around the globe who focus on ESKAPE pathogens, we will mention the following:

- In 2012, a European Innovative Medicine Initiative called "New Drugs for Bad Bugs" (ND4BB) assembled an unprecedented consortium of academic, biotech, and big pharma players to combat antibacterial resistance [66]. The EU pledged €109 million funding, while other members provided in-kind and tangible assets, raising the total value of the program up to €224 million. The three currently established programs include COMBACTE (clinical development), TRANSLOCATION (study of Gram-negative membrane permeability and efflux), and ENABLE (support for Gram-negative discovery and early development projects).
- The Broad Spectrum Antimicrobials program, funded by the US Biomedical Advanced Research and Development Authority (BARDA), provides discovery and development funding for selected antibacterial programs since 2010 [67]. Active programs include collaborations with Cempra, Tetraphase, Rempex, GSK, Achaogen, and Basilea (see Sect. 3 for details on their pipeline assets).
- In the USA, the National Institute of Allergy and Infectious Diseases (NIAID) continues to be a critical resource for basic, translational, and clinical research in the field of antibacterial resistance. As such, NIAID plays a key role in advising, collaborating, and funding discovery programs for academia and biotechs [68].

In the never-ending battle against bacterial resistance, 2014 could well be a turnaround year, with the resurgence of antibiotic investment, regulatory progress, and four FDA approvals. Several major pharma companies such as Roche and Sanofi took notice of the changing landscape and reentered the field [69], pursuing in-house and licensing activities to build a new antibacterial franchise. In addition, mid-sized players such as Cubist [70], Actavis, or The Medicines Company have several marketed or late-stage products in their pipelines; doing so they acquired enough critical mass to become long-term players. Finally, a favorable deal flow since 2010 prompted venture capital firms to reinvest in small antibacterial companies on both sides of the Atlantic.

4 Overview of Clinical Pipeline and Recent Drug Approvals

Since 2010, there has been substantial progress in clinical development of antibacterial agents against serious Gram-positive and Gram-negative infections. These efforts culminated in four FDA approvals and a New Drug Application (NDA) in 2014. Furthermore, several Phase 2 and 3 studies were underway or completed in 2014. As many of these agents have recently been expertly reviewed [71–74], only brief updates on agents in active or recently completed, late-stage clinical development for bacterial infections, not including mycobacterial infections, will be presented here. On a quarterly basis, The Pew Charitable Trusts revises its comprehensive summary of antibiotics in the clinical development pipeline [75]; a condensed and updated version of their summary is presented in Table 4.1.

Table 4.1 Antibacterial agents approved in 2014 or in active late-stage clinical development

Antibacterial agent	Development phase	Current sponsor	Drug class	Activity vs. Gram-negatives	QIDP indications	Additional/potential indication(s)
Tedizolid	Approved for ABSSSI (2014)	Cubist [70]	Oxazolidinone	No	ABSSSI, HABP, VABP	
Dalbavancin	Approved for ABSSSI (2014)	Actavis	Lipoglycopeptide	No	ABSSSI	CABP
Oritavancin	Approved for ABSSSI (2014)	The Medicines Company	Lipoglycopeptide	No	ABSSSI	
Ceftolozane-tazobactam	Approved for cUTI and cIAI (2014)	Cubist [70]	Cephalosporin-BLI	Yes	cUTI, cIAI, HABP, VABP	
Ceftazidime-avibactam	NDA for cUTI and cIAI under FDA review	Actavis	Cephalosporin-diazabicyclooctane BLI	Yes	cUTI, cIAI, HABP, VABP	
Carbavance	Phase 3	The Medicines Company	Meropenem-cyclic boronate BLI	Yes	cUTI, cIAI, HABP, VABP, febrile neutropenia	Bacteremia due to CRE, Gram-negative biothreat pathogens
Delafloxacin	Phase 3	Melinta	Fluoroquinolone	Yes	ABSSSI, CABP, uncomplicated gonorrhea	
Nemonoxacin	Phase 3	TaiGen	Nonfluorinated quinolone	Yes	CABP, ABSSSI	diabetic foot infection
Zabofloxacin	Phase 3	Dong Wha	Fluoroquinolone			ABE-COPD, CABP
Plazomicin	Phase 3	Achaogen	Neoglycoside	Yes		Bloodstream infections and nosocomial pneumonia caused by CRE, cUTI, AP, biothreat pathogens
Eravacycline	Phase 3	Tetraphase	Fluorocycline	Yes	cIAI, cUTI	HABP, biothreat pathogens
Solithromycin	Phase 3	Cempra	Fluoroketolide	Yes	CABP	Uncomplicated urogenital gonorrhea, biothreat pathogens
Cadazolid	Phase 3	Actelion	Quinolonyl-oxazolidinone		CDAD	
Surotomycin	Phase 3	Cubist [70]	Cyclic lipopeptide	No	CDAD	
Relebactam-imipenem	Phase 2	Merck	Carbapenem-diazabicyclooctane BLI	Yes	cUTI, cIAI, HABP, VABP	
S-649266	Phase 2	Shionogi	Catechol-substituted siderophore cephalosporin	Yes		
Finafloxacin	Phase 2	MerLion	Fluoroquinolone	Yes	cUTI, cIAI, ABSSSI	Exacerbations of chronic cystic fibrosis infection and COPD
GSK2140944	Phase 2	GSK	Type II topoisomerase inhibitor	Yes		ABSSSI, urogenital gonorrhea, biothreat pathogens
AZD0914	Phase 2	AstraZeneca	Benzisoxazole DNA gyrase inhibitor	Yes	Uncomplicated gonorrhea	
MRX-I	Phase 2	MicuRx	Oxazolidinone	No		ABSSSI
Omadacycline	Phase 2	Paratek (merged with Transcept)	Aminomethylcycline	Yes	CABP, ABSSSI, cUTI	
Lefamulin	Phase 2	Nabriva	Pleuromutilin	Yes	ABSSSI, CABP	
Fusidic acid	Phase 2	Cempra	Fusidane	No		PJI, ABSSSI
Brilacidin	Phase 2	Cellceutix	Defensin-mimetic	No		ABSSSI
GSK1322322	Phase 2	GSK/IMI (Combacte)	Peptide deformylase inhibitor	No		ABSSSI, CABP
POL7080	Phase 2	Polyphor (licensed to Roche)	Protein epitope mimetic LptD inhibitor	Yes	VABP caused by *P. aeruginosa*	Lower respiratory tract infection, bronchiectasis
SMT19969	Phase 2	Summit	Bis(benzimidazole)	No	CDAD	

Abbreviations: ABSSSI, acute bacterial skin and skin structure infection; CABP, community-acquired bacterial pneumonia; HABP, hospital-acquired bacterial pneumonia; VABP, ventilator-associated bacterial pneumonia; cUTI, complicated urinary tract infections; cIAI, complicated intra-abdominal infections; AP, acute pyelonephritis; CDAD, *Clostridium difficile*-associated diarrhea; BLI, beta-lactamase inhibitor; NDA, New Drug Application; PJI, prosthetic joint infection; ABE-COPD, acute bacterial exacerbation of chronic obstructive pulmonary disease

4.1 Agents Approved by the FDA in 2014

Tedizolid gained FDA approval in June 2014 for treatment of adults with Gram-positive acute bacterial skin and skin structure infections (ABSSSI). In Phase 3 studies, tedizolid 200 mg once daily for 6 days was non-inferior to linezolid 600 mg every 12 h for 10 days for early clinical response at 48–72 h after the first dose and for investigator-assessed clinical response at post-therapy evaluation, 7–14 days after the end of therapy [76, 77]. Indicated organisms include *Staphylococcus aureus* (both MRSA and Methicillin-sensitive), *Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus anginosus* Group (including *S. anginosus*, *S. intermedius*, and *S. constellatus*), and *Enterococcus faecalis*. As of December 2014, an additional efficacy study with dalbavancin was underway, comparing the efficacy of a single 1500 mg dose of dalbavancin with the FDA-approved, two dose dalbavancin regimen (NCT02127970).

Dalbavancin exerts time-dependent bactericidal activity in vitro, similar to vancomycin. It was approved by the FDA in May 2014 for treatment of adults with ABSSSI. The long elimination half-life of dalbavancin allows for once-weekly dosing and this dose regimen (1 g on Day 1 followed by 500 mg on Day 8) was found to be non-inferior to IV vancomycin (1 g every 12 h or 15 mg/kg every 12 h) with optional switch to oral linezolid (600 mg every 12 h), for 10–14 days [78]. Indicated organisms are the same as for tedizolid except for *E. faecalis*, which is infrequently isolated in ABSSSI. As of December 2014, two additional efficacy studies with dalbavancin were underway, one in community-acquired bacterial pneumonia (CABP, NCT02269644) and another in ABSSSI which compares the efficacy of a single 1500 mg dose of dalbavancin with the FDA-approved, two dose dalbavancin regimen (NCT02127970).

Oritavancin was approved by the FDA in August 2014 for the treatment of adults with ABSSSI. Indicated organisms include those in the tedizolid label as well as *Streptococcus dysgalactiae*. Like dalbavancin, oritavancin has a long elimination half-life. However, unlike dalbavancin, oritavancin has three mechanisms of action (disruption of bacterial membrane integrity and inhibition of both transglycosylation and transpeptidation) and concentration-dependent bactericidal activity in vitro [79, 80]. In Phase 3 studies comparing a single 1200 mg intravenous dose of oritavancin to intravenous vancomycin (1 g or 15 mg/kg every 12 h) for 7–10 days, oritavancin was non-inferior to vancomycin for both the early clinical response endpoint at 48–72 h and for the investigator-assessed clinical response endpoint at 7–14 days after the end of therapy [81, 82]. A PK/safety study with oritavancin in pediatric patients (NCT02134301) is currently underway.

Ceftolozane-tazobactam is an anti-pseudomonal cephalosporin combined with the beta-lactamase inhibitor (BLI) tazobactam. Against a collection of over 7000 isolates of Enterobacteriaceae and *P. aeruginosa* with a variety of resistance phenotypes, ceftolozane-tazobactam was generally as active as cefepime but had limited in vitro activity against ESBL-producing *K. pneumoniae* [83]. Overall, the combination inhibited more than 96 % of *P. aeruginosa* isolates at ≤4 μg/mL. In recently completed Phase 3 studies, the clinical efficacy of ceftolozane-tazobactam was superior to high-dose levofloxacin in patients with complicated UTI (cUTI) and was similar to meropenem in patients with complicated intra-abdominal infections (cIAI) [84]. Ceftolozane-tazobactam was approved for cUTI and cIAI by the FDA in December 2014, while a Phase 3 study to treat VABP is underway (NCT02070757).

4.2 Agents with NDA Under FDA Review in 2014

Ceftazidime-avibactam combines ceftazidime, a third-generation cephalosporin, with avibactam, a non-β lactam (diazabicyclooctane) BLI. Avibactam restores the antibacterial activity of ceftazidime against isolates of Enterobacteriaceae and *Pseudomonas aeruginosa* that express several types of serine β-lactamases. In particular, avibactam inhibits Ambler Class A ESBLs and *K. pneumoniae* carbapenemases (KPC), AmpC, and some Class D enzymes but is not active against Class B (metallo β-lactamase) enzymes [74]. In Phase 3 studies in patients with cIAI, ceftazidime-avibactam combined with metronidazole was non-inferior to meropenem for clinical cure at 28–35 days [85]. Ceftazidime-avibactam also demonstrated non-inferiority to comparator in a smaller cUTI study, as well as documented clinical activity against ceftazidime non-sensitive strains [86]. In December 2014, the FDA's Anti-Infective Drugs Advisory Committee [65] recommended approval of ceftazidime-avibactam for cIAI and cUTI caused by Gram-negative pathogens. A Phase 3 study comparing ceftazidime-avibactam to meropenem in HABP / VABP (NCT01808092) is currently in progress.

4.3 Agents in Phase 3 Development

Carbavance is a combination of the carbapenem antibiotic meropenem and a novel cyclic boronate BLI, RPX7009. RPX7009 is a potent and selective inhibitor of class A carbapenemases. The combination is under development for infections due to resistant gram-negative bacteria, particularly KPC-producing carbapenem-resistant enterobacteriaceae

(CRE). In a recent in vitro study 98.5 % of 200 isolates of enterobacteriaceae, including 100 KPC-producing strains, were inhibited by ≤2 μg/mL of meropenem with RPX7009 fixed at 8 μg/mL [87]. Carbavance was also efficacious in murine infection models against a variety of meropenem-resistant KPC-producing strains [88]. As with the β-lactam-BLI combinations listed above, potentiation of in vitro activity of the β-lactam component is not seen with enterobacteriaceae expressing metallo β-lactamases and activity against OXA-48-expressing strains is variable. A Phase 3 study of carbavance compared to piperacillin/tazobactam in patients with cUTI or acute pyelonephritis was initiated in November 2014 (NCT02166476), with a second Phase 3 study of carbavance in 150 patients with serious infections due to CRE across multiple indications also underway (NCT02168946).

A number of quinolone antibiotics are being developed with activity against some fluoroquinolone-resistant strains. Delafloxacin broadly covers Gram-positive organisms in vitro, including resistant strains, and is available in both IV and oral presentations. In a Phase 2 study, for the primary endpoint of Investigator-assessed clinical cure, both the 300 mg and 450 mg delafloxacin 12-h regimens provided comparable efficacy to vancomycin, with the low-dose delafloxacin arm being selected for future study due to its favorable safety profile [89]. An additional Phase 2 study, with enrolment criteria and endpoints more reflective of current FDA guidance for ABSSSI, has also been completed [90]. In that study, delafloxacin had a similar efficacy and safety profile compared to linezolid and had a higher response rate than vancomycin-treated patients at 14 days. A Phase 3 study in ABSSSI, comparing 300 mg delafloxacin IV every 12 h for 6 doses followed by 450 mg oral tablet every 12 h for 10–28 doses to vancomycin 15 mg/kg intravenously plus 2 g aztreonam every 12 h for 10–28 doses is currently underway (NCT01984684). Another open Phase 3 trial evaluates a single, 900 mg oral dose of delafloxacin versus intramuscular ceftriaxone for the treatment of uncomplicated gonorrhea (NCT02015637).

Nemonoxacin is a non-fluorinated quinolone with activity against Gram-positive and atypical pathogens, having somewhat reduced activity relative to fluoroquinolones [91]. In a randomized, double-blind, Phase 2 study in adults with mild-to-moderate CABP, clinical efficacy outcomes at test-of-cure were comparable across nemonoxacin 500 mg or 750 mg, or levofloxacin 500 mg, with each treatment administered once daily for 7 days [92]. Currently, a Phase 3 study in patients with CABP is recruiting (NCT02205112); patients are randomized 1:1 to receive either 500 mg nemonoxacin IV or 500 mg levofloxacin IV once daily for 7–14 days. Results of a completed Phase 2 study of nemonoxacin in patients with diabetic foot infections (NCT00685698) are not yet available. Oral nemonoxacin was approved in Taiwan for the treatment of CABP in adults.

Dong Wha Pharmaceuticals completed a Phase 3 study of the efficacy of 5-day zabofloxacin treatment compared to 7-day moxifloxacin treatment for mild-to-moderate CABP [93]; however, results are not yet available. A prior dose-finding study led to the selection of the 5-day regimen [94]. A Phase 2 study in patients with acute bacterial exacerbation of chronic obstructive pulmonary disease (NCT01658020) showed that a 3-day regimen of once-daily oral zabofloxacin and a 7-day regimen of once-daily moxifloxacin provided similar clinical and microbiological responses.

Plazomicin is a novel aminoglycoside designed to address aminoglycoside resistance. It exhibits in vitro activity against enterobacteriaceae including isolates expressing certain carbapenem resistance mechanisms or resistance to other aminoglycosides, to colistin, and to tigecycline [95]. A Phase 3 study comparing plazomicin in combination with meropenem or tigecycline to colistin in combination with meropenem or tigecycline is currently enrolling patients with bloodstream infections or HABP due to CRE (NCT01970371). Along with the Phase 3 study of carbavance, noted above, the plazomicin Phase 3 study is one of just two studies that are currently enrolling patients prospectively for treatment of CRE infections. A Phase 2 study assessing the safety, efficacy, and PK of plazomicin in patients with cUTI or AP has been completed (NCT01096849).

Eravacycline is a new, IV, and oral fluorocycline agent with broad spectrum activity including activity against tetracycline-resistant isolates. Its in vitro potency is equal or better to that of tigecycline. Enrolment has been completed in a Phase 3 study which compared eravacycline 1 mg/kg IV every 12 h to ertapenem 1 g IV once daily for patients with cIAI (NCT01844856); the eravacycline 1 mg/kg every 12 h dose was selected based on results from a previous Phase 2 study in cIAI [96]. A separate Phase 3 study in patients with cUTI is evaluating eravacycline 1.5 mg/kg IV once daily followed by eravacycline either 200 mg or 250 mg PO every 12 h versus levofloxacin 750 mg IV once daily followed by 750 mg orally once daily (NCT01978938). Analysis of data from the lead-in portion of the study suggested that responses were comparable across the three treatment arms [97].

Solithromycin is a fluoroketolide being developed in both oral and IV formulations for CABP and uncomplicated gonococcal infections. Its spectrum of activity is well suited for community-acquired respiratory infections. In vitro, solithromycin displays greater potency than erythromycin and clindamycin and similar potency to azithromycin against most relevant organisms [98]. A Phase 3 study of efficacy and safety study of IV to oral solithromycin versus IV to oral moxifloxacin for patients with CABP is presently underway (NCT01968733).

Cadazolid is being developed for oral treatment of *C. difficile* associated diarrhea (CDAD) based on its gram-positive spectrum and negligible absorption, resulting in high gut lumen

concentrations and low systemic exposure [99]. In a Phase 2 study, cadazolid provided similar efficacy to vancomycin on key efficacy endpoints including clinical cure and sustained cure [100]. A Phase 3 study in CDAD is currently underway (NCT01987895). A key challenge will be to demonstrate superiority to vancomycin in sustained clinical response 25 days after the end of treatment, as has been shown in pivotal studies for the recently approved CDAD agent, fidaxomicin [101].

Surotomycin is a lipopeptide oral antibiotic that is structurally related to daptomycin. It exhibits approximately fourfold increased in vitro activity against *C. difficile* compared to vancomycin, with minimal systemic absorption. In Phase 2 studies, it demonstrated comparable cure rates to vancomycin but with reduced rates of recurrence [102]. Presently, two Phase 3 studies of surotomycin versus vancomycin are underway in CDAD (NCT01598311 and NCT01597505).

4.4 Agents in Phase 2 Development

Several cell wall inhibitors are in Phase 2 development. Relebactam (formerly, MK-7655) is a non-β lactam (diazabicyclooctane) inhibitor of class A and C β-lactamases evaluated in combination with imipenem/cilastatin in an ongoing Phase 2 study in cUTI (NCT01505634). A Phase 2 study in cIAI (NCT01506271) has recently completed enrolment. S-649266, a cephalosporin-siderophore conjugate, is actively transported through the outer membrane into the periplasmic space where it binds to penicillin-binding proteins (PBPs) and disrupts cell wall synthesis. S-649266 is more stable to carbapenemases than ceftazidime, cefepime, and meropenem and is active in vitro against *P. aeruginosa, A. baumannii,* and enterobacteriaceae. Phase 2 development is underway [103], although the type of infections targeted in the study has not been disclosed.

The fluoroquinolone finafloxacin offers a similar spectrum of activity to existing quinolones and is being evaluated in a phase 2 study for treatment of cUTI and AP (NCT01928433). GSK2140944 is a bacterial Type II topoisomerase inhibitor with a distinct binding mode compared to fluoroquinolones, providing in vitro activity against fluoroquinolone-resistant isolates. It is presently in Phase 2 development for Gram-positive ABSSSI (NCT02045797) and uncomplicated urogenital gonorrhea (NCT02294682). AZD0914 is a benzisoxazole DNA gyrase inhibitor [104] under evaluation as a single oral dose compared to intramuscular treatment with ceftriaxone in a Phase 2 study in adults with uncomplicated urogenital gonorrhea (NCT02257918).

Several protein synthesis inhibitors are also under development. A Phase 2 study of the oxazolidinone MRX-I compared to linezolid is currently underway in adult patients with ABSSSI (NCT02269319). However, any potential benefits of the proposed 10-day, twice-daily oral course of therapy are unclear given the recent approval of tedizolid, with its 5-day, once-daily regimen. Omadacycline is an aminomethylcycline with potential utility in treatment of ABSSSI on the basis of a completed Phase 2 study [105]. A Phase 3 study to compare the safety and efficacy of omadacycline with linezolid in the treatment of adults with ABSSSI was terminated in 2013 but new pivotal studies are slated to begin in 2015 [106]. Lefamulin is the first systemically available pleuromutilin studied for the treatment of ABSSSI and CABP owing to in vitro activity against Gram-positives, fastidious Gram-negatives, and atypicals. With a Phase 2 ABSSSI study now completed [107], a Phase 3 study is being planned [108]. Fusidic acid (CEM-102) demonstrated similar safety and efficacy to linezolid in a Phase 2 study of adults with ABSSSI [109]. A Phase 2 study of fusidic acid in combination with rifampin versus standard of care for prosthetic joint/spacer infection was recently terminated (NCT01756924).

Brilacidin, a defensin, was recently evaluated in a Phase 2b study versus daptomycin for treatment of ABSSSI (NCT02052388). Subjects randomized to brilacidin in this study received either a single IV infusion (low dose, 0.6 mg/kg or high dose, 0.8 mg/kg) or a 3 day IV regimen (0.6 mg/kg on Day 1 followed by 0.3 mg/kg on Days 2 and 3). The clinical success rates for each regimen were comparable to that of daptomycin administered once daily for 7 days [110].

GSK1322322 is one of a new class of agents that inhibit the bacterial peptide deformylase enzyme. A Phase 2a study in patients with ABSSSI has been completed. However, results showed less favorable safety and efficacy outcomes compared to linezolid [111]. Further clinical development is to occur through the Innovative Medicines Initiative COMBACTE program.

POL7080 belongs to a class of protein epitope-mimetics. It is a fully synthetic cyclic peptide that interacts with the outer membrane protein LptD in *P. aeruginosa*, resulting in down-regulation of lipopolysaccharide biosynthesis. In 2013, Roche obtained the license rights to develop and commercialize POL7080 globally. A Phase 2 study in adults with VABP caused by *P. aeruginosa* is underway assessing efficacy of POL7080 when co-administered with standard-of-care (NCT02096328).

SMT19969 has similar in vitro activity to fidaxomicin against *C. difficile* and limited activity compared to vancomycin and metronidazole against other Gram-positive aerobes and anaerobes, suggesting that it may have little impact on normal gut microbiota during CDAD treatment [112]. A Phase 2 study in CDAD is underway (NCT02092935).

5 Conclusion

Small-molecule antibiotic therapy had, together with vaccination, a tremendous impact on life expectancy in the twentieth century. After the "Golden Age," it felt like bacteria were winning the battle against science, while society appeared unable to respond to the challenge of resistance.

Fortunately, over the past few years, governments, regulators, and the scientific and medical communities started to truly cooperate in order to define a new future. This effort led to new investment in antibacterial R&D, the emergence of innovative regulatory pathways, as well as financial incentives to discover and launch new antibacterials. While a lot remains to be done, late-stage development now appears to proceed at a rate higher than that of the past decade, and four new drugs were approved by the FDA in 2014. However, one should note that the number of truly novel classes represented amongst the late-stage global development pipeline remains small. This sobering fact comes as a reminder that investment in basic antibacterial research needs to remain a priority for many years to come.

Acknowledgments The authors wish to acknowledge David Shlaes and Steven Projan who wrote the first version of this chapter entitled "Antimicrobial Resistance vs. the Discovery and Development of New Antimicrobials," published in 2009 by the same editor. Their contribution represented a very useful starting point. We also would like to thank Olga Lomovskaya and Doug Mayers for their kind review and editorial comments.

References

1. Kardos N, Demain AL. Penicillin: the medicine with the greatest impact on therapeutic outcomes. Appl Microbiol Biotechnol. 2011;92:677–87.
2. Fleming A. On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of B. influenza. Br J Exp Path. 1929;10:226–36.
3. Lax E. The mold in Dr. Florey's coat. Henry Holt and Co; 2004.
4. American Chemical Society. 2014. The discovery and development of penicillin 1928–1945 Commemorative Booklet. http://www.acs.org/content/acs/en/education/whatischemistry/landmarks/flemingpenicillin.html. Accessed 6 Dec 2014.
5. Nobel Media AB. 2014. Nobel Prize in physiology or medicine 1945 http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/ Accessed 6 Dec 2014.
6. Otten H. Domagk and the development of the sulphonamides. J Antimicrob Chemother. 1986;17:689–96.
7. Woods D. The relation of p-aminobenzoic acid to the mechanism of action of sulphanilamide. Brit J Exp Pathol. 1940;21:74–90.
8. Rolinson GN, Geddes AM. The 50th anniversary of the discovery of 6-aminopenicillanic acid (6-APA). Int J Antimicrob Agents. 2007;29:3–8.
9. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. Nature. 1940;146:837.
10. Spink WW, Ferris V. Penicillin-resistant staphylococci: mechanisms involved in the development of resistance. J Clin Invest. 1947;26:379–83.
11. Finland M, Haight TH. Antibiotic resistance of pathogenic staphylococci: study of five hundred strains isolated at Boston City Hospital from October, 1951, to February, 1952. AMA Arch Intern Med. 1953;91:143–58.
12. Jacoby G, Bush K. Beta-lactam resistance in the 21st century. In: White DG, Alekshun MN, McDermott PF, editors. Frontiers in antimicrobial resistance. Washington, DC: ASM Press; 2005. p. 53–65.
13. Fair RJ, Tor Y. Antibiotics and Bacterial Resistance in the 21st Century. Perspect Med Chem. 2014;6:25–64.
14. Centers for Disease Control and Prevention. 2013. Antibiotic resistance threats in the United States; 2013. http://www.cdc.gov/media/releases/2013/p0916-untreatable.html. Accessed 6 Dec 2014.
15. Walsh CT, Wencewicz TA. Prospects for new antibiotics: a molecule-centered perspective. J Antibiotics. 2014;67:7–22.
16. Davies J. Where have all the antibiotics gone? Can J Infect Dis Med Microbiol. 2006;17:287–90.
17. Walsh C. Where will new antibiotics come from? Nat Rev Microbiol. 2003;1:65–70.
18. Silver LL. Challenges of antibacterial discovery. Clin Microbiol Rev. 2011;24:71–109.
19. Schatz A, Bugie E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc Soc Exptl Biol Med. 1944;55:66–9.
20. Umezawa S, Tsuchiya T. Total synthesis and chemical modification of the aminoglycoside antibiotics. In: Umezawa H, Hooper IR, editors. Aminoglycoside antibiotics. Berlin: Springer; 1982. p. 37–110.
21. BerlinWaitz JA, Moss Jr E, Drube CG, Weinstein MJ. Comparative activity of sisomicin, gentamicin, kanamycin, and tobramycin. Antimicrob Agents Chemother. 1972;2:431–7.
22. Vakulenko SB, Mobashery S. Versatility of aminoglycosides and prospects for their future. Clin Microbiol Rev. 2003;16:430–50.
23. Hermann T. Aminoglycoside antibiotics: old drugs and new therapeutic approaches. Cell Mol Life Sci. 2007;64:1841–52.
24. Reynolds AV, Hamilton-Miller JM, Brumfitt W. Newer aminoglycosides--amikacin and tobramycin: an in-vitro comparison with kanamycin and gentamicin. Br Med J. 1974;3:778–870.
25. Nelson ML, Levy SB. The history of the tetracyclines. Ann NY Acad Sci. 2011;241:17–32.
26. Zhanel GG, Homenuik K, Nichol K, et al. The glycylcyclines: a comparative review with the tetracyclines. Drugs. 2004;64:63–88.
27. Andersson MI, MacGowan AP. Development of the quinolones. J Antimicrob Chemother. 2003;51(Suppl S1):1–11.
28. Hoshino K, Kitamura A, Morrissey I, et al. Comparison of inhibition of Escherichia coli topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob Agents Chemother. 1994;38:2623–7.
29. Van Bambeke F, Michot JM, Van Eldere J, Tulkens PM. Quinolones in 2005: an update. Clin Microbiol Infect. 2005;11:256–80.
30. Fleischmann RD, Adams MD, White O, et al. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science. 1995;269:496–512.
31. Mori H. From the sequence to cell modeling: comprehensive functional genomics in Escherichia coli. J Biochem Mol Biol. 2004;37:83–92.
32. Pucci MJ. Novel genetic techniques and approaches in the microbial genomics era: identification and/or validation of targets for the discovery of new antibacterial agents. Drugs R D. 2007;8:201–12.
33. Hensel M, Shea JE, Gleeson C, et al. Simultaneous identification of bacterial virulence genes by negative selection. Science. 1995;269:400–3.
34. Slauch JM, Mahan MJ, Mekalanos JJ. In vivo expression technology for selection of bacterial genes specifically induced in host tissues. Methods Enzymol. 1994;235:481–92.
35. Pucci MJ. Use of genomics to select antibacterial targets. Biochem Pharmacol. 2006;71:1066–72.
36. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov. 2007;6:29–40.
37. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev. 2001;46:3–26.

38. DeVito JA, Mills JA, Liu VG, et al. An array of target-specific screening strains for antibacterial discovery. Nat Biotechnol. 2002;20:478–83.
39. Brötz-Oesterhelt H, Sass P. Postgenomic strategies in antibacterial drug discovery. Future Microbiol. 2010;5:1553–79.
40. Rice L. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis. 2008;197:1079–81.
41. Boucher H, Talbot GH, Bradley JS. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48:1–12.
42. Spellberg B, Lipsky BA. Systemic antibiotic therapy for chronic osteomyelitis in adults. Clin Infect Dis. 2012;54:393–407.
43. Nordman P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect Dis. 2009;9:228–36.
44. Dortet L, Poirel L, Nordman P. Worldwide dissemination of the NDM-type carbapenemases in gram-negative bacteria. BioMed Res Int. 2014. Article ID 249856.
45. Evans BA, Amyes SGB. OXA beta-lacatamases. Clin Microbiol Rev. 2014;27:241–63.
46. David MZ, Daum RS. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010;23:613–87.
47. Doyle Y, Park YS, Rivera JI. Community-associated Extended-Spectrum β-Lactamase–producing *Escherichia coli* infection in the United States. Clin Inf Dis. 2013;56:641–8.
48. Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Future Microbiol. 2012;7:1401–22.
49. Projan S. Why is big Pharma getting out of antibacterial drug discovery? Curr Opin Microbiol. 2003;6:427–30.
50. White AR, Kaye C, Poupard J, et al. Augmentin® (amoxicillin/clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent. J Antimicrob Chemother. 2004;53:i3–i20.
51. Peters DH, Friedel HA, McTavish D. Azithromycin. A review of its antimicrobial activity, pharmacokinetic properties and clinical efficacy. Drugs. 1992;44:750–99.
52. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. Carbapenems: past, present and future. Antimicrob Agents Chemother. 2011;55:4943–60.
53. Livermore DM. Discovery research: the scientific challenge of finding new antibiotics. J Antimicrob Chemother. 2011;66:1941–4.
54. Ledford H. FDA under pressure to relax drug rules. Nature. 2012;492:19.
55. Jarvis LM. A bacterial battle. Chem Eng News. 2014;92:9–14.
56. Sertkaya A, Eyraud J, Birkenbach A, Franz C, et al. 2014. Analytical framework for examining the value of antibacterial products. http://aspe.hhs.gov/sp/reports/2014/antibacterials/rpt_antibacterials.cfm. Accessed 20 Dec 2014.
57. Staton T. It's official: Gilead's Sovaldi zooms past previous records with fastest-ever drug launch. Fierce Pharma Market . 2014;22:2014.
58. Mauldin PD, Salgado CD, Hansen IS, et al. Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant gram-negative bacteria. Antimicrob Agents Chemother. 2010;54:109–15.
59. Spellberg B, Rex JH. The value of single-pathogen antibacterial agents. Nat Rev Drug Discov. 2013;12:963.
60. Murray PR, Masur H. Current approaches to the diagnosis of bacterial and fungal bloodstream infections for the ICU. Crit Care Med. 2012;40:3277–82.
61. US Public law 112–144 (2012) www.gpo.gov/fdsys/pkg/PLAW-112publ144/pdf/PLAW-112publ144.pdf. Accessed 20 Dec 2014.
62. Rex JH, Eisenstein BI, Alder J, Goldberger M, et al. A comprehensive regulatory framework to address the unmet need for new antibacterial treatments. Lancet Infect Dis. 2013;13:269–75.
63. Antibiotic Development to Advance Patient Treatment Act of 2013; 2013. www.hpm.com/pdf/blog/GINGRE_057_xml.pdf. Accessed 20 Dec 2014.
64. Antibacterial Task Force: How CDER is establishing and promoting new approaches to antibacterial drug development. http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/ucm406769.htm. Accessed 20 Dec 2014.
65. December 4, 2014: Anti-infective drugs advisory committee meeting announcement (2014) www.fda.gov/AdvisoryCommittees/Calendar/ucm420134.htm. Accessed 20 Dec 2014.
66. Rex JH. ND4BB: addressing the antimicrobial resistance crisis. Nat Rev Microbiol. 2014;12:231–2.
67. Jarrett E. BARDA Broad Spectrum Antimicrobials (BSA) Program; 2014. http://www.phe.gov/ASPRBlog/Lists/Posts/Post.aspx?ID=97. Accessed 20 Dec 2014.
68. NIAID antibacterial resistance program: current status and future directions; 2014. http://www.niaid.nih.gov/topics/antimicrobialResistance/documents/arstrategicplan2014.pdf. Accessed 20 Dec 2014.
69. Plumridge H. Drug makers tiptoe back into antibiotic R&D. Wall Street J. January 23, 2014.
70. Merck to acquire cubist pharmaceuticals for $102 per share in cash. Company press release, December 8, 2014. http://www.merck.com/licensing/our-partnership/cubist-press-release.html.
71. Boucher HW, Talbot GH, Benjamin Jr DK, et al. 10×'20 Progress--development of new drugs active against gram-negative bacilli: an update from the Infectious Diseases Society of America. Clin Infect Dis. 2013;56:1685–94.
72. Pucci MJ, Bush K. Investigational antimicrobial agents of 2013. Clin Microbiol Rev. 2013;26:792–821.
73. Butler MS, Blaskovich MA, Cooper MA. Antibiotics in the clinical pipeline in 2013. J Antibiot (Tokyo). 2013;66:571–91.
74. Drawz SM, Papp-Wallace KM, Bonomo RA. New β-lactamase inhibitors: a therapeutic renaissance in an MDR world. Antimicrob Agents Chemother. 2014;58:1835–46.
75. The Pew Charitable Trusts. 2014. Antibiotics Currently in Clinical Development. http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development. Accessed 11 Dec 2014.
76. Prokocimer P, De Anda C, Fang E, et al. Tedizolid phosphate vs linezolid for treatment of acute bacterial skin and skin structure infections: the ESTABLISH-1 randomized trial. J Am Med Assoc. 2013;309:559–69.
77. Moran GJ, Fang E, Corey GR, et al. Tedizolid for 6 days versus linezolid for 10 days for acute bacterial skin and skin-structure infections (ESTABLISH-2): a randomised, double-blind, phase 3, non-inferiority trial. Lancet Infect Dis. 2014;14:696–705.
78. Boucher HW, Wilcox M, Talbot GH, et al. Once-weekly dalbavancin versus daily conventional therapy for skin infection. N Engl J Med. 2014;370:2169–79.
79. Zhanel GG, Calic D, Schweizer F, et al. New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. Drugs. 2010;70:859–86.
80. Zhanel GG, Schweizer F, Karlowsky JA. Oritavancin: mechanism of action. Clin Infect Dis. 2012;54 Suppl 3:S214–9.
81. Corey GR, Good S, Jiang H, et al. Single-dose oritavancin versus 7–10 days of vancomycin in the treatment of gram-positive acute bacterial skin and skin structure infections: The SOLO II noninferiority study. Clin Infect Dis. 2014. pii: ciu778 [Epub ahead of print].
82. Corey GR, Kabler H, Mehra P, et al. Single-dose oritavancin in the treatment of acute bacterial skin infections. N Engl J Med. 2014;370:2180–90.

83. Farrell DJ, Flamm RK, Sader HS, Jones RN. Antimicrobial activity of ceftolozane-tazobactam tested against enterobacteriaceae and Pseudomonas aeruginosa with various resistance patterns isolated in U.S. hospitals (2011–2012). Antimicrob Agents Chemother. 2013;57:6305–10.
84. Popejoy MW, Cloutier D, Huntington JA, et al. Ceftolozane/tazobactam for the treatment of cUTI and cIAI caused by ESBL-producing Enterobacteriaceae. In: Paper presented at IDWeek, Philadelphia, 8-12 October 2014; 2014.
85. Actavis announces positive topline results from the Phase III program of ceftazidime-avibactam in patients with complicated intra-abdominal infections (cIAI). http://www.actavis.com/news/news/thomson-reuters/actavis-announces-positive-topline-results-from-th. Accessed 11 Dec 2014.
86. FDA Division of Anti-Infective Products. Briefing package NDA 206494 ceftazidime-avibactam. http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/Anti-InfectiveDrugsAdvisoryCommittee/UCM425458.pdf. Accessed 11 Dec 2014.
87. Castanheira M, Rhomberg PR, Watters A, Jones RN. In vitro activity of meropenem/RPX7009, a carbapenem/β-lactamase inhibitor combination tested against contemporary populations of Enterobacteriaceae and KPC-producing strains. In: Paper presented at IDWeek, Philadelphia, 8–12 October 2014; 2014.
88. Sabet M, Tarazi Z, Nolan T, et al. In Vivo Efficacy of Carbavance (meropenem/RPX7009) against KPC-producing Enterobacteriaceae. In: Paper presented at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, 5–9 September 2014; 2014.
89. O'Riordan W, Mehra P, Manos P, et al. A randomized phase 2 study comparing two doses of delafloxacin with tigecycline in adults with complicated skin and skin-structure infections. Int J Infect Dis. 2014. pii: S1201-9712(14)01655-5. doi: 10.1016/j.ijid.2014.10.009 [Epub ahead of print].
90. Longcor J, Hopkins S, Lawrence L et al (2012) Results of a phase 2 study of delafloxacin (DLX) compared to vancomycin (VAN) and linezolid (LNZ) in acute bacterial skin and skin structure infections (ABSSSI). In: Paper presented at the 52nd interscience conference on antimicrobial agents and chemotherapy, San Francisco, 9–12 September 2012.
91. Qin X, Huang H. Review of nemonoxacin with special focus on clinical development. Drug Des Devel Ther. 2014;8:765–74.
92. van Rensburg DJ, Perng RP, Mitha IH, et al. Efficacy and safety of nemonoxacin versus levofloxacin for community-acquired pneumonia. Antimicrob Agents Chemother. 2010;54:4098–106.
93. Dong Wha Pharmaceuticals. Dong Wha completes Phase III study of quinolone antibacterial compound zabofloxacin; 2014. https://www.dong-wha.co.kr/english/customer/dnews/content.asp?t_idx=239. Accessed 11 Dec 2014.
94. Tillotson G, Kim YS, Kim MJ, et al. Zabofloxacin for the treatment of mild to moderate community-acquired bacterial pneumonia compared to moxifloxacin; a phase 2 study. In: Paper presented at the 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, 10–13 May 2014; 2014.
95. Livermore DM, Mushtaq S, Warner M, et al. Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant enterobacteriaceae isolates. J Antimicrob Chemother. 2011;66:48–53.
96. Solomkin JS, Ramesh MK, Cesnauskas G, et al. Phase 2, randomized, double-blind study of the efficacy and safety of two dose regimens of eravacycline versus ertapenem for adult community-acquired complicated intra-abdominal infections. Antimicrob Agents Chemother. 2014;58:1847–54.
97. Tetraphase Pharmaceuticals. Tetraphase Pharmaceuticals announces positive oral dosing data from lead-in of IGNITE 2 Phase 3 trial of eravacycline in cUTI support advancement to pivotal portion; 2014. http://ir.tphase.com/releasedetail.cfm?ReleaseID=868654. Accessed 11 Dec 2014.
98. Biedenbach DJ, Castanheira M, Jones RN (2012) Activity of the fluoroketolide, solithromycin tested against bacterial species associated with significant community-acquired bacterial pneumonia and other gram-positive organisms. In: Paper presented at IDWeek, San Diego, 17–21 October 2012
99. Locher HH, Seiler P, Chen X, et al. In vitro and in vivo antibacterial evaluation of cadazolid, a new antibiotic for treatment of *Clostridium difficile* infections. Antimicrob Agents Chemother. 2014;58:892–900.
100. Louie TJ, Buitrago M, Cornely OA et al (2013) Multicentre, double-blind, randomised, phase 2 study evaluating the novel antibiotic, cadazolid, in subjects with *Clostridium difficile*-associated diarrhea. In: Paper presented at the 53rd European Congress of Clinical Microbiology and Infectious Diseases, Berlin, 27–30 April 2013
101. DIFICID Prescribing Information 2014. http://www.dificid.com/downloads/Dificid_PI.pdf. Accessed 11 Dec 2014
102. Mascio CTM, Mortin LI, Howland KT, et al. *In Vitro* and *In Vivo* characterization of CB-183,315, a novel lipopeptide antibiotic for treatment of *Clostridium difficile*. Antimicrob Agents Chemother. 2012;56:5023–30.
103. Shionogi & Co., Ltd. Shionogi presents pre-clinical and Phase 1 results for S-649266, a novel siderophore cephalosporin antibiotic with potential to treat multidrug-resistant gram-negative pathogens; 2014. http://www.prnewswire.com/news-releases/shionogi-presents-pre-clinical-and-phase-i-results-for-s-649266-a-novel-siderophore-cephalosporine-antibiotic-with-potential-to-treat-multidrug-resistant-gram-negative-pathogens-274336081.html. Accessed 11 Dec 2014.
104. Jacobsson S, Golparian D, Alm RA, et al. High *in-vitro* activity of the novel spiropyrimidinetrione AZD0914, a DNA gyrase inhibitor, against multidrug resistant *Neisseria gonorrhoeae* isolates suggests a new effective option for oral treatment of gonorrhea. Antimicrob Agent Chemother. 2014;58:5585–8.
105. Noel GJ, Draper MP, Hait H, et al. A randomized, evaluator-blind, phase 2 study comparing the safety and efficacy of omadacycline to those of linezolid for treatment of complicated skin and skin structure infections. Antimicrob Agents Chemother. 2012;56:5650–4.
106. Paratek Pharmaceuticals. http://www.paratekpharm.com/content/aboutus/index.htm. Accessed 11 Dec 2014.
107. Prince WT, Ivezic-Schoenfeld Z, Lell C, et al. Phase II clinical study of BC-3781, a pleuromutilin antibiotic, in treatment of patients with acute bacterial skin and skin structure infections. Antimicrob Agents Chemother. 2013;57:2087–94.
108. Nabriva Therapeutics. Nabriva Therapeutics announces Qualified Infectious Disease Product and Fast Track status granted by the FDA for lefamulin; 2014. http://www.nabriva.com/news-publications/press-releases/fast-track/. Accessed 11 Dec 2014
109. Craft JC, Moriarty SR, Clark K, et al. A randomized, double-blind phase 2 study comparing the efficacy and safety of an oral fusidic acid loading-dose regimen to oral linezolid for the treatment of acute bacterial skin and skin structure infections. Clin Infect Dis. 2011;52 Suppl 7:S520–6.
110. Cellceutix Corporation (2014) Cellceutix announces positive top-line data from Phase 2b ABSSSI trial; single-dose brilacidin comparable to 7-days of daptomycin. http://cellceutix.com/cellceutix-announces-positive-top-line-data-from-phase-2b-absssi-trial-single-dose-brilacidin-comparable-to-7-days-of-daptomycin/#sthash.SpaWi7VQ.XuEVN9NE.dpbs. Accessed 11 Dec 2014.
111. Corey R, Naderer OJ, O'Riordan WD, et al. Safety, tolerability, and efficacy of GSK1322322 in the treatment of acute bacterial skin and skin structure infections. Antimicrob Agents Chemother. 2014;58:6518–27.
112. Goldstein EJ, Citron DM, Tyrrell KL, Merriam CV. Comparative in vitro activities of SMT19969, a new antimicrobial agent, against *Clostridium difficile* and 350 gram-positive and gram-negative aerobic and anaerobic intestinal flora isolates. Antimicrob Agents Chemother. 2013;57:4872–6.

Part II

General Mechanisms of Drug Resistance

Genetic Mechanisms of Transfer of Drug Resistance

5

Paul H. Roy and Sally R. Partridge

1 Introduction

Resistance to antibiotics in clinical bacteria has closely followed the introduction of each antibiotic. Resistance to sulfa drugs and penicillin was known in the 1940s, and the transmissibility of resistance to sulfa drugs, streptomycin, chloramphenicol, and tetracycline became known during the following decade. In the course of studies on bacillary dysentery in Japan, it was found that several drug resistances could be transferred together from *Shigella* to *Escherichia coli*. Many of these studies were published in Japanese. A key review by Watanabe [1] summarized these studies and, in retrospect, was exceptionally insightful. Developed in the late 1950s and early 1960s, the studies introduced the concept of "R factor" episomes made up of RTF (resistance transfer factor) and individual resistance genes. R factors were recognized as plasmids, and even the phenomenon of fertility inhibition of F factor by some R factors (now known to be IncF plasmids) was observed. The RTF is now known to be composed of the replication and transfer functions of the plasmids, and the resistance genes in these studies are now known to be associated with mobile elements (simple and composite transposons and integrons). Genomic islands, replicating as part of the chromosome, also carry multiresistance regions. The 1960s and 1970s saw a rapid increase in the number of antibiotics available (particularly aminoglycosides and β-lactams) and a concomitant increase in the number and types of resistance genes. Mapping of plasmids by restriction enzyme digests and electron microscopy of heteroduplexes gave an idea of how DNA rearrangements were taking place, but only after the advent of DNA sequencing in the late 1970s could the variety and complexity of genetic mechanisms of resistance gene dissemination be appreciated.

While isolates from the 1960s often carried a single resistance gene associated with a single mobile element, recent isolates often carry complex multiresistance regions (MRRs) formed by clustering of a wide variety of mobile elements and associated resistance genes. Many clinically important resistance genes, notably those encoding extended-spectrum β-lactamases (ESBLs) and carbapenemases, are found in MRRs.

2 Conjugative and Mobilizable Plasmids

Most antibiotic resistance genes, in both Gram-negative and Gram-positive bacteria, reside on conjugative plasmids. Conjugative plasmids have four groups of "backbone" genes, for DNA replication, partition, control of copy number, and conjugative transfer, plus auxiliary genes encoding functions such as antibiotic and heavy metal resistance, or degradation of complex organic chemicals [2, 3]. Gene names and functions vary from plasmid to plasmid; the well-studied IncFII plasmid R100 is used here as an example.

2.1 DNA Replication

Plasmids are divided into several incompatibility groups based on their DNA replication machinery, which determines the host range of the plasmid. For example, IncF plasmids are limited to Enterobacteriaceae while IncP-1 plasmids can also

P.H. Roy, Ph.D. (✉)
Centre de recherche en Infectiologie, Université Laval, Québec, QC G1V 0A6, Canada
e-mail: paul.roy@crchul.ulaval.ca

S.R. Partridge, D.Phil.
Centre for Infectious Diseases and Microbiology, The Westmead Institute for Medical Research, The University of Sydney and Westmead Hospital, Sydney, NSW 2145, Australia
e-mail: sally.partridge@sydney.edu.au

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_5

be maintained in a wide variety of Pseudomonadaceae and many other Gram-negative bacteria. The term "incompatibility (Inc) group" arises from the fact that two plasmids of the same group are incompatible, i.e., are not stably maintained together since the replication and partition machinery cannot tell them apart, and daughter cells will, after a few generations, end up with all of one or all of the other plasmid.

Plasmids have an *oriV* site as the origin of replication [4]. Replication of plasmids of Gram-negative bacteria is usually by a theta structure intermediate and may be unidirectional or bidirectional, while Gram-positive plasmids may use either a theta-intermediate or rolling-circle method of replication. A plasmid-borne replication initiation protein is often involved in replication, although most replication functions (DnaA, DnaB, DnaC, DNA polymerase III, and DNA gyrase) are furnished by the host cell [5].

2.2 Partition and Control of Copy Number

Plasmids often have membrane-attachment mechanisms (in IncP-1, ParA and ParB, and a centromere-like site) that segregate the plasmid molecules into daughter cells. Some plasmids share the XerCD dimer resolution system used by the bacterial chromosome, but use different auxiliary proteins. Even if the daughter cells receive unequal numbers of plasmid molecules, the expression of the plasmid replication initiation protein (TrfA in IncP-1) is tightly controlled to assure the proper number of plasmid copies [6]. Additionally, plasmids may contain one or more "plasmid addiction systems" encoding a stable "toxin" and labile "antidote." In plasmid-free segregants, the "antidote" is degraded and the "toxin" kills the plasmid-free daughter cell, thus maintaining the plasmid in the cell population. The "toxin" and "antidote" may both be proteins or, as in the IncF plasmid R1, the "antidote" can be an antisense RNA (*sok*) whose degradation allows expression of the *hok* gene encoding the "toxin" [7].

2.3 Conjugative Transfer

The conjugative transfer system includes a distinct site, *oriT*, as the origin of conjugative replication, within which a single strand is produced by displacement synthesis and transferred into the recipient cell. Several genes are involved in the synthesis of pili and in membrane modifications involved in mating pair formation [8]. Among these are *trbC*, coding for the pilin subunit, *traI*, coding for a nickase (also called relaxase) acting at *oriT* to begin DNA replication, and *traJ* and *traK*, coding for components of the relaxosome. DNA is transferred as a single strand through the mating pore, and the second strand is synthesized in the recipient cell.

2.4 Mobilizable Plasmids

Plasmids do not necessarily need to encode the entire conjugative machinery in order to be transferred. They need an *oriT* site and DNA processing genes but can depend on another plasmid's mating pair formation genes [8]. When conjugative and mobilizable plasmids are present in the same cell the conjugation machinery may promote transfer of either or both plasmid types.

2.5 Antibiotic Resistance and Other Auxiliary Genes

Antibiotic resistance genes on conjugative plasmids are often associated with smaller mobile elements: certain insertion sequences, composite transposons, simple transposons, and integrons. In order for a plasmid to remain successful, insertion of one of these elements must not interrupt vital functions and so insertions in or close to existing mobile elements appear common. This often results in the formation of complex multiresistance regions (MRRs; described in detail by Partridge [9]) and certain insertions seem to have created "winner" combinations with large numbers of variants.

Plasmid R100 [10] carries a good example of an MRR and the winner combination of an integron within transposon Tn*21* (Fig. 5.1). Chloramphenicol resistance is carried on a composite transposon similar to Tn*9*. Within this element is a simple transposon, Tn*21*, coding for mercury resistance, and the whole element is called Tn*2670*. Within Tn*21* is a defective site-specific simple transposon similar to Tn*402*, and this element contains an integron containing antiseptic and sulfonamide resistance and additionally a streptomycin resistance gene cassette, *aadA1*. The defective transposon also carries the insertion sequence IS*1326* with another insertion sequence, IS*1353,* inserted within it. Tetracycline resistance is carried elsewhere on the plasmid as part of the composite transposon Tn*10*.

In addition to favoring rapid evolution of conjugative plasmids, association with mobile elements gives an additional advantage for resistance genes: if the plasmid is unable to replicate in the recipient cell due to host range limitations, the resistance gene can "hop" to another plasmid or to the chromosome, either as part of a transposon or as an integron cassette.

3 Genomic Islands

3.1 *Salmonella* Genomic Islands (SGI)

The first genomic resistance island described was SGI1 from *Salmonella enterica* serovar Typhimurium DT104 and many variants (designated SGI1-A, SGI1-B, etc.) have since been

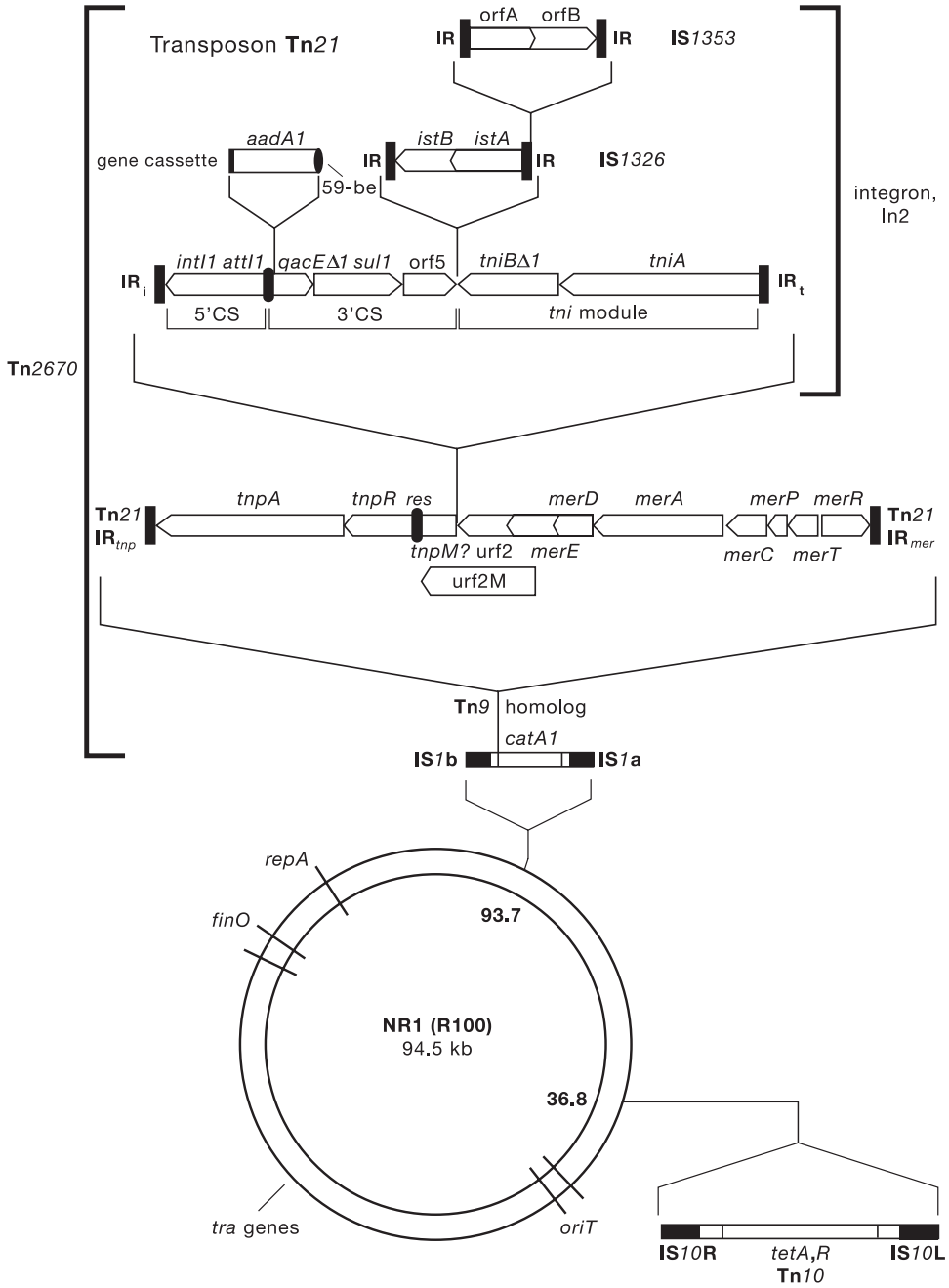


Fig. 5.1 The conjugative plasmid R100. This plasmid contains several embedded mobile elements including Tn*10* and Tn*2670*, which is made up of Tn*21* within a Tn*9*-like transposon. Tn*21* contains an integron within a defective Tn*402*-like element. The integron contains one mobile gene cassette, and the Tn*402*-like element contains two insertion sequences, one within the other. Adapted from [10]

identified in different *Salmonella* serovars [11–13] and also in *Proteus mirabilis* [14, 15]. The 27.4 kb "backbone" of SGI1 contains 28 open reading frames of mostly unknown function: int_{SGI} (S001), S002-026, *resG* (S027, also called *tnpR*) and S044 (Fig. 5.2A). SGI1 is found inserted into a specific site in the chromosome, close to the end of the *trmE* (formerly called *thdF*) gene. The Int_{SGI} recombinase is responsible for insertion and also excision of SGI1 to create a circularized form. This cannot transfer itself horizontally to new cells, as it lacks a full set of conjugation genes, but it can be mobilized by IncA/C plasmids [16].

SGI1 and variants carry a transposon-borne class 1 integron (see Sect. 7.1 below) inserted between *resG* and S044, flanked by 5 bp direct repeats indicative of transpositional insertion. The integron in SGI1 itself (designated In104) carries five resistance genes, *sul1* gene (sulfonamide resistance), the bla_{CARB-1} (*blaP1*; ampicillin) and *aadA2* (streptomycin-spectinomycin) gene cassettes separated by a region containing the *floR* (chloramphenicol-florfenicol) and *tetA*(G) (tetracycline) genes and an IS*CR3* element (Sect. 8.3). The integrons in SGI1 variants have differences

that can be explained by homologous recombination, including exchange of gene cassettes, loss of a gene cassette array plus the central *floR*-*tetA*(G) region, or acquistion of additional genes, e.g., *dfrA10* (trimethoprim resistance) [12, 13]. In SGI1-H a short region of the backbone is missing and in derivatives, such as SGI1-K, extra resistance genes and a mercury resistance operon are present [12, 17].

SGI2 (formerly SGI1-J) is related to SGI1 (144 single nucleotide differences between backbones) and has been found in several *Salmonella* serovars [18]. SGI2 is inserted in the same chromosomal location as SGI1 but the integron is inserted in S023 (Fig. 5.2A), rather than between *resG* and S044, indicative of independent acquisition. The integron in the original SGI2 is related to In104 but has a variant of *floR*, called *cmlA9*, and only one cassette array (*dfrA1-gcuC*). Variants of this arrangement, equivalent to those seen for SGI1, have been identified. The integron in SGI2 has also been found in *Pseudomonas aeruginosa* [19].

3.2 *Acinetobacter baumannii* Resistance Islands

The first example of an *Acinetobacter baumannii* resistance island (AbaR1) was found by whole genome sequencing of an *A. baumannii* isolate [20] and a number of variants have now been identified in global clonal complex 1 (GC1) isolates [21, 22]. These islands have a 16 kb backbone corresponding to transposon Tn*6019*, which is related to Tn*7*/Tn*5053*-like site-specific transposons (see Sect. 5.3), leading to an alternative designation of AbaR1 as Tn*AbaR1* [23]. Tn*6019* is bounded by inverted repeats and is inserted in the chromosomal *comM* gene, flanked by 5 bp direct repeats, and includes an arsenate resistance operon (*ars*) and other genes (Fig. 5.2B).

A composite transposon (see Sect. 4) bounded by two copies of the cadmium and zinc resistance transposon Tn*6018* is inserted in Tn*6019*, flanked by 8 bp direct repeats. The region

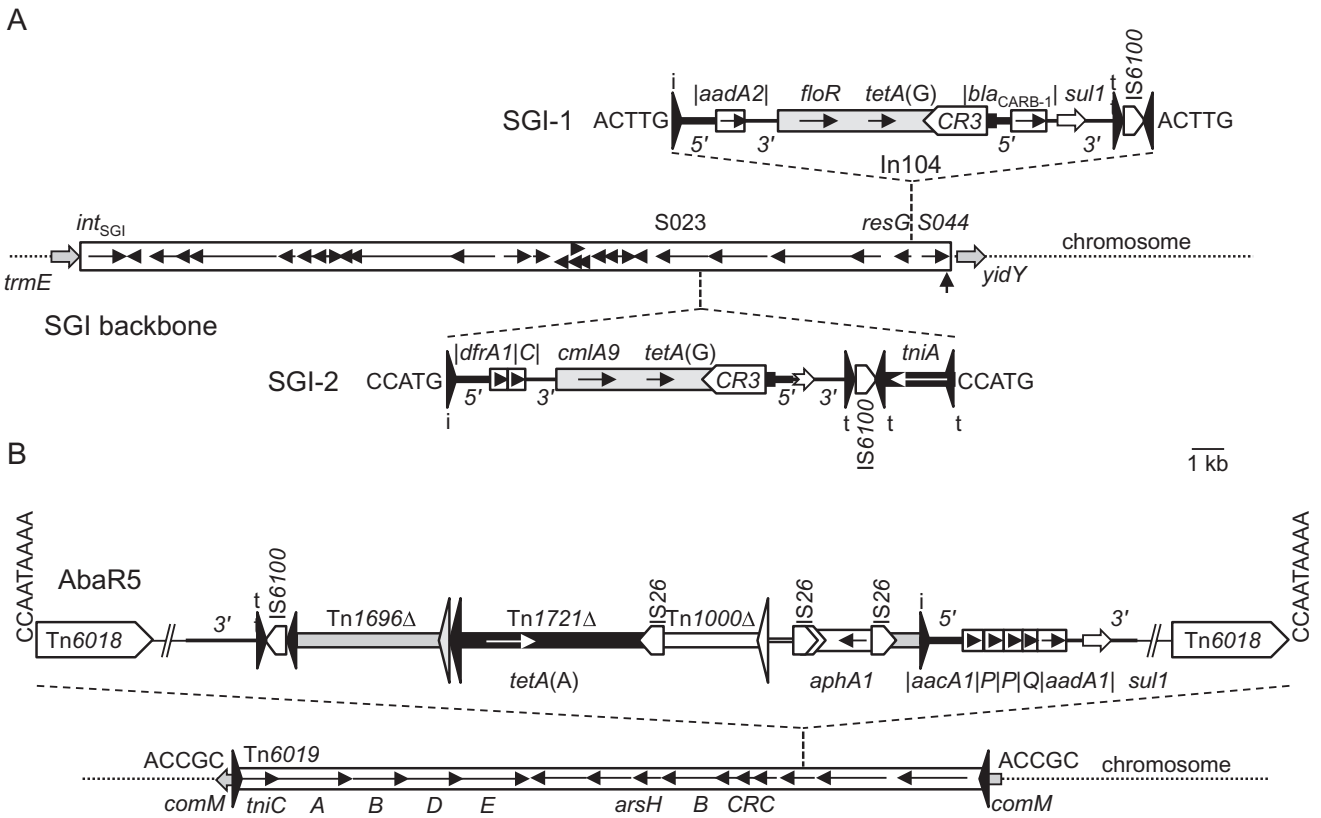


Fig. 5.2 Examples of genomic resistance islands. Resistance and backbone genes are indicated by arrows and selected genes are named. Transposon fragments are indicated, IS are shown as pointed boxes, inverted repeats as tall triangles, gene cassettes as small boxes (with C, P, and Q indicating *gcu),* and the 5′-CS (5′) and 3′-CS (3′) of class 1 integrons are indicated. The sequences of flanking direct repeats are shown. (**a**) SGI1 and SG12 are inserted in the same chromosomal location and have very similar backbones, but different class 1 integrons are inserted in different places. (**b**) AbaR5 as an example of an AbaR island, consisting of a complex MRR containing different transposon fragments, IS and resistance genes in a Tn*6018*-mediated composite transposon inserted in a Tn*6019* backbone, which is inserted in the chromosomal *comM* gene

between the two Tn*6018* contains different combinations of antibiotic resistance genes, mobile elements and other genes in different AbaR variants. For example, AbaR5 [21] has the *tetA*(A) (tetracycline) and *aphA1* (kanamycin) resistance genes plus the *aacC1* (gentamicin) and *aadA1* gene cassettes and the *sul1* gene, fragments of several transposons and some insertion sequences (Fig. 5.2B). Other variants have additional resistance genes and mobile elements or deletions compared with AbaR5 [22, 24] and AbaR variants have been identified in isolates that are not part of GC1 [25]. A simpler island carrying only the bla_{OXA-23} gene, based on a Tn*6019*-like transposon called Tn*6022*, is known as AbaR4 [26].

In multi-resistant isolates belonging to *A. baumannii* global clonal complex 2 (GC2), a distinct (but related) type of resistance island (named AbGRI1; [27]) is inserted in the *comM* gene. The backbone is a Tn*6019*-like transposon, but this island lacks the Tn*6018* composite transposon and has a different set of resistance genes, although these encode resistance to a similar set of antibiotics, namely sulfonamides (*sul2*), streptomycin (*strAB*), and tetracycline (*tetA*(B)), and sometimes the bla_{OXA-23} carbapenemase gene. A second resistance island, named AbGRI2, is also found inserted in another location in GC2 isolates and can include the *aphA1*, *aacC1,* and *aadA1* genes found in some AbaR variants [27].

3.3 *Pseudomonas aeruginosa* Genomic Islands

Comparison of the sequences of the first few complete genome sequences of *P. aeruginosa* led to the definition of numerous genomic islands containing various nonessential genes, including virulence factors, transporters, regulatory genes, novel metabolic pathways, restriction-modification systems, and resistance genes [28, 29]. Some of these islands contain an integrase gene at one end and some are inserted next to tRNA genes. Several resistance genes are found in these genomic islands [30]. In a recent study of resistance genes from 390 *P. aeruginosa* genomes, numerous multiresistance integrons were found to be on chromosomal resistance islands [31].

4 Composite Transposons and Insertion Sequences

Many of the earliest described mobile elements encoding antibiotic resistance are composite transposons. Among these are Tn*9*, encoding chloramphenicol resistance, Tn*10*, encoding tetracycline resistance [32], and Tn*5*, encoding kanamycin, bleomycin, and streptomycin resistance [33] (Fig. 5.3). Composite transposons consist of a region of DNA flanked by insertion sequences, in either direct or inverse orientation.

4.1 Insertion Sequences

Insertion sequences (ISs) are cryptic mobile DNA elements, i.e., coding only for their own mobility. Different IS were originally numbered, but are now assigned names reflecting the species in which they were first identified (e.g., IS*Aba1* for *Acinetobacter baumanni;* https://www-is.biotoul.fr/), but this does not necessarily imply any particular association with that species. They typically encode a transposase that acts at short inverted repeats (IR) at the ends of the element, designated left (IR_L) and right (IR_R) in relation to the direction of transcription of the transposase gene.

IS can move either conservatively or replicatively. In conservative transposition, the transposase cuts at each end of the IS, holding the ends together while it finds a target site. The target site is then cut and the ends of the IS are ligated to the site. In replicative transposition, the transposase binds to the target site, cuts it and attaches the ends to the ends of the IS so as to create a structure that resembles a nearly completed round of replication, with two replication forks approaching each other. Replication of the IS then occurs, creating a cointegrate structure in which the donor and recipient molecules are joined by two copies of the IS, each of the latter consisting of one parental and one newly synthesized strand. Site-specific recombination at a *res* site within the IS then separates the donor and recipient molecules, leaving each with a copy of the IS. The mechanism of replicative transposition was worked out for bacteriophage Mu [34] and also applies to unit transposons of the Tn*3* family (see Sect. 5.1 below).

4.2 Formation and Movement of Composite Transposons

When two copies of the same IS insert on either side of a short region of DNA such as an antibiotic resistance gene, a composite transposon is created. The transposase can then act at the extremities of the entire element, rather than the extremities of a single IS, thus moving the two IS copies and the central region together. For example, Tn*9* (Fig. 5.3) consists of two directly repeated copies of the insertion sequence IS*1* flanking the *catA1* gene coding for a chloramphenicol acetyltransferase. Tn*10* consists of two copies of IS*10* in inverted orientation flanking a tetracycline resistance determinant. Since the short inverted repeat sequences of the two IS ends are identical, the transposase can recognize the entire composite transposon as if it were a single copy of the IS.

Tn*10* undergoes conservative transposition [35] and possesses an interesting mechanism of regulation. The DNA ends contain GATC sequences that are target sites for the Dam methylase. Newly replicated DNA is methylated on only the parental strand (since the Dam methylase lags behind the DNA polymerase) and this hemimethylated state

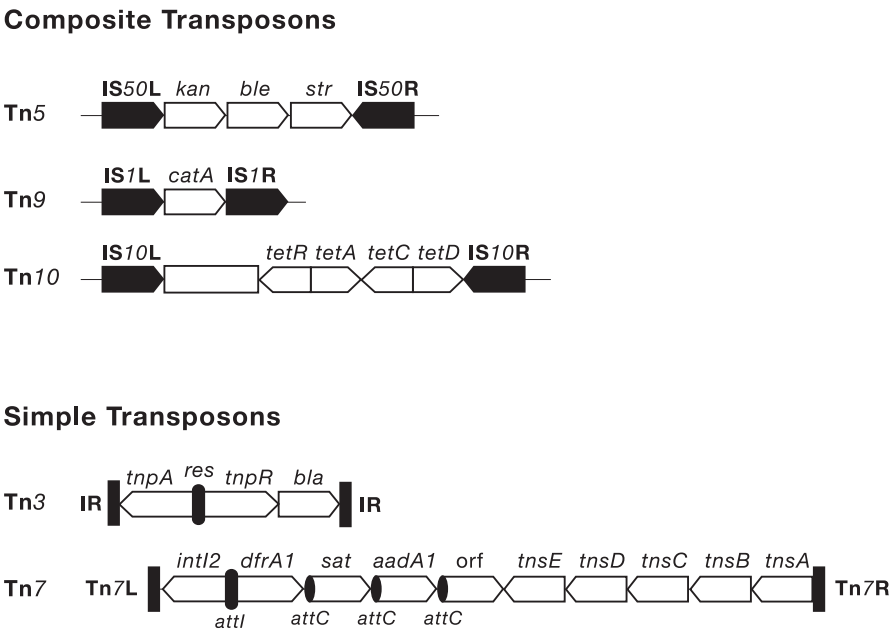


Fig. 5.3 Composite and unit transposons. Composite transposons have a central region with one or more genes flanked by insertion sequences in direct (Tn*9*) or inverted (Tn*5*, Tn*10*) orientation. Unit transposons are flanked by short (38 bp for Tn*3*) inverted repeats. Tn*3* has two transposition genes and carries the TEM-1 β-lactamase gene; Tn*7* has five transposition genes and carries an integron with three mobile cassettes

favors transposition. Tn*5* also undergoes conservative transposition [33, 36] and has a central region with three resistance genes, *kan* (*aph*(*6*)*-IIc)* encoding an aminoglycoside phosphotransferase, *ble* encoding bleomycin resistance, and *str* encoding a streptomycin phosphotransferase, flanked by two copies of IS*50* in inverted orientation. These copies are nearly identical; one copy furnishes the active transposase while in the other copy a point mutation creates a premature stop codon in the transposase but at the same time provides a promoter for the transcription of the antibiotic resistance gene cluster.

Genes encoding several recently identified and clinically important carbapenemases have been found as part of composite transposons. The $bla_{\text{OXA-48}}$ gene appears to have been mobilized from *Shewanella* species by IS*1999* as part of the composite transposon Tn*1999*, found inserted in the IncL/M plasmid pOXA-48 and close relatives [37]. The $bla_{\text{OXA-23}}$ gene is found within an IS*Aba1*-mediated composite transposon (named Tn*2006*) in *A. baumannii* [38]. *Acinetobacter* spp. may also have acted as an intermediate in the transfer of the bla_{NDM} metallo-β-lactamase gene from an unknown progenitor organism to the *Enterobacteriaceae*: in plasmids from various *Acinetobacter* spp. $bla_{\text{NDM-1}}$ is found in an IS*Aba125*-mediated composite transposon, referred to as Tn*125* [39], and fragments of this structure are present in plasmids found in *Enterobacteriaceae* [40].

Composite transposons are also common in Gram-positive bacteria. An example is Tn*4001*, where a bifunctional *aac-aph* gene encoding gentamicin resistance is flanked by two copies of IS*256* [41]. Another is Tn*1547*, in which a chromosomal *vanB* operon is known to have undergone first one and then another insertion of IS*256*, forming an element in which the *vanB* operon, including the *vanRS* two-component regulator, became a transposon that then "hopped" onto a conjugative plasmid [42].

4.3 IS*Ecp1* and Related Elements

A single copy of the insertion sequence IS*Ecp1* has been demonstrated to move regions adjacent to its right-hand (IR_R) end, creating direct repeats of five (or sometimes six) bp flanking the whole transposition unit [43]. The right end of the mobilized regions have some resemblance to the IS*Ecp1* IR, but the precise mechanism of mobilization is not yet known. IS*Ecp1* is also able to pick up different lengths of adjacent DNA in different transposition events [9]. IS*Ecp1* appears to have been responsible for capturing the progenitors of several of the $bla_{\text{CTX-M}}$ sub-families from the chromosomes of different *Kluyvera* spp. and transferring them to plasmids [44]. IS*Ecp1* is also responsible for mobilization of the *Citrobacter freundii* ($bla_{\text{CMY-2}}$) chromosomal *ampC* gene onto plasmids [45]. Several other IS, including IS*1247*, IS*Enca1,* and IS*Sm2*, appear to have mobilized adjacent resistance genes in a similar fashion [9].

4.4 Transposition by a Single Copy of IS*26*

The insertion sequence IS*26* is often identified in multiple copies in MRR and resistance plasmids. Historically, several IS*26*-mediated composite transposons have been identified, but in many MRR single copies of IS*26* separate segments carrying different resistance genes and these IS*26* are not

flanked by the 8 bp direct repeats characteristic of this IS. Recently, it was demonstrated that a single copy of IS*26* can mobilize an adjacent region containing a resistance gene by a mechanism that requires the IS*26* transposase and is not dependent on homologous recombination [46]. IS*26* was found to preferentially insert these "translocatable units" (TU) adjacent to another copy of itself, which can explain the observed chains of resistance modules.

4.5 Resistance Gene Expression From IS

In composite transposons the IS upstream of the captured resistance genes often provides a promoter (or at least the −35 component) for expression of these genes. The *aacC3* aminoglycoside-(3)-acetyltransferase was found both in its "original" plasmid context and in a composite transposon formed by flanking insertions of IS*26*. In the latter, the −35 element of the promoter is replaced by a stronger −35 element present in the end of IS*26*. Moreover, the correct spacing of the −35 and −10 elements is maintained, resulting in increased expression of *aacC3* in its transposon context [47]. IS*Aba1* in Tn*2006* provides the promoter for expression of $bla_{\text{OXA-23}}$ [38]. IS*Aba125* provides the −35 element for bla_{NDM} genes in the Tn*125* composite transposon [48] and this relationship is preserved in the fragments of Tn*125* found on plasmids in *Enterobacteriaceae* [40]. IS*Ecp1* also includes a promoter near IR_R that has been demonstrated to be responsible for expression of several adjacent resistance genes [43].

Insertions of a single copy of an IS can also alter expression of nearby antibiotic resistance genes. The TEM-6 extended spectrum β-lactamase is overexpressed by the insertion of an IS*1*-like element upstream, furnishing a −35 element to form a stronger promoter than in the original context [49]. As well as providing a promoter for $bla_{\text{OXA-23}}$ in Tn*2006*, insertion of IS*Aba1* upstream of intrinsic *Acinetobacter baumannii* $bla_{\text{OXA-51}}$-like genes provides a promoter that increases expression, resulting in measurable carbapenem resistance [50]. Similarly, IS*Aba1* insertions upstream of the *A. baumannii* chromosomal *ampC* gene, known as bla_{ADC}, can increase expression and thus β-lactam resistance [51].

5 Unit ("Simple") Transposons

5.1 Tn*3* and bla_{TEM} β-Lactamase Genes

Perhaps the best-known unit transposon is Tn*3*, which encodes the TEM-1 β-lactamase. Unlike composite transposons, simple transposons have no long repeats, only short inverted repeats at their extremities (38 bp in the case of Tn*3* and related Tn) (Fig. 5.3). Simple transposons of the Tn*3* family carry a *tnpA* gene encoding a transposase and a *tnpR* gene encoding a resolvase. These genes may be either divergently transcribed as in Tn*3* or co-transcribed as in Tn*21* [10]. These transposons also contain a *res* site. They undergo replicative transposition [34, 52], in which the transposase carries out the first step (nicking of the transposon ends and of the target site) and the resolvase carries out the final step (site-specific recombination at the *res* site to resolve the cointegrate structure) [53]. The bla_{TEM} gene of Tn*3* has undergone significant evolution and selection of point mutants to yield extended-spectrum varieties resistant to cefotaxime and ceftriaxone (e.g., TEM-3), ceftazidime (e.g., TEM-5) and/or β-lactamase inhibitors [54]. Currently there are >200 TEM variants (http://www.lahey.org/Studies), nearly half with an ESBL phenotype [55].

The appearance of penicillin-resistant *Haemophilus* and *Neisseria* beginning in 1974 [56] appears to have been due to transfer of Tn*3*, either by transformation or conjugation, onto a plasmid unable to replicate in these species. Tn*3* appears to have transposed onto resident cryptic plasmids before the original plasmid was degraded. Versions of the recipient plasmid with an intact Tn*3* have been found in *Haemophilus ducreyi*, while in *Haemophilus parainfluenzae* and *Neisseria gonorrhoeae* truncated versions lacking *tnpA* and *tnpR* are found [57]. Remarkably, after 30 years and despite widespread use of ceftriaxone for treatment of gonorrhea, there is still no report of mutant, extended-spectrum, β-lactamases from *N. gonorrhoeae*. Could this be related to the fidelity of its DNA polymerase?

5.2 Tn*1546* and Vancomycin Resistance

Tn*1546* was first found in vancomycin-resistant enterococci (VRE) and contains *tnpR* and *tnpA* genes at its left end, followed by a complete gene cluster encoding vancomycin resistance. The *vanRS* two-component system encodes a sensor and a regulator that respond to the presence of vancomycin in the medium [58, 59] and induce expression of the *vanHAX* operon encoding a D-Ala-D-lac depsipeptide in place of D-Ala-D-Ala at the termini of the pentapeptide of the peptidoglycan layer, thereby altering the target of vancomycin [58, 60]. Auxiliary genes recycle the components of the wild-type termini. Tn*1546* has now been found in *Staphylococcus aureus*, producing a fully vancomycin-resistant VRSA [61].

5.3 Site-Specific Transposons: Tn*7* and the Tn*5053* Family

Tn*7* is a site-specific transposon that was found to insert into unique sites in the chromosome of *E. coli* and in bacteriophage lambda. It carries short inverted repeats at its ends, five transposition genes (*tnsA-E*) in its right half and a class 2 integron (see

below) with *dfrA1, sat*, and *aadA1* gene cassettes encoding trimethoprim, streptothricin, and streptomycin/spectinomycin resistance. Tn*7* usually undergoes conservative transposition, but also has a replicative transposition mode [62].

A related family of site-specific transposons is the Tn*5053* family, including Tn*402*. They have four transposition genes, *tniA, tniB, tniQ*, and *tniR*, and a *res* site. The *tniA* gene encodes a D,D(35)E protein and is homologous to *tnsB* of Tn*7*. The *tniB* gene has an ATP-binding motif and is homologous to *tnsC* of Tn*7*. The *tniR* gene encodes a serine recombinase of the invertase-resolvase family. Tn*402* carries a class 1 integron (see below) with *dfrB3* and *qacE* gene cassettes encoding trimethoprim and quaternary-ammonium-compound resistance, respectively. Tn*402* and the mercury resistance transposon Tn*5053* are closely related, and the products of their transposition genes can complement each other in *trans* [63]. These transposons have been characterized as *res* site hunters; they preferentially insert in *res* sites (e.g., in Tn*1696*), except in Tn*21* where insertion has occurred close to, but not in, *res* (Fig. 5.1), which may help to explain the success of variants of this structure.

5.4 Tn*4401*

Tn*4401* is a Tn*3*-family element in which a bla_{KPC} class A carbapenemase gene is flanked by two distinct insertion sequences, IS*Kpn6* and IS*Kpn7*. Different variants of Tn*4401*, with different deletions in the promoter region, appear to influence KPC expression [64] and some plasmids with bla_{KPC} carry only part of Tn*4401*. bla_{KPC} genes, originally found in *Klebsiella pneumoniae*, have now spread to other species.

5.5 Miniature Inverted-Repeat Transposable Elements (MITES)

MITE elements appear to be transposon derivatives consisting of little more that the two terminal IR separated by a short region, i.e., they lack a transposase, but they may carry a promoter or otherwise influence expression of adjacent genes [65]. MITES are presumably mobilized by transposition proteins present in the same cell. Two copies of a 439 bp MITE have been found flanking and truncating class 1 integrons associated with different gene cassettes and other resistance genes in *Acinetobacter* spp. [66]. Some MITES appear to be derivatives of Tn*3*-family transposons and the name TIME (Tn*3*-derived inverted-repeat miniature elements) has been coined for these [67]. Two copies of a 288-bp TIME are found truncating a class 1 integron fragment carrying the bla_{GES-5} gene cassette [68].

6 ICE (Conjugative Transposons)

Integrative conjugative elements or ICEs (formerly called conjugative transposons) are elements that can reside either on the bacterial chromosome or on plasmids. They have a broad host range and are important vehicles of antibiotic resistance in Gram-positive bacteria, notably in streptococci, but also occur in *Bacteroides* and Enterobacteriaceae. They are capable of excision from the donor chromosome or plasmid to form a non-replicative circle, which then undergoes conjugal transfer into the recipient bacteria [69] (Fig. 5.4).

6.1 Tn*916*-Like Elements and Their Antibiotic Resistance Genes

The type element of this group is Tn*916*, discovered in 1981 [70], that carries the tetracycline resistance gene *tet*(M). Tn*916* is 18 kb in size and has 18 ORFs, among which are an integrase and an excisase similar to those of lambdoid phages, as well as genes coding for intracellular and extracellular transposition functions. There is also an *oriT* site for transfer of single-stranded DNA from the excised circle. The frequency of transfer is relatively low and may be related to the DNA sequences flanking the donor element [71]. A closely related element, Tn*1545* [72], carries kanamycin and erythromycin resistance genes in addition to *tet*(M). A newer conjugative transposon, Tn*5382* [73], also called Tn*1549* [74], was first isolated from *Enterococcus faecium* and carries the *vanB* vancomycin resistance operon in place of *tet*(M). Although *E. faecium* carrying *vanB* represents a smaller proportion of vancomycin-resistant enterococci than *E. faecalis* carrying *vanA* mediated by the simple transposon Tn*1546*, the former is clinically significant.

Several novel ICEs similar to Tn*916* and to Tn*1549* have been described in *Clostridium difficile*, and notably carry genes encoding ABC transporters [75].

6.2 Mechanism of Transfer

Transfer is initiated by excision of the conjugative transposon into a non-replicative circular intermediate, mediated by the *int* and *xis* genes located at one extremity of the element. These genes are similar to the *int* and *xis* genes of phage lambda, and the process is analogous to phage excision, except that the "sticky ends" produced are not complementary, as there is no equivalent of an *attB* site. The *oriT* site is then nicked, and DNA replication by strand displacement then begins. Conjugal transfer resembles that of conjugative plasmids [76]. The second

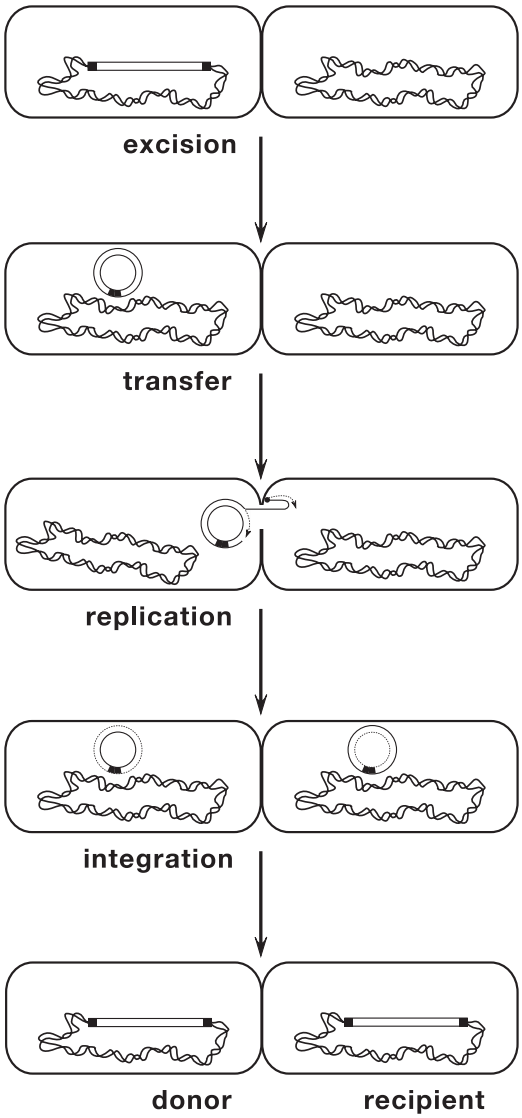


Fig. 5.4 Transfer of an ICE. The ICE excises and forms a circle; a single strand is then transferred into the recipient while replacement synthesis takes place in the donor. The second strand is synthesized in the recipient; circular copies are then able to integrate into the chromosome. Adapted from [78]

strand is synthesized in the recipient, and integration involves the Int protein. In contrast to phage lambda, integration, while showing site preferences, is not site-specific [69, 77].

6.3 Related Elements

CTnDOT is an ICE from *Bacteroides* that carries the *tet*(Q) gene coding for a ribosomal protection mechanism and the *erm*(F) gene for macrolide-lincosamide-streptogramin (MLS) resistance [78]. The latter gene, instead of being in the usual central region, is at the extremity, beyond the *int* gene. The *tet*(Q) and *erm*(F) genes are also found in *Prevotella* and *Porphyromonas*, providing evidence for horizontal transfer. CTnDOT has a greater degree of site specificity than Tn*916*.

Bacteroides also has mobilizable transposons such as NBU1. This element has its own integrase and *oriT* site and, analogously to mobilizable plasmids, can be transferred by the transfer genes of a conjugative transposon such as CTnDOT. Integration of NBU1 is site-specific, with a 14-bp recognition site [79]. Tn*5398* of *Clostridium difficile* is another mobilizable transposon, and carries the *erm*(B) MLS resistance gene [80].

Another group of site-specific ICEs are the SXT elements of *Vibrio*. They were previously referred to as constins (conjugative, self-transmissible, integrating elements). The resistance gene content varies, but they often encode resistance to trimethoprim-sulfa as well as streptomycin and chloramphenicol. These elements are site-specific. Most of the resistance genes are in a cluster but the trimethoprim resistance gene is a cassette, next to an integrase gene, in a distinct class of integron [81].

7 Integrons

7.1 Class 1 Integrons

Integrons are elements composed of an *intI* gene encoding an integrase of the tyrosine recombinase (phage integrase) family, an *attI* site, and one or more gene cassettes. A cassette is usually composed of a single, promoterless structural gene and a palindromic *attC* site (previously called a 59-base element) with conserved consensus sequences at its ends and conserved structure (but not sequence) in the center [82]. The integrase mediates the site-specific excision and integration of the mobile gene cassettes [83]. In the early 1980s, restriction enzyme digests and electron microscopy heteroduplex experiments pointed to the existence of gene-sized insertions in otherwise identical plasmids. DNA sequencing resulted in the discovery of gene cassettes flanked by a "5′-conserved segment" (5′-CS) with the *intI1* gene and *attI1* site, and a "3′-conserved segment" (3′-CS) that turns out to contain a truncated *qacE* gene cassette followed by a nonspecifically inserted *sul1* gene encoding a sulfonamide resistant dihydropteroate synthase [84]. The 5′-CS also contains a promoter (Pc) directed toward the cassettes that is responsible for their expression [85, 86]. Class 1 integrons are often found within transposons related to Tn*21* and are often (erroneously) referred to as "Tn*21*-like elements." Many Tn*21*-family transposons in fact differ only by the cassette content of their integrons, e.g., Tn*2603* that differs from Tn*21* only by a single additional cassette encoding the OXA-1 β-lactamase [87]. Most class 1 integrons are in fact on defective simple transposons of the Tn*402* family; some of these have found their way onto competent transposons related to Tn*21* while others occur on plasmids such as R46 and R388, etc. where they do not "piggyback" on another mobile element.

7.2 Recent Evolution of Class 1 Integrons

Sequence analysis gives us some clues concerning the evolutionary history of class 1 integrons. This suggests an ancient association of *intI1* and *attI1* with a Tn*402*-like transposon containing only the four *tni* transposition genes and associated 25-base inverted repeats (called IRi, at the integrase end, and IRt, at the *tni* end) [88]. The first cassette to be integrated would have contained the *qacE* gene, encoding resistance to quaternary-ammonium compounds by an *smr* family efflux mechanism. This cassette, unlike the rest of the elements, is AT-rich and has a very different codon usage pattern from the other five genes, indicating that *qacE* was laterally transferred, probably from a low-GC Gram-positive organism (class Firmicutes). Two subsequent events provided the ancestor of ca. 95 % of the class 1 integrons seen today: (1) addition of the *sul1* sulfonamide resistance gene by a non-site-specific event that also removed part of the *qacE* cassette including the *attC* site, effectively locking it into place as -part of the 3′-CS -, and (2) deletion of the *tniQ* and *tniR* genes as well as part of the *tniB* gene. This resulted in a defective transposon as a vehicle for most class 1 integrons. The movement of such transposons with intact IRi and IRt catalyzed by Tni proteins provided in *trans* has now been shown to occur, with RecA-mediated cointegrate resolution in the absence of the *res* site [89]. Subsequent events included insertion of IS*1326* with or without IS*1353* between the *sul1* and *tniB* genes [90], and the acquisition of the *aadA1* cassette encoding an adenylyltransferase conferring streptomycin and spectinomycin resistance. This cassette is very common, but not ubiquitous, in class 1 integrons [91]. It is interesting to speculate that the *qacE* cassette may have arrived in the integron early in the twentieth century with the use of antiseptics, incorporation of *sul1* may have taken place in the late 1930s, and the *aadA1* cassette was added in the 1950s. Several recent class 1 integrons, notably some carrying bla_{VIM} class B carbapenemase genes, have an intact *tniABQR* module. These may or may not have had an intact *qacE* cassette during their evolution; in any case they represent a separate lineage from the *sul1*-containing integrons (Fig. 5.5).

7.3 Antibiotic Resistance Genes Carried by Integrons

Class 1 integrons contain a great variety of antibiotic resistance gene cassettes [91], and the order of the first occurrence of these cassettes closely mirrors that of the first clinical use of the corresponding antibiotics. Chloramphenicol resistance is often mediated by *cmlA*, which encodes a specific efflux protein of the major facilitator family [92, 93]. Chloramphenicol resistance can also be mediated by *catB* genes such as *catB2* from Tn*2424*, which encodes a chloramphenicol acetyltransferase that is very different from *catA1* encoded by Tn*9* [94]. CatB2 is in fact a member of the xenobiotic acetyltransferase family, and is closely related to Vat and Sat proteins that mediate virginiamycin and streptogramin resistance in Gram-positive bacteria [95, 96]. The *catB2, catB3*, etc., genes in integron cassettes are closely related to the chromosomal *catB1* and *catB7* genes of *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*, respectively [97].

Aminoglycoside resistance genes include various adenylyltransferases and acetyltransferases whose spectrum depends on the availability of specific target positions on the aminoglycoside molecule. The *aadA3* genes encode aminoglycoside-(2″) adenylyltransferases giving resistance to streptomycin and spectinomycin. Gentamicin resistance is commonly mediated by gene cassettes such as *aadB2* encoding an aminoglycoside-(3″) adenylyltransferase [98], or *aacC1* encoding an aminoglycoside-(3) acetyltransferase [99]. Gentamicin resistance can also be conferred by a version of the *aacA4* gene. This gene differs from a version conferring amikacin resistance by a single point mutation, with a serine codon in the gentamicin-resistant, amikacin-sensitive version and a leucine codon in the amikacin-resistant, gentamicin-sensitive version [100]. While the latter was discovered first, the former is the more probable ancestor. As in the case of the ESBLs mentioned above, a point mutation resulted in resistance to the more recent, semisynthetic antibiotic amikacin. Mutations at two additional positions have led to creation of the *aac(6′)-Ib*-cr ("ciprofoxacin resistant") variant, which confers additional low-level resistance to fluoroquinolones, but is less effective against aminoglycosides [101]. An extremely large variety of other *aacA* gene cassettes have now been found in integrons [91], including *aacA7* that encodes resistance to both gentamicin and amikacin [102].

Integrons carry a wide variety of genes encoding β-lactamases. Although less common than the widespread TEM- and SHV-β-lactamases (including their extended-spectrum variants), gene cassettes encode OXA- and CARB-β-lactamases. A few years ago, relatively few novel resistance genes were appearing in integrons as compared to other mobile elements. Except for a variety of *aacA* amikacin resistance genes, most of the new genes found in integrons were for resistance to older antibiotics such as trimethoprim and streptomycin. This situation changed dramatically with the discovery of a gene encoding a class B metallo-β-lactamase conferring resistance to carbapenems. The bla_{IMP-1} gene was first found in a class 3 integron (with a distinct IntI3 integrase and *attI3* site) in *Serratia marcescens* in Japan [103], but then spread to class 1 integrons as well as to other genera (*Klebsiella* and *Pseudomonas*) and geographically. A gene for another class B β-lactamase, bla_{VIM-1}, was first found in an isolate from Italy [104] and a close relative, bla_{VIM-2}, has also undergone worldwide dissemination.

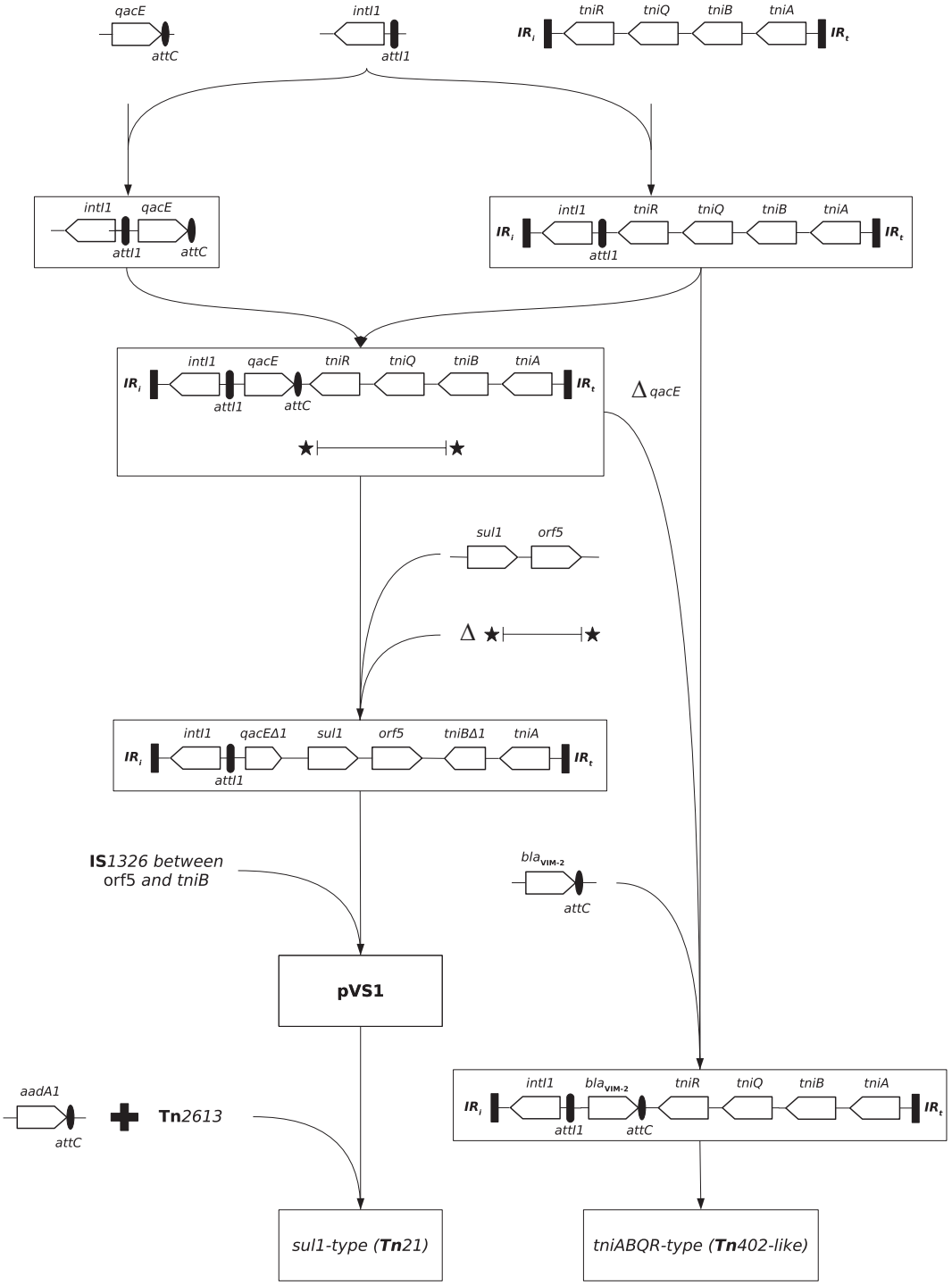


Fig. 5.5 A schema for integron evolution. There was probably an ancient association of the *intI1* gene and adjacent *attI1* site with a Tn*5053*-family transposon to create an immediate precursor of Tn*402* (without gene cassettes). A first cassette, *qacE*, encoding antiseptic resistance, differs greatly from the Tn*402*-precursor in G+C content and codon usage, may have arrived from a Gram-positive organism in the late nineteenth or early twentieth century, and may have become associated with *intI1-attI1* before association of the latter with the transposon. Two subsequent events, the truncation of the *qacE* gene by the arrival of *sul1* and orf5 and the deletion of *tniQ* and *tniR*, immobilized *qacE* and resulted in a defective transposon. These events may have occurred in the 1930s or 1940s. Subsequently, insertion sequences and gene cassettes (beginning with the common but not ubiquitous *aadA1*) may have occurred in the 1950s. The defective transposon can "piggyback" on a competent transposon (the mercury resistance transposon Tn*2613* of the Tn*3* family) to create Tn*21*, thus reacquiring mobility. Recently, a significant fraction of class 1 integrons, notably those carrying genes encoding metallo-β-lactamases, have been found with intact *tniABQR* genes, suggesting a parallel line of evolution from ancestral forms

7.4 Gene Expression in Class 1 Integrons

Class 1 integrons are a sort of natural expression vector, analogous to constructed plasmids used in recombinant DNA experiments, for the inserted cassette genes. The *attI* site in an integron is analogous to a multiple cloning site in an expression vector, and both integron and vector plasmid possess an upstream promoter for expression of the inserted genes. Most of the small number of polymorphisms in the 5′-CS are related to the Pc promoter directed toward the cassette array. At least five common versions of Pc of varying strength exist [105]. In addition, a second promoter (P2), 110 bp downstream of the first, can be created by an insertion of three G residues, changing the spacing of –35 and –10 elements from 14 to 17 bp [85, 86]. Again, just as some expression vectors permit the expression of cloned genes as fusion proteins, integrons can do the same. A 19-bp insert permits the AAC (3′)-I protein to be expressed as a fusion protein from a start codon in the 5′-CS, using an efficient ribosome-binding site to maximize its translational expression [99].

7.5 Chromosomal Integrons

Chromosomal integrons were first observed in *Vibrio cholerae* [106] and genome sequencing projects have revealed their presence in several β-, γ-, and Δ-Proteobacteria, as well as a few taxonomically distant bacteria (spirochetes and planctomycetes). Chromosomal integrons typically have many more cassettes than class 1 integrons, but few, if any, antibiotic resistance genes. The functions of most cassette genes in chromosomal integrons are not known, but they include nonessential genes such as those encoding virulence factors, restriction-modification systems, and plasmid addiction (toxin–antitoxin) systems [107]. Some chromosomal integron integrases have been shown to be active in cassette excision and integration [108–110]. Chromosomal integrons tend to have uniform *attC* sites, and cassette codon usage and G+C content reflects that of the parent organism. In contrast, class 1 integron cassettes have a wide variety of codon usage patterns and G+C contents, indicating a wide variety of origins. Thus, different chromosomal integrons may serve as a reservoir for some antibiotic resistance gene cassettes.

7.6 Origin of Integron Cassettes

A major unanswered question is how genes are recruited into cassettes and become attached to their *attC* sites. Recent evidence suggests a role for an RNA element called a group II intron in this process. Some group II introns (class C) target transcriptional terminators [111]. A group II intron was found in a class 1 integron, inserted exactly at the junction of the *aadB* gene and its *attC* site [112]. This structural gene-intron-*attC* may represent a "frozen" intermediate in cassette formation, along with structural gene-intron and intron-*attC* intermediates found in chromosomal integrons. Group II introns may thus target separately to the ends of structural genes and to *attC* sites, and subsequent steps of homologous recombination, transcription, RNA splicing, and reverse transcription may lead to the formation of novel cassettes [113].

8 CR Elements

8.1 IS*CR1* and Class 1 Integrons

In some class 1 integrons, a region downstream of *sul1* is replaced by a region containing an open reading frame that was first called orf513. A short distance beyond the end of orf513 sequences diverged at a specific site and were followed by antibiotic resistance genes such as *catA2* and *dfrA10* [114], defining a "common region" (CR). These results recalled the discovery of the *attI1* site in the early 1980s. The antibiotic resistance genes are not in cassettes, and the region is followed by a partial duplication of the 3′-CS. The Orf513 protein (now also called Rcr1, for rolling circle replicase) is a transposase of the IS*91* family, a group of IS which transpose by a rolling circle mechanism, and the point at which the sequences diverge has been identified as an extremity (*ori*IS) of an insertion sequence, now called IS*CR1*. The other extremity has not been identified; and it is probable that replication into the adjacent region results in the acquisition of part of the 3′-CS, allowing subsequent insertion, by homologous recombination, into other class 1 integrons [115].

8.2 Antibiotic Resistance Genes Mobilized by IS*CR* Elements

The first two genes found associated with the region now called IS*CR1* were the *catA2* gene coding for a chloramphenicol acetyltransferase and the *dfrA10* gene coding for a trimethoprim-resistant dihydrofolate reductase (DHFR) [114]. CTX-M extended-spectrum type A β-lactamases, beginning with CTX-M-2 [116], the most widespread ESBL in Argentina, have been found in IS*CR1* elements. This gene is virtually identical to the chromosomal β-lactamase of *Kluyvera ascorbata*, and the precise extent of the DNA sequence mobilized by the IS*CR1* element is evident. In contrast, while some resistance gene cassettes in integrons are very similar to chromosomal genes (compare *catB2,* etc. with *catB1* of *Agrobacterium tumefaciens* or *catB7* of *Pseudomonas aeruginosa*, or *aac(6′)-Id* of Tn*4000* with *aac(6′)-Ic* of *Serratia marcescens*), no cassette gene in its "original" chromosomal context has yet been found.

A series of class C β-lactamases (referred to as cephamycinases), including DHA-1 [117] and CMY-9 [118], are also encoded by genes associated with IS*CR1* elements. The bla_{DHA-1} β-lactamase gene is accompanied by the divergently transcribed *ampR* regulatory gene. A novel quinolone resistance determinant, *qnrA*, mediating a gyrase protection mechanism, is associated with an IS*CR1* element [119]. The *armA* gene, one of the relatively recently identified genes encoding a 16S rRNA methylase (high-level resistance to all clinically relevant aminoglycosides) is also associated with IS*CR1* [120].

8.3 Other IS*CR*s

A number of other IS*CR*s have been described. Regions including IS*CR2* have mainly been identified on IncA/C plasmids and their relatives, the SXT element [121]. IS*CR2* is associated with genes conferring resistance to trimethoprim, florfenicol, streptomycin, sulfonamides, and tetracycline. IS*CR3* is principally associated with SGI and resistance to florfenicol and tetracycline (see Sect. 3.1 above). Elements closely related to IS*CR3* are associated with other important resistance genes, including IS*CR27* with bla_{NDM-1} and IS*CR14* with *rmtD* 16S rRNA methylase genes [9]. IS*CR4* is associated with the bla_{SPM-1} carbapenemase gene.

9 Outlook

In the years following the introduction of each new antibiotic, resistance has appeared, either by point mutations of chromosomal genes or by recruitment and lateral transfer from antibiotic producers or other bacteria sharing their environment (e.g., CTX-M-2, CTX-M-3, CMY-2, see Sect. 8.2, or *qnrA* from *Shewanella algae* [122]). Nonetheless, many antibiotics have had many years of useful life before the emergence of resistance reduced their utility. Certain species have still not developed resistance to certain antibiotics, e.g., *Haemophilus* is still susceptible to third-generation cephalosporins. Many broad-spectrum antibiotics have seen their spectrum narrowed by resistance, but can still be useful when the use of rapid molecular diagnostics becomes common practice.

Novel classes of antibiotics, acting against new targets, are sorely needed. Resistance against new antibiotics of existing classes will be quicker to emerge, often by point mutation of an existing resistance gene. This is illustrated by the multiplicity of TEM β-lactamases resistant to third-generation cephalosporins and to β-lactamase inhibitors. Existing resistance genes have to be recognized and taken into account, as in the case of resistance to quinupristin-dalfopristin conferred by the combination of *satA* and *vgb* genes [123]. On the other hand, resistance to new classes of antibiotics should take longer to emerge and be disseminated. Linezolid resistance, for example, has been limited to point mutants in rRNA genes, although another mechanism is suspected to exist. Unpleasant surprises do occur, however. Fluoroquinolone resistance was for a long time limited to accumulation of multiple point mutations in DNA gyrase and topoisomerase (although even this chromosomal resistance has been disseminated by lateral transfer). Only later did plasmid-specified gyrase protection mechanisms, mediated by *qnr* genes, emerge [124]. Another unexpected event was the adaptation of an aminoglycoside acetyltransferase to confer fluoroquinolone resistance (see Sect. 7.3 above) [101].

In the future we can expect to see the continuation of the recruitment of resistance genes from environmental bacteria, by flanking insertions of ISs to create composite transposons, by recombination into conjugative transposons, by nearby insertion of IS*CR*, IS*Ecp1,* or IS*26* elements, by movement of cassettes from chromosomal integrons, and *de novo* formation of new integron cassettes by acquisition of *attC* sites. Again, agents directed against new targets, while not "magic bullets," can nonetheless slow down the process of emergence and dissemination of resistance.

References

1. Watanabe T. Infective heredity of multiple drug resistance in bacteria. Bacteriol Rev. 1963;27:87–115.
2. Adamczyk M, Jagura-Burdzy G. Spread and survival of promiscuous IncP-1 plasmids. Acta Biochim Pol. 2003;50(2):425–53.
3. Grohmann E, Muth G, Espinosa M. Conjugative plasmid transfer in Gram-positive bacteria. Microbiol Mol Biol Rev. 2003;67(2):277–301.
4. Pansegrau W, Lanka E, Barth PT, et al. Complete nucleotide sequence of Birmingham IncPα plasmids. Compilation and comparative analysis. J Mol Biol. 1994;239(5):623–63.
5. Pinkney M, Diaz R, Lanka E, Thomas CM. Replication of mini RK2 plasmid in extracts of *Escherichia coli* requires plasmid-encoded protein TrfA and host-encoded proteins DnaA, B, G DNA gyrase and DNA polymerase III. J Mol Biol. 1988;203(4):927–38.
6. Smith CA, Shingler V, Thomas CM. The *trfA* and *trfB* promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. Nucleic Acids Res. 1984;12(8):3619–30.
7. Jensen RB, Gerdes K. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol Microbiol. 1995;17(2):205–10.
8. Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F. Mobility of plasmids. Microbiol Mol Biol Rev. 2010;74(3):434–52.
9. Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol Rev. 2011;35(5):820–55.
10. Liebert CA, Hall RM, Summers AO. Transposon Tn*21*, flagship of the floating genome. Microbiol Mol Biol Rev. 1999;63(3):507–22.
11. Quinn T, O'Mahony R, Baird AW, Drudy D, Whyte P, Fanning S. Multi-drug resistance in *Salmonella* enterica: efflux mechanisms and their relationships with the development of chromosomal resistance gene clusters. Curr Drug Targets. 2006;7(7):849–60.
12. Hall RM. *Salmonella* genomic islands and antibiotic resistance in *Salmonella enterica*. Future Microbiol. 2010;5:1525–38.
13. Mulvey MR, Boyd DA, Olson AB, Doublet B, Cloeckaert A. The genetics of *Salmonella* genomic island 1. Microbes Infect. 2006;8(7):1915–22.

14. Ahmed AM, Hussein AI, Shimamoto T. *Proteus mirabilis* clinical isolate harbouring a new variant of *Salmonella* genomic island 1 containing the multiple antibiotic resistance region. J Antimicrob Chemother. 2007;59(2):184–90.
15. Siebor E, Neuwirth C. Emergence of *Salmonella* genomic island 1 (SGI1) among *Proteus mirabilis* clinical isolates in Dijon, France. J Antimicrob Chemother. 2013;68(8):1750–6.
16. Doublet B, Boyd D, Mulvey MR, Cloeckaert A. The *Salmonella* genomic island 1 is an integrative mobilizable element. Mol Microbiol. 2005;55(6):1911–24.
17. Hamidian M, Holt KE, Hall RM. The complete sequence of *Salmonella* genomic island SGI1-K. J Antimicrob Chemother. 2015;70(1):305–6.
18. Hamidian M, Holt KE, Hall RM. The complete sequence of *Salmonella* genomic island SGI2. J Antimicrob Chemother. 2015; 70(2):617–9.
19. Perez F, Hujer AM, Marshall SH, et al. Extensively drug-resistant *Pseudomonas aeruginosa* isolates containing $bla_{\text{VIM-2}}$ and elements of *Salmonella* genomic island 2: a new genetic resistance determinant in Northeast Ohio. Antimicrob Agents Chemother. 2014;58(10):5929–35.
20. Fournier PE, Vallenet D, Barbe V, et al. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. PLoS Genet. 2006;2(1), e7.
21. Post V, Hall RM. AbaR5, a large multiple-antibiotic resistance region found in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2009;53(6):2667–71.
22. Adams MD, Chan ER, Molyneaux ND, Bonomo RA. Genomewide analysis of divergence of antibiotic resistance determinants in closely related isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2010;54(9):3569–77.
23. Rose A. Tn*AbaR1*: a novel Tn*7*-related transposon in *Acinetobacter baumannii* that contributes to the accumulation and dissemination of large repertoires of resistance genes. Biosci Horizons. 2010;3(1):40–8.
24. Post V, White PA, Hall RM. Evolution of AbaR-type genomic resistance islands in multiply antibiotic-resistant *Acinetobacter baumannii*. J Antimicrob Chemother. 2010;65(6):1162–70.
25. Ramirez MS, Vilacoba E, Stietz MS, et al. Spreading of AbaR-type genomic islands in multidrug resistance *Acinetobacter baumannii* strains belonging to different clonal complexes. Curr Microbiol. 2013;67(1):9–14.
26. Hamidian M, Hall RM. AbaR4 replaces AbaR3 in a carbapenem-resistant *Acinetobacter baumannii* isolate belonging to global clone 1 from an Australian hospital. J Antimicrob Chemother. 2011;66(11):2484–91.
27. Nigro SJ, Farrugia DN, Paulsen IT, Hall RM. A novel family of genomic resistance islands, AbGRI2, contributing to aminoglycoside resistance in *Acinetobacter baumannii* isolates belonging to global clone 2. J Antimicrob Chemother. 2013;68(3):554–7.
28. Mathee K, Narasimhan G, Valdes C, et al. Dynamics of *Pseudomonas aeruginosa* genome evolution. Proc Natl Acad Sci U S A. 2008;105(8):3100–5.
29. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tummler B. *Pseudomonas aeruginosa* genomic structure and diversity. Front Microbiol. 2011;2:150.
30. Roy PH, Tetu SG, Larouche A, et al. Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. PLoS ONE. 2010;5(1), e8842.
31. Kos VN, Deraspe M, McLaughlin RE, et al. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. Antimicrob Agents Chemother; 2014;59(1):427–36.
32. Haniford DB. Transposon Tn*10*. In: Craig NL et al., editors. Mobile DNA II. Washington, DC: ASM Press; 2002. p. 457–83.
33. Reznikoff WS. Tn*5* transposition. In: Craig NL et al., editors. Mobile DNA II. Washington, DC: ASM Press; 2002. p. 403–22.
34. Shapiro JA. Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc Natl Acad Sci U S A. 1979;76(4):1933–7.
35. Kennedy AK, Guhathakurta A, Kleckner N, Haniford DB. Tn*10* transposition via a DNA hairpin intermediate. Cell. 1998;95(1):125–34.
36. Davies DR, Goryshin IY, Reznikoff WS, Rayment I. Three-dimensional structure of the Tn*5* synaptic complex transposition intermediate. Science. 2000;289(5476):77–85.
37. Poirel L, Bonnin RA, Nordmann P. Genetic features of the widespread plasmid coding for the carbapenemase OXA-48. Antimicrob Agents Chemother. 2012;56(1):559–62.
38. Corvec S, Poirel L, Naas T, Drugeon H, Nordmann P. Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene $bla_{\text{OXA-23}}$ in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2007;51(4):1530–3.
39. Poirel L, Bonnin RA, Boulanger A, Schrenzel J, Kaase M, Nordmann P. Tn*125*-related acquisition of bla_{NDM}-like genes in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2012;56(2):1087–9.
40. Partridge SR, Iredell JR. Genetic contexts of $bla_{\text{NDM-1}}$. Antimicrob Agents Chemother. 2012;56(11):6065–7.
41. Byrne ME, Rouch DA, Skurray RA. Nucleotide sequence analysis of IS*256* from the *Staphylococcus aureus* gentamicin-tobramycin-kanamycin-resistance transposon Tn*4001*. Gene. 1989;81(2):361–7.
42. Quintiliani Jr R, Courvalin P. Characterization of Tn*1547*, a composite transposon flanked by the IS*16* and IS*256*-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. Gene. 1996;172(1):1–8.
43. Poirel L, Decousser JW, Nordmann P. Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a $bla_{\text{CTX-M}}$ β-lactamase gene. Antimicrob Agents Chemother. 2003;47(9):2938–45.
44. D'Andrea MM, Arena F, Pallecchi L, Rossolini GM. CTX-M-type β-lactamases: a successful story of antibiotic resistance. Int J Med Microbiol. 2013;303(6–7):305–17.
45. Jacoby GA. AmpC β-lactamases. Clin Microbiol Rev. 2009;22(1):161–82.
46. Harmer CJ, Moran RA, Hall RM. Movement of IS*26*-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS*26* and preferentially inserts adjacent to another IS*26*. MBio. 2014;5(5):e01801–14.
47. Allmansberger R, Brau B, Piepersberg W. Genes for gentamicin-(3)-N-acetyl-transferases III and IV. II. Nucleotide sequences of three AAC(3)-III genes and evolutionary aspects. Mol Gen Genet. 1985;198(3):514–20.
48. Poirel L, Bonnin RA, Nordmann P. Analysis of the resistome of a multidrug-resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome sequencing. Antimicrob Agents Chemother. 2011;55(9):4224–9.
49. Goussard S, Sougakoff W, Mabilat C, Bauernfeind A, Courvalin P. An IS*1*-like element is responsible for high-level synthesis of extended-spectrum β-lactamase TEM-6 in *Enterobacteriaceae*. J Gen Microbiol. 1991;137(12):2681–7.
50. Turton JF, Ward ME, Woodford N, et al. The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. FEMS Microbiol Lett. 2006;258(1):72–7.
51. Heritier C, Poirel L, Nordmann P. Cephalosporinase over-expression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*. Clin Microbiol Infect. 2006;12(2):123–30.
52. Grindley NDF. The movement of Tn3-like elements: transposition and cointegrate resolution. In: Craig NL et al., editors. Mobile DNA II. Washington, DC: ASM Press; 2002. p. 272–302.
53. Stark WM, Boocock MR, Sherratt DJ. Site-specific recombination by Tn*3* resolvase. Trends Genet. 1989;5(9):304–9.
54. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. Clin Microbiol Rev. 2005;18(4):657–86.

55. Poirel L, Bonnin RA, Nordmann P. Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. Infect Genet Evol. 2012;12(5):883–93.
56. Brunton JL, Maclean I, Ronald AR, Albritton WL. Plasmid-mediated ampicillin resistance in *Haemophilus ducreyi*. Antimicrob Agents Chemother. 1979;15(2):294–9.
57. Elwell LP, Roberts M, Mayer LW, Falkow S. Plasmid-mediated β-lactamase production in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 1977;11(3):528–33.
58. Arthur M, Molinas C, Courvalin P. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol. 1992;174(8):2582–91.
59. Wright GD, Holman TR, Walsh CT. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry. 1993;32(19):5057–63.
60. Arthur M, Molinas C, Depardieu F, Courvalin P. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol. 1993;175(1):117–27.
61. Flannagan SE, Chow JW, Donabedian SM, et al. Plasmid content of a vancomycin-resistant *Enterococcus faecalis* isolate from a patient also colonized by *Staphylococcus aureus* with a VanA phenotype. Antimicrob Agents Chemother. 2003;47(12):3954–9.
62. Craig NL. Tn*7*. In: N.L. Craig, et al. eds. Mobile DNA II. Washington, DC: ASM Press; 2002. p. 423–56.
63. Kholodii GY, Mindlin SZ, Bass IA, Yurieva OV, Minakhina SV, Nikiforov VG. Four genes, two ends, and a *res* region are involved in transposition of Tn*5053*: a paradigm for a novel family of transposons carrying either a *mer* operon or an integron. Mol Microbiol. 1995;17(6):1189–200.
64. Naas T, Cuzon G, Truong HV, Nordmann P. Role of IS*Kpn7* and deletions in bla_{KPC} gene expression. Antimicrob Agents Chemother. 2012;56(9):4753–9.
65. Delihas N. Small mobile sequences in bacteria display diverse structure/function motifs. Mol Microbiol. 2008;67(3):475–81.
66. Zong Z. The complex genetic context of bla_{PER-1} flanked by miniature inverted-repeat transposable elements in *Acinetobacter johnsonii*. PLoS ONE. 2014;9(2), e90046.
67. Szuplewska M, Ludwiczak M, Lyzwa K, Czarnecki J, Bartosik D. Mobility and generation of mosaic non-autonomous transposons by Tn*3*-derived inverted-repeat miniature elements (TIMEs). PLoS ONE. 2014;9(8), e105010.
68. Poirel L, Carrer A, Pitout JD, Nordmann P. Integron mobilization unit as a source of mobility of antibiotic resistance genes. Antimicrob Agents Chemother. 2009;53(6):2492–8.
69. Churchward G. Conjugative transposons and related mobile elements. In: Craig NL et al., editors. Mobile DNA II. Washington, DC: ASM Press; 2002. p. 177–91.
70. Franke AE, Clewell DB. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J Bacteriol. 1981;145(1):494–502.
71. Clewell DB, Flannagan SE, Jaworski DD. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. Trends Microbiol. 1995;3(6):229–36.
72. Courvalin P, Carlier C. Transposable multiple antibiotic resistance in *Streptococcus pneumoniae*. Mol Gen Genet. 1986;205(2):291–7.
73. Carias LL, Rudin SD, Donskey CJ, Rice LB. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J Bacteriol. 1998;180(17):4426–34.
74. Garnier F, Taourit S, Glaser P, Courvalin P, Galimand M. Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. Microbiology. 2000;146(Pt 6):1481–9.
75. Sebaihia M, Wren BW, Mullany P, et al. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat Genet. 2006;38(7):779–86.
76. Wozniak RA, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat Rev Microbiol. 2010;8(8):552–63.
77. Caparon MG, Scott JR. Excision and insertion of the conjugative transposon Tn*916* involves a novel recombination mechanism. Cell. 1989;59(6):1027–34.
78. Whittle G, Shoemaker NB, Salyers AA. Characterization of genes involved in modulation of conjugal transfer of the *Bacteroides* conjugative transposon CTnDOT. J Bacteriol. 2002;184(14):3839–47.
79. Shoemaker NB, Wang GR, Stevens AM, Salyers AA. Excision, transfer, and integration of NBU1, a mobilizable site-selective insertion element. J Bacteriol. 1993;175(20):6578–87.
80. Farrow KA, Lyras D, Rood JI. Genomic analysis of the erythromycin resistance element Tn*5398* from *Clostridium difficile*. Microbiology. 2001;147(Pt 10):2717–28.
81. Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R, Waldor MK. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob Agents Chemother. 2001;45(11):2991–3000.
82. Hall RM, Stokes HW. Integrons: novel DNA elements which capture genes by site-specific recombination. Genetica. 1993;90(2–3):115–32.
83. Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol. 1995;15(4):593–600.
84. Hall RM, Vockler C. The region of the IncN plasmid R46 coding for resistance to β-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found in IncW plasmids and in Tn*21*-like transposons. Nucleic Acids Res. 1987;15(18):7491–501.
85. Levesque C, Brassard S, Lapointe J, Roy PH. Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. Gene. 1994;142(1):49–54.
86. Collis CM, Hall RM. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob Agents Chemother. 1995;39(1):155–62.
87. Ouellette M, Bissonnette L, Roy PH. Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: nucleotide sequence of the OXA-1 β-lactamase gene. Proc Natl Acad Sci U S A. 1987;84(21):7378–82.
88. Radstrom P, Skold O, Swedberg G, Flensburg J, Roy PH, Sundstrom L. Transposon Tn*5090* of plasmid R751, which carries an integron, is related to Tn*7*, Mu, and the retroelements. J Bacteriol. 1994;176(11):3257–68.
89. Petrovski S, Stanisich VA. Tn*502* and Tn*512* are *res* site hunters that provide evidence of resolvase-independent transposition to random sites. J Bacteriol. 2010;192(7):1865–74.
90. Brown HJ, Stokes HW, Hall RM. The integrons In0, In2, and In5 are defective transposon derivatives. J Bacteriol. 1996;178(15):4429–37.
91. Partridge SR, Tsafnat G, Coiera E, Iredell JR. Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiol Rev. 2009;33(4):757–84.
92. Bissonnette L, Champetier S, Buisson JP, Roy PH. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In4 integron of Tn*1696*: similarity of the product to transmembrane transport proteins. J Bacteriol. 1991;173(14):4493–502.
93. Stokes HW, Hall RM. Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn*1696* integron suggests regulation by translational attenuation. Plasmid. 1991;26(1):10–9.
94. Parent R, Roy PH. The chloramphenicol acetyltransferase gene of Tn*2424*: a new breed of *cat*. J Bacteriol. 1992;174(9):2891–7.
95. Allignet J, Loncle V, Simenel C, Delepierre M, el Solh N. Sequence of a staphylococcal gene, *vat,* encoding an acetyltransferase

inactivating the A-type compounds of virginiamycin-like antibiotics. Gene. 1993;130(1):91–8.
96. Rende-Fournier R, Leclercq R, Galimand M, Duval J, Courvalin P. Identification of the *satA* gene encoding a streptogramin A acetyltransferase in *Enterococcus faecium* BM4145. Antimicrob Agents Chemother. 1993;37(10):2119–25.
97. Tennigkeit J, Matzura H. Nucleotide sequence analysis of a chloramphenicol-resistance determinant from *Agrobacterium tumefaciens* and identification of its gene product. Gene. 1991;98(1):113–6.
98. Cameron FH, Groot Obbink DJ, Ackerman VP, Hall RM. Nucleotide sequence of the AAD(2″) aminoglycoside adenylyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. Nucleic Acids Res. 1986;14(21):8625–35.
99. Wohlleben W, Arnold W, Bissonnette L, et al. On the evolution of Tn*21*-like multiresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I(AAC(3)-I), another member of the Tn*21*-based expression cassette. Mol Gen Genet. 1989;217(2–3):202–8.
100. Rather PN, Munayyer H, Mann PA, Hare RS, Miller GH, Shaw KJ. Genetic analysis of bacterial acetyltransferases: identification of amino acids determining the specificities of the aminoglycoside 6′-*N*-acetyltransferase Ib and IIa proteins. J Bacteriol. 1992;174(10):3196–203.
101. Robicsek A, Strahilevitz J, Jacoby GA, et al. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12(1):83–8.
102. Bunny KL, Hall RM, Stokes HW. New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene, *catB3*, in an integron in pBWH301. Antimicrob Agents Chemother. 1995;39(3):686–93.
103. Arakawa Y, Murakami M, Suzuki K, et al. A novel integron-like element carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob Agents Chemother. 1995;39(7):1612–5.
104. Lauretti L, Riccio ML, Mazzariol A, et al. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother. 1999;43(7):1584–90.
105. Jové T, Da Re S, Denis F, Mazel D, Ploy MC. Inverse correlation between promoter strength and excision activity in class 1 integrons. PLoS Genet. 2010;6(1), e1000793.
106. Mazel D, Dychinco B, Webb VA, Davies J. A distinctive class of integron in the *Vibrio cholerae* genome. Science. 1998;280(5363):605–8.
107. Rowe-Magnus DA, Guerout AM, Biskri L, Bouige P, Mazel D. Comparative analysis of superintegrons: engineering extensive genetic diversity in the Vibrionaceae. Genome Res. 2003;13(3):428–42.
108. Drouin F, Melancon J, Roy PH. The IntI-like tyrosine recombinase of *Shewanella oneidensis* is active as an integron integrase. J Bacteriol. 2002;184(6):1811–5.
109. Leon G, Roy PH. Excision and integration of cassettes by an integron integrase of *Nitrosomonas europaea*. J Bacteriol. 2003;185(6):2036–41.
110. Biskri L, Bouvier M, Guerout AM, Boisnard S, Mazel D. Comparative study of class 1 integron and *Vibrio cholerae* superintegron integrase activities. J Bacteriol. 2005;187(5):1740–50.
111. Toor N, Robart AR, Christianson J, Zimmerly S. Self-splicing of a group IIC intron: 5′ exon recognition and alternative 5′ splicing events implicate the stem-loop motif of a transcriptional terminator. Nucleic Acids Res. 2006;34(22):6461–71.
112. Centron D, Roy PH. Presence of a group II intron in a multiresistant *Serratia marcescens* strain that harbors three integrons and a novel gene fusion. Antimicrob Agents Chemother. 2002;46(5):1402–9.
113. Léon G, Roy PH. Potential role of group IIC-*attC* introns in integron cassette formation. J Bacteriol. 2009;191(19):6040–51.
114. Stokes HW, Tomaras C, Parsons Y, Hall RM. The partial 3′-conserved segment duplications in the integrons In6 from pSa and In7 from pDGO100 have a common origin. Plasmid. 1993;30(1):39–50.
115. Toleman MA, Bennett PM, Walsh TR. IS*CR* elements: novel gene-capturing systems of the 21st century? Microbiol Mol Biol Rev. 2006;70(2):296–316.
116. Arduino SM, Roy PH, Jacoby GA, Orman BE, Pineiro SA, Centron D. $bla_{CTX-M-2}$ is located in an unusual class 1 integron (In35) which includes Orf513. Antimicrob Agents Chemother. 2002;46(7):2303–6.
117. Gaillot O, Clement C, Simonet M, Philippon A. Novel transferable β-lactam resistance with cephalosporinase characteristics in *Salmonella enteritidis*. J Antimicrob Chemother. 1997;39(1):85–7.
118. Doi Y, Shibata N, Shibayama K, et al. Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother. 2002;46(8):2427–34.
119. Wang M, Tran JH, Jacoby GA, Zhang Y, Wang F, Hooper DC. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob Agents Chemother. 2003;47(7):2242–8.
120. Galimand M, Sabtcheva S, Courvalin P, Lambert T. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. Antimicrob Agents Chemother. 2005;49(7):2949–53.
121. Wozniak RA, Fouts DE, Spagnoletti M, et al. Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. PLoS Genet. 2009;5(12), e1000786.
122. Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob Agents Chemother. 2005;49(8):3523–5.
123. Bozdogan B, Leclercq R. Effects of genes encoding resistance to streptogramins A and B on the activity of quinupristin-dalfopristin against *Enterococcus faecium*. Antimicrob Agents Chemother. 1999;43(11):2720–5.
124. Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci U S A. 2002;99(8):5638–42.

Mutations as a Basis of Antimicrobial Resistance

6

Robert A. Bonomo

1 Introduction

The past three decades have witnessed a disturbing increase in antimicrobial resistance. Bacterial isolates are emerging that are resistant to all currently available antimicrobial agents. Bacteria with this phenotype are designated multidrug-resistant (MDR) or "pan-drug" resistant (PDR) strains. What is the genetic basis of this remarkable survival skill? Are advantageous changes in the genome always random? Is antibiotic pressure the cause of growing resistance rates or does it merely serve as a trigger that selects the archived defense armamentarium within bacteria? In this review we will explore these concepts and discuss: (1) genetic diversity and mutations as its basis and (2) "hyper-mutators" and the mechanisms responsible for high mutation rates. Our review will conclude with examples of specific point mutations in bacterial enzymes that confer resistance to certain antibiotic classes.

2 Genetic Diversity and Mutator Strains

Nearly six decades ago, Luria and Delbrück developed the field of modern bacterial genetics. Until then, it was believed that mutations (as defined by antibiotic resistance) emerged by an unknown process in which the antibiotic "trained" the bacteria. In a classical set of experiments called "fluctuation analysis" Luria and Delbrück demonstrated the role of chance and selection in the recovery of a novel phenotype [1]. An example of "fluctuation analysis" as it relates to antimicrobial resistance follows. One inoculates streptomycin susceptible cells of *Escherichia coli* in a flask containing 100 mL of broth, and also in 100 tubes each containing 1 mL of broth. After reaching full growth, 1 mL samples of both groups are plated on streptomycin-containing medium and incubated overnight. If resistant mutations arise spontaneously, before exposure to antibiotics, parallel cultures in liquid medium should have their first mutation at different times resulting in a wide variation in the colony count of resistant bacteria. If however, resistance does not arise until "directed" by the antibiotic, the samples from different tubes should all be equivalent, just like the aliquots from a single flask. The numbers of streptomycin-resistant colonies on the hundred plates from the flask are all similar. On the other hand, the number of colonies "fluctuated" significantly on the plates originating from the hundred different tubes. This experiment showed that the resistant mutants appeared before antibiotic exposure and were only selected, not directed by the agent [2]. Statistically, these random outcomes follow a Poisson distribution. This experiment is based upon earlier studies examining the susceptibility of *E. coli* bacteria to bacteriophage lysis.

We recognize now that genetic diversity is based on mutations. DNA polymerases, the enzymes that replicate bacterial genomes, possess limited fidelity. If a polymerase introduces the incorrect nucleotide, repair enzymes generally correct the "mistake" [3, 4]. If the incorrect nucleotide is introduced without "correction," a point mutation occurs. Nucleotide sequences in a codon are permanently changed as a result of substitutions, deletions, or additions. Point mutations can be "silent" if the new codon encodes the same amino acid. They can be "nonsense mutations" if the new codon is one of the chain-terminating ones, or they can be "missense mutations" which encode a different amino acid in the peptide chain. Missense mutations are point mutations that can sometimes confer resistance, since point mutations can affect the key amino acid residues important in protein function. Deletions

R.A. Bonomo, M.D. (✉)
Pharmacology, Molecular Biology and Microbiology, University Hospitals Case Medical Center, Cleveland, OH, USA

Chief, Medical Service, Louis Stokes Cleveland Department of Veteran Affairs Medical Cente, University Hospitals Case Medical Center, Cleveland, OH, USA

Vice Chair for Veteran Affairs, Department of Medicine, University Hospitals Case Medical Center, Cleveland, OH, USA
e-mail: robert.bonomo@va.gov

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_6

or insertions usually cause "frame shifting" mutations that are deleterious to the tertiary structure of a protein and may also result in premature chain termination. In general, mutations that are "harmful" or deleterious to a particular bacterial phenotype do not get passed to the next generation [5].

In times of normal growth, a perfectly adapted clonal population has a mutation rate close to zero. The absence of mutations, however, may prevent adaptation to the environment should something suddenly change. A high mutation rate is desirable in times of stress or drastic changes in the environment (e.g., antibiotic selection pressure), where advantageous mutations (drug resistance) are selected and rapidly propagated to ensure survival. Conversely, a very high mutation rate can introduce lethal changes. This "fine-tuning" of the global mutation rate is postulated to be a function of "hyper mutators." It has been estimated that hyper mutators represent approximately 0.0001–0.001 % of some bacterial populations. Under selective pressure this percentage can increase up to 0.5 %. Many natural isolates of *E. coli* and *Salmonella* spp. were found to have even higher numbers of mutators, 1–5 % [6]. A high proportion of bacteria with increased mutation frequencies has recently been described in *Pseudomonas aeruginosa* isolates from sputum of cystic fibrosis patients [7]. Two distinct types of hypermutators have been described: constitutive or permanent hypermutators and transient hypermutators [8]. In the next section we discuss the differences between constitutive and transient hypermutators and their evolutionary significance.

3 Mismatch Repair-Deficient Permanent Hypermutators

During evolution, bacteria have developed safety mechanisms that recognize mismatched bases and remove them. In that way the genetic information is kept intact and passed on to daughter cells unchanged. One of the best described DNA repair mechanisms in bacteria is the methyl-dependent mismatch repair system (MMR) in *E. coli*. The MMR system consists of three proteins; MutS, MutL, and MutH. Once MutS recognizes a distorted double helix caused by a mismatched base (e.g., an insertion or a deletion) it undergoes an ATP-dependent conformational change and binds to MutL. This MutS-MutL complex activates MutH, which functions as an endonuclease and nicks the unmethylated nascent DNA strand upstream from the mismatch. Helicase II then unwinds the DNA toward the mismatch and a specific exonuclease excises the nascent strand. This is followed by resynthesis and ligation. Bacteria that have an inactive MMR system have an increased mutation rate since they do not repair mismatches efficiently. These MMR-deficient strains are permanent hypermutators and they exhibit up to a 10,000-fold increase in mutation rates compared to wild-type bacteria [6]. According to recent evidence, permanent hypermutators are responsible for pre-exposure mutations that are present in the population prior to selective antibiotic pressure. In terms of survival value, MMR inefficiency may come at too high a price for the random protection it offers against noxious agents [7, 9].

Hypermutators are utilized in evaluating the frequency at which resistant genotypes arise in vitro while assessing a novel antimicrobial agent. The recovered mutants can provide insight into a likely mechanism of resistance. Hypermutators can potentially be utilized for selecting rare, interesting mutations with modified metabolic capabilities of biotechnological relevance [10]. For example, taking a culture of fully grown *E. coli* with a density of 10^{10} CFU/mL and resuspending this culture in 1/10 the volume, followed by incorporation of 1-mL aliquots on 10 agar plates, will detect mutants that arise at a frequency of about 10^{12}. If hypermutators of *E. coli* exhibiting a 1000-fold higher mutation rate are used, mutants that arise at frequencies as low as 10^{-15} can potentially be identified. This approach has been used to detect rare *ampC* promoter mutations in *E. coli* that confer increased ampicillin resistance [10].

4 Transient Hypermutators and the *SOS* System

Transient hypermutators have an inducible, genetically programmed *SOS* system that allows them to mutate at a higher frequency only under times of stress. The SOS system is composed of a number of polymerases—"mutases"—that introduce errors at high rates. One of the best known DNA mutase groups is the SOS system in *E. coli* [3]. In response to DNA damage, for example, brought about by exposure to fluoroquinolones, a protein designated RecA activates and wraps around the single-stranded DNA (ssDNA) forming a nucleoprotein filament [11, 12]. This nucleoprotein filament is a poor substrate for the chromosomal replicase. However, this nucleoprotein filament triggers the specific proteolytic cleavage of a suppressor protein called LexA. Under conditions of normal bacterial growth, LexA suppresses a group of nearly 40 genes involved in the "SOS response." In the absence of LexA, the SOS system is activated.

"SOS" consists of three major polymerases—Pol II, Pol IV, and Pol V—that actively generate mutations in the genome [13, 14]. All three polymerases collaborate in generating nucleotide substitutions, so-called "translesions," by dNTP mis-insertions followed by mis-pair extension [15]. See Scheme 6.1.

As a result of exposure to DNA-damaging antibiotics, SOS^+ bacteria actively increase the number of mutations. Therefore, transient hypermutators are responsible for post-exposure mutations, arising under selective antibiotic pressure and offering a better evolutionary tool for diversity, incurring an overall lower cost [9]. The SOS system renders

Scheme 6.1 dNTP mis-insertions followed by mis-pair extension

DNA damage→RecA activation→LexA proteolysis→Derepression of SOS genesPol II+PolIV+PolVTranslesions

itself a suitable target for new antimicrobial agent development as the inhibition of mutation could serve as a novel strategy in combating the evolution of antibiotic resistance [9].

5 Antimicrobial Resistance Determinants

Mutations that confer antimicrobial resistance can occur in different parts of the genome and are spread among the population by diverse mechanisms. Based on the origin of the mutated gene, antimicrobial resistance determinants can be classified into three distinct groups [5]:

1. Acquisition of foreign DNA
2. Mutations of preexisting genetic determinants
3. Mutations in acquired genes

Acquisition of foreign DNA in bacteria can occur by transduction, transformation, and conjugation. Briefly, transformation refers to the uptake of naked DNA; conjugation is plasmid-mediated mating between cells in contact; and transduction involves infection of the bacteria by a nonlethal bacteriophage carrying bacterial genes [2]. These topics are covered elsewhere in this book. In this section of the chapter we will focus primarily on point mutations in both preexisting and acquired genes.

Mutations of preexisting genetic determinants can affect either **structural** or **regulatory** genes. Select examples of antimicrobial resistance acquired through a one-step mutation in a **structural** gene are effective resistance mechanisms for β-lactams, fluoroquinolones, streptomycin, and rifampin. Mutations involving **regulatory** genes in a number of different species are known to confer resistance to various classes of antimicrobials, including fluoroquinolones, tetracyclines, and β-lactams.

6 β-Lactam Resistance Mediated by Low-Affinity Penicillin Binding Proteins (PBPs)

In most Gram-negative bacteria resistance to β-lactam antibiotics generally involves inactivation of β-lactam antibiotics by β-lactamases. The majority of clinically important Gram-positive bacteria along with a handful of Gram-negative organisms demonstrate low-affinity penicillin binding proteins (PBPs) that confer resistance to β-lactam agents. PBPs are cell wall synthesizing enzymes. Based on size, PBPs are divided into high molecular weight and low molecular weight enzymes. The high molecular weight group comprises transpeptidases and transglycosidases, which are essential for cell wall synthesis. Low molecular weight enzymes are carboxypeptidases, which rearrange and degrade the three-dimensional murein structure. Low molecular weight PBPs serve some regulatory functions but are not essential. See Table 6.1. All cell wall containing organisms described to date have from four to eight PBPs. To illustrate, *Staphylococcus aureus* has five PBPs, whereas *E coli* has eight different PBPs.

Table 6.1 Major PBP characteristics[a]

PBP	Size	Function
Essential	HMW	Transpeptidases
1, 2, 3, 4		Transglucosidases
Nonessential	LMW	Endopeptidases
5, 6, 7, 8		Carboxypeptidases

[a]*PBP* penicillin binding proteins, *HMW* high molecular weight, *LMW* low molecular weight

All PBPs have a highly conserved serine residue in their active site that forms an ester with the carboxyl group of an "opened" β-lactam ring [16, 17]. This serine ester is a structural analogue of the PBP's actual substrate, the C terminal D-Ala-D-Ala that is excised from the disaccharide-pentapeptide building block of the cell wall. Unlike the natural substrate, the β-lactam formed ester is hydrolyzed very slowly rendering the PBP nonfunctional.

The bactericidal activity of β-lactams is based on their effective inhibition of high molecular weight essential PBPs. Some bacteria manage to escape this action by the presence of PBPs that do not readily bind to the β-lactam and are thus not inactivated by the drug. The origins of these "low affinity PBPs" are very diverse. Point mutations have been described only in the high molecular weight essential PBPs [18, 19]. In transformable species like S. *pneumoniae,* "mosaic genes" acquired through homologous recombination and natural transformation from neighboring intrinsically resistant organisms have given rise to highly resistant strains. PBP2b, 2x, and 1a are encoded by mosaic genes that can be transferred between *Streptococcus sanguis, Streptococcus oralis, Streptococcus mitis,* and *S. pneumoniae* [20]. A succession of seven amino acid substitutions in PBP2b is responsible for penicillin resistance [21]. An interesting point mutation that also causes significant modification of PBP2b affinity codes for the substitution of Thr446 by an Ala. This mutation alone

confers significant resistance to penicillin when found in wild-type *S. pneumoniae* strains. PBP2b production is associated with much slower cell wall hydrolysis at high β-lactam concentrations. While all other PBPs are inhibited, PBP2b continues active synthesis of the cell wall and thereby counters the action of cell wall autolytic enzymes, which are activated by a process unleashed by interference with cell wall synthesis. This effect is great enough to slow the hydrolysis down and prevent cell lysis. Resistant PBP2x variants differ from the wild type by only 8–10 amino acids. Apart from the major mutation involving a Thr to Ala substitution immediately following the active-site Ser337, the Thr550 to Ala change is noteworthy for conferring resistance to extended spectrum cephalosporins and also for producing increased susceptibility to oxacillin.

Methicillin-resistant *S. aureus* (MRSA) possesses the *mecA* gene, which has probably evolved from a closely related gene by point mutations and codes for PBP2a, a novel additional PBP. This low-affinity PBP functions as a transpeptidase and mediates the cell wall synthesis in lieu of other PBPs, which are all inhibited by β-lactam concentrations that do not inhibit PBP2a [22].

Enterococci are intrinsically resistant to all cephalosporins. This resistance is based on the structure of enterococcal PBP5, which does not bind cephalosporins. The mechanism for resistance toward penicillins among enterococci is somewhat more complex. In certain enterococcal species, a point mutation in the regulatory gene (*psr*) causes hyperproduction of PBP5 that translates into high-level penicillin resistance [23]. This does not appear to be the case in *Enterococcus faecium* where highly ampicillin-resistant clinical isolates do not have increased level of PBP5 expression, but achieve higher MIC values to ampicillin by point mutation in the *pbp5* gene, thereby lowering the affinity of PBP5 for ampicillin binding [24].

7 Quinolone-Resistance Determining Region (QRDR) in Fluoroquinolone-Resistant Bacteria

Fluoroquinolones are inhibitors of DNA replication. Quinolones target prokaryotic topoisomerase enzymes whose major function is unwinding of DNA [25]. In binding to the enzyme-DNA complex, they stabilize it. This inhibits the movement of proteins such as DNA and RNA polymerases along the DNA chains, thus arresting the replication fork. In Gram-negative bacteria resistance to fluoroquinolones arises from alterations in the DNA gyrase (topoisomerase II), an enzyme responsible for the relaxation of supercoiled DNA. The DNA gyrase enzyme has two subunits (A and B). Amino acid substitutions resulting in quinolone resistance usually occur in Gyrase A. In *E. coli* these mutations are clustered between amino acid positions 67 and 106 at the amino terminus of the polypeptide chain. This domain is called the quinolone-resistance determining region (QRDR).

The most common mutations encountered in resistant strains involve Ser83 and Asp87. It appears that the above amino acid changes caused by point mutations in the QRDR region of Gyrase A alter the structure of the quinolone binding area at the interface of the enzyme-DNA complex, thereby reducing its affinity for the drug. Many other Gram-negative bacteria, *Mycobacteria,* and atypical pathogens with amino acid substitutions in positions equivalent to Ser83 and Asp87 display fluoroquinolone resistance. Amino acid substitutions in Gyrase B usually result in low-level resistance. In Gram-positive bacteria like *S. aureus*, resistance to fluoroquinolones usually involves point mutations in Topoisomerase IV, which separates intertwined DNA rings. Topoisomerase IV also has two subunits (ParC and ParE). High-grade resistance to fluoroquinolones is linked to amino acid substitutions in ParC [26].

8 Streptomycin Resistance and *Mycobacteria*

In *Mycobacteria,* point mutations in genes that encode ribosomal proteins confer resistance to streptomycin. Most resistant strains have one isolated nucleotide change from adenine to guanine in codon 43 of the *rpsL* gene. This changes the tertiary structure of the ribosomal protein S12, resulting in inability of streptomycin to bind to the ribosome and inhibit protein synthesis [27]. It is intriguing that *Mycobacteria* rely on generating resistance solely by de novo mutations and vertical transmission. *Mycobacteria* seem not to exchange genetic determinants horizontally, i.e., by conjugation or transformation.

9 Rifampin Resistance

In *E. coli*, rifampin resistance arises from point mutations in highly conserved regions of the *rpoB* gene which encodes the β subunit of RNA polymerase. One amino acid change in the β subunit causes a large change in the binding affinity of rifampin to the DNA-dependent RNA polymerase, thereby hindering rifampin's inhibition of mRNA transcription. Curiously, resistance to rifampin occurs at high frequency in many genera of bacteria. *M. tuberculosis, Neisseria meningitidis,* and *Mycobacterium leprae* develop rifampin resistance by accumulating point mutations in the same highly conserved regions of the *rpoB* gene [28]. Thus, rifampin is

never used as monotherapy primarily because of the high frequency at which resistant mutants arise. Combining rifampin with a second agent significantly reduces the chances of rifampin resistance arising on therapy. This paradigm forms the basis of our therapy against *M. tuberculosis*. Point mutations which confer resistance to two antibiotics are separate events and the chance of both mutations occurring in one organism is the product of the frequencies of each of them occurring alone [29].

10 Fluoroquinolone Resistance Caused by Over-Expression of Active Efflux Pumps

Multidrug-resistant (MDR) strains of *P. aeruginosa* display cross-resistance to a number of structurally unrelated antimicrobial agents. A major role for this type of resistance has recently been attributed to an active efflux pump system encoded by the *mexA-mexB-OprM* operon. The MexA-MexB-OprM efflux pump system has wide substrate specificity including β-lactams, β-lactamase inhibitors, tetracyclines, quinolones, macrolides, chloramphenicol, trimethoprim, and novobiocin [30]. Expression of the efflux operon is under control of the *mexR* regulator gene. A point mutation in *mexR* (substitution of Trp to Arg at position 69) alters the function of the MexR protein causing overexpression of the MexA-MexB-OprM efflux system. This in turn leads to higher levels of resistance to a variety of antibiotics, as seen in the *nalB* multidrug-resistant mutant, OCR1 [31].

11 Constitutive Tetracycline Resistance due to a Mutated Repressor Gene

Tetracycline antibiotics are bacteriostatic agents that inhibit protein synthesis by blocking the attachment of aminoacyl-tRNA to the acceptor site on the 30S ribosomal subunit (as reviewed in [32]). Resistance to tetracyclines is mediated by either ribosomal protection proteins or efflux pumps. The tetracycline efflux pumps belong to the Major Facilitator Superfamily (MFS). MFS efflux pumps are approximately 46-kDa membrane bound proteins that expel tetracyclines against a concentration gradient. In Gram-negative organisms the efflux system determinants comprise two genes—a gene coding for the efflux pump and another one coding for a repressor molecule. Both are regulated by the presence of tetracyclines. In the absence of tetracycline, the repressor binds to the operator and blocks the transcription of the efflux pump [33]. In certain strains of *H. influenzae* constitutive expression of the efflux protein has been attributed to a single omission of thymidine causing a frame shift mutation The resultant truncated repressor molecule is half the usual size and nonfunctional. The constitutive expression can be reversed by addition of functional repressor molecules [34].

12 Constitutive and Inducible Glycopeptide Resistance Caused by Point Mutations in the Regulatory System

Glycopeptide antibiotics, both vancomycin and teicoplanin, act as inhibitors of cell wall synthesis by binding to the D-Ala-D-Ala terminus of the pentapeptide precursor of the peptidoglycan molecule [35]. Two types of gene clusters, designated *vanA* and *vanB,* account for the majority of acquired resistance to glycopeptides [36]. The gene clusters include three genes, v*anH, vanA,* and *vanX*, which encode enzymes involved in incorporating D-Ala-D-Lac instead of D-Ala-D-Ala into the peptidoglycan precursors thereby reducing the binding affinity of glycopeptides by roughly a 1000-fold. Though the number of genes in the *Van* cluster is variable, there are five "core genes" present as illustrated in Scheme 6.2. The expression of the *VanA* and *VanB* gene clusters are regulated on the transcriptional level by a set of two other genes: *VanS* and *VanR* whose products comprise the VanRS and VanRbSb regulatory system. VanS and VanSb are transmembrane kinases that autophosphorylate a histidine residue in the presence of glycopeptides and thereupon transfer the phosphoryl group to an aspartate residue on the VanR regulator protein. The phosphorylated regulator protein activates transcription of both the resistance and the regulatory genes. VanS also functions as a phosphatase, switching off the VanR regulator protein in the absence of glycopeptides. Alterations in the functions of VanS and VanSb give rise to a variety of phenotypical expressions of vancomycin (or glycopeptide) resistance. The phenotypes fall into several major categories: (1) constitutive expression; (2) inducible expression by vancomycin and teicoplanin; (3) inducible expression by vancomycin alone; and (4) repressed under all conditions. Mutations in the transmembrane segments of VanSb affect signal transduction and lead to inducible expression of resistance genes. Mutations in *VanSb* causing substitutions at two specific positions located on either side of the His 233 give rise to constitutive expression of VanB by conveying resistance to the dephosphorylation of VanR.

Scheme 6.2 Vancomycin resistance *VanA* operon with gene function table

Gene	Product
VanR	Response Regulator
VanS	Histidine Kinase
VanH	Dehydrogenase
VanA	Ligase
VanX	D-D dipeptidase
VanY	D-D carboxypeptidase
VanZ	Unknown

13 Unique Regulation of Inducible Macrolide Resistance by Translational Attenuation

Macrolide antibiotics inhibit protein synthesis by binding to the peptidyl-tRNA binding region of the larger ribosomal subunit, thereby preventing translocation of the peptidyl-tRNA molecule from the donor to the acceptor site on ribosome. In Gram-positive organisms there are two major mechanisms of resistance to macrolides: (1) methylation of the ribosome and (2) macrolide efflux pumps (*mef*). The ribosomal methylation is accomplished by "erythromycin ribosomal methylases" (*erm*) which are products of a variety of *erm* genes [37]. Posttranscriptional methylation of a single adenine residue in 23S rRNA confers resistance to macrolides, the related lincosamides (clindamycin and lincomycin), and streptogramin B (MLSb resistance) [38]. This type of resistance is inducible by erythromycin, but not clindamycin, and it is regulated by a proposed unique mechanism of translational attenuation. This unusual regulatory mechanism does not involve repressor genes but relies on conformational isomerization of the *ermC* message to a translationally active form. Mutations in the messenger RNA cause different conformational changes which result in constitutive expression of MLS resistance [39]. In the clinical microbiology laboratory setting, inducible resistance to clindamycin brought about by erythromycin is detected by the so-called "D-test" [40]. The D-test is used to alert clinicians to avoid the use of clindamycin in treating staphylococcal and streptococcal infections.

14 β-Lactam Resistance Caused by AmpC β-Lactamase Hyperproduction

β-Lactam antibiotics are therapeutically important bactericidal agents. However, both Gram-negative and Gram-positive organisms have developed enzymes able to degrade the β-lactam ring, thereby rendering the β-lactam inactive. Gram-positive organisms produce extracellular β-lactam hydrolyzing enzymes only when needed, i.e., by induction upon exposure to the agent. The majority of Gram-negative beta-lactamases are expressed constitutively and are contained in the periplasmic space, where they inactivate incoming β-lactams. In addition to the constitutive β-lactamases in Gram-negative bacteria, AmpC is an inducible chromosomally encoded β-lactamase. The *ampC* gene is found in all Gram-negative bacteria. Its product, the AmpC β-lactamase, is primarily a cephalosporinase, but when produced in large amounts in the presence of an efficient "inducer" like cefoxitin or imipenem, it can confer resistance to all cephalosporins, penicillins, β-lactam-β-lactamase inhibitor combinations and the monobactam, aztreonam.

The regulatory system responsible for the induction mechanism is rather complex and under strict control of several other genes: *ampR, ampD, ampE,* and *ampG*. The most widely accepted explanation of how AmpC production is regulated postulates that the gene product of AmpR has a dual function. It serves as a repressor of *ampC* transcription at baseline, but turns into an activator upon exposure to β-lactams. Current experimental evidence suggests that peptidoglycan breakdown products, i.e., muramyl peptides, and not the β-lactam molecule itself, serve as the activation trigger [41].

Table 6.2 AmpC regulatory system-specific protein functions

Protein	Function
AmpR	Repressor
AmpG	Permease
AmpE	Transmembrane protein
AmpD	Amidase
AmpC	Cephalosporinase

The product of *ampG* is a transmembrane protein through which peptidoglycan breakdown products enter the cytoplasm. The *ampD* product linked to the transmembrane AmpE protein is a soluble cytosolic *N*-acetylmuramyl-L-alanine amidase that helps to recycle the breakdown products. In the presence of agents whose actions lead to cell wall destruction, like certain β-lactams, the recycling capacity of the amidase is exceeded and AmpR activates the production of AmpC. As a consequence of point mutations in AmpD that render it inactive, the regulatory system breaks down and AmpC production is permanently switched on, conferring resistance to all penicillins and cephalosporins. Strains that hyperproduce AmpC as a consequence of AmpD mutations are designated "derepressed mutants" [42, 43]. The functions of individual proteins of the AmpC regulatory system are summarized in Table 6.2.

15 Point Mutations in Acquired Resistance Genes—The "New-Generation" β-Lactamases

Ampicillin was the first synthetic aminopenicillin active against *E. coli* and other Gram-negative bacteria. Before long, enzymes capable of hydrolyzing ampicillin and first-generation cephalosporins were discovered. The genes encoding these β-lactamases were transferred onto plasmids and propagated with astonishing rapidity among *E. coli* and other *Enterobacteriaceae*. These first "broad-spectrum" β-lactamases were the TEM- and SHV-type. Their mechanism of action is based on catalytically disrupting the amide bond in the β-lactam ring by forming an acyl-enzyme complex. With the help of a strategically positioned water molecule in the active site, the covalent ester link is disrupted, the free enzyme released, and the β-lactam transformed into inactive penicilloyl and cephalosporyl moieties.

The need for antibiotics resistant to hydrolysis by plasmid-borne β-lactamases of Gram-negative bacilli, namely TEM-1 and SHV-1, was the stimulus for the development of extended-spectrum cephalosporins. These newer-generation "extended-spectrum" cephalosporins managed to avoid hydrolysis by alterations in the β-lactam molecule that interfered with effective interaction with the β-lactamase, while still retaining their binding affinity to target PBPs. Since the modifications in the β-lactam molecule were relatively minor, it was reasonable to predict that β-lactamases able to hydrolyze these new β-lactams would soon evolve. No one, however, expected they would do so as easily and rapidly as they did—threatening the utility of the entire class of extended-spectrum cephalosporins. These novel β-lactamases were called "extended-spectrum β-lactamases" or ESBLs.

Table 6.3 Sites for phenotype-altering amino acid substitutions in TEM and SHV β-Lactamases[a]

Phenotype	Position of amino acid (Ambler numbering) substitutions [45]	
	TEM	SHV
ESBL	Gly 104 Arg 164 Gly 238 Glu 240	Gly 238 Glu 240
IRT	Met 69 Ser 130 Arg244 Arg275 Asp276	Met 69 Ser 130
CMT	(Gly 238 or Glu 240) + (Met 69 or Ser 130 or Arg 275Leu, −Asn276Asp)	SHV-10, -129

[a]*ESBL* extended-spectrum β-lactamases, *IRT* inhibitor-resistant TEMs, *CMT* complex mutants of TEM

Another strategy of battling the growing problem of β-lactamase-mediated resistance to penicillins was the development of effective inhibitors of the enzymes to protect the penicillins from inactivation. Clavulanic acid, sulbactam, and tazobactam are β-lactam compounds that occupy the active site of the β-lactamase and act as "suicide" substrates (or β-lactamase inhibitors) resulting in inactivation of the enzyme. When combined with a penicillin, these β-lactamase inhibitors protect the penicillin from inactivation by the β-lactamase. The success of β-lactamase inhibitors was compromised by subsequent mutational resistance. As early as 1992, an ampicillin-resistant clinical isolate of *E coli* resistant to the ampicillin-sulbactam inhibitor combination was discovered, possessing a β-lactamase with reduced affinity for the inhibitor molecule [44].

The bases for resistance to extended-spectrum cephalosporins by ESBLs and resistance to β-lactam β-lactamase inhibitor combinations are point mutations in the β-lactamase gene that cause amino acid substitutions which alter the structure or dynamics of the enzyme. The majority of β-lactamases have more than one amino acid substitution compared to the wild-type enzyme. Interestingly, only a few point mutations at selected loci in the β-lactamase gene give rise to the abovementioned phenotypes. The corresponding major amino acid positions at which substitutions conferring new resistance occur most frequently are summarized in Table 6.3.

16 The Gly238Ser ESBL Mutation

One of the most frequently encountered and, therefore, most studied mutations codes for the G238S amino acid substitution. In nature, the substitutions of -Ser, -Ala, or -Asp for Gly at the Ambler position ABL 238 are mutations in SHV β-lactamase that confer resistance to extended-spectrum cephalosporins. There are currently more than 30 TEM and more than 25 SHV β-lactamase variants with the substitution Gly238Ser (www.lahey.org). Numerous hypotheses have been advanced to explain why the Gly238Ser substitution results in significant resistance to broad-spectrum cephalosporins. In 2003, the crystallographic structure of SHV-2 was elucidated and compared to the structure of SHV-1 from which it differs in only the one G238S substitution [46].

17 IRTs (Inhibitor-Resistant TEMs)

There are currently 20 inhibitor-resistant TEM and an increasing number inhibitor-resistant SHV mutants. In general the inhibitor-resistant mutants are devoid of ESBL activity and are less active than classical TEM against narrow-spectrum cephalosporins [47]. The number of IRTs in TEM far exceeds the number in the SHV series, although the mutations sites are the same. The reason for this is a subject of ongoing studies.

SHV-10 was the first Inhibitor-Resistant SHV (IRS) enzyme discovered in 1997 in a clinical isolate of *E. coli*. It is a derivative of SHV-5, an ESBL enzyme. As a result of a single point mutation in which adenine transitioned to guanine, a glycine is substituted for a serine at Ambler position 130. The enzyme partially retained its ability to hydrolyze penicillins, but lost significant activity against cephalosporins. Only recently, a second inhibitor-resistant SHV was discovered, SHV-49. This novel β-lactamase was found to be a derivative of chromosomal SHV-1 and differs from the original gene only by the substitution of guanine by adenine at position 195, leading to the amino acid substitution M69I. Experiments using site-directed mutagenesis have shown that this change in the SHV-1 conveys inhibitor resistance [48].

Inhibitor-resistant variants of TEM are more numerous [49, 50]. The largest group involves changes at position 69, where Met is substituted with one of the hydrophobic, aliphatic amino acids Leu, Ile, or Val. Although distant from the cross-linking S130, the majority of IRT mutations cause a change in the local environment of S130. For example, in TEM-32 the M69I substitution distorts S70 causing S130 into a new conformation, moving its O γ 2.3 Å away from where the inhibitor would bind. Similarly, in TEM-34 the M69V substitution leads to a conformational change in Ser-130, causing it to hydrogen bond with K73 and K234 and reducing its nucleophilicity for cross-linking [51].

18 CMT (Complex Mutants of TEM)

Both ESBLs and IRTs arose from the common plasmid-mediated TEM and SHV-1 penicillinases by single point mutations. These substitutions either conferred resistance to inhibitors or resulted in the ability to hydrolyze oxyimino-β-lactams, but not both. Since the 1990s, a new subgroup of enzymes has emerged in different species of the *Enterobacteriaceae* family that combine mutations responsible for inhibitor resistance (i.e., Leu-69 and Asp-276) with those responsible for the extended-spectrum phenotype (Lys-104 and Ser-238). These mutants were termed Complex Mutants of TEM [52, 53].To date, there have been five CMTs described.

19 CTX-M

CTX-Ms are a growing group of plasmid-borne enzymes that belong to the same class as SHVs and TEMs (class A). They share only 40 % sequence identity with TEM and SHV and are thought to be derived from the chromosomal *ampC* gene of the *Kluvyera spp.* [54]. In general, CTX-Ms confer resistance to most oxyimino-cephalosporins and cefepime, but do not efficiently hydrolyze ceftazidime. Therefore, when screening for ESBL production, in addition to checking for susceptibility to ceftazidime, cefotaxime should also be tested to reduce the risk of overlooking a CTX-M enzyme. Recently, new members of this group have evolved with a point mutation resulting in an Asp240Gly or a Pro167Ser substitution. These new mutants phenotypically display increased resistance to ceftazidime and higher susceptibility to cefepime and must have evolved under ceftazidime selection pressure. Neither one of the substitutions has ever been found in naturally occurring TEM or SHV ESBLs, which may suggest that CTX-Ms have a distinct evolutionary potential [55].

20 Global Suppressors

In the mid-1980s experimental work was carried out in an attempt to elucidate genes that would code for the tertiary structure of a protein. Using random gap misrepair mutagenesis, a number of missense mutations were introduced into the gene for staphylococcal nuclease, rendering the mutant strains nuclease negative (*nuc*−). Most of the detrimental mutations, as expected, affected amino acids located in the active site of the enzyme or in close proximity to it. There were, however, several distinct mutations involving remote sites. Surprisingly, after subjecting these "remote-site mutants" to another round of mutagenesis, nuclease activity was restored. Introducing

the remote site mutations into other *nuc*− mutants had the same protein restoring effect. The term "global suppressors" was applied to outlying mutations capable of suppressing the deleterious effects of active-site mutations.

At that time, it was hypothesized that in some way the peripherally located amino acid substitutions were involved in preserving the tertiary structure of the protein [56]. One striking example is the unique mutation involving the substitution of Met with Thr at position 182. Residue 182 is located in the hinge region between two different domains of the protein. Amino acids in this area, around position 182 and leading to the catalytic site, generally do not tolerate substitutions well. They are believed to play an essential role in core packing and catalytic site orientation. M182T is found in several different TEM enzymes (TEM-32, TEM-43, TEM-52) [57]. It is thought that M182T functions as a global suppressor by affecting protein folding and thereby stabilizing the enzyme. This ability of M182T to compensate for deleterious effects of other mutations makes it a powerful tool in acquiring resistance. As a natural polymorphism in β-lactamases, it will permit sampling of a much greater number of positions that tolerate substitutions. On the other hand, small inhibitor molecules could be designed against the hinge region of a β-lactamase, hindering folding to the active conformation of the enzyme and opening a new avenue for antimicrobial development [58]. Investigations were undertaken to find a global suppressor in other class A β-lactamases, such as SHV. This was recently found in SHV-129 (G238S-E240K-R275L-N276D). In addition to expanding the spectrum of β-lactamase activity to include the hydrolysis of cefepime, the amino acid substitutions found in SHV-129 provide the enzyme with an excess of stability, which expands the evolutionary landscape of this enzyme and may result in further evolution to potentially include resistance to carbapenems or β-lactamase inhibitors [59].

21 OXA

The OXA-type enzymes are classified as a group of ESBLs that are different from SHV, TEM, and CTX-M but share a common substrate spectrum. They are not inhibited by clavulanic acid and they hydrolyze oxacillin and cloxacillin very efficiently. OXAs are primarily found in *P. aeruginosa* and *Acinetobacter baumannii*. Although the group is genotypically diverse, the most recent additions show some degree of homology to the existing members [60]. Most OXAs are derivatives of OXA-10, including OXA-11, -14, -16, and -17. They differ from the parental enzyme by one to several amino acid substitutions. The two most important substitutions in OXA-10 derivatives are Ser73Asn and Gly157Asp. The latter appears to be necessary for high-level ceftazidime resistance. This substitution is lacking in OXA-17, which, in contrast to the rest of the group, hydrolyzes cefotaxime and ceftriaxone much better than ceftazidime. OXA-31 differs from OXA-1 in only three amino acid substitutions and was found to confer a rather unusual susceptibility pattern. OXA-31 hydrolyzes cefepime but not ceftazidime. It is therefore important to avoid reporting ceftazidime resistance solely on the basis of cefepime resistance, as is the routine in most clinical laboratories [61].

22 Concluding Remarks

To summarize, emerging antibiotic resistance is often a consequence of chance mutations. The vast majority are detrimental to the host bacterium and do not spread. The ones that offer a survival advantage are selected. From a Darwinian standpoint, antibiotics function as a "selection tool." By killing the susceptible bacteria, antibiotics provide a new niche for the resistant organisms. Yet, mutations come at a "price." They usually confer decreased fitness upon the mutant compared to the wild parental strain. This is readily seen among β-lactamases. We are learning how to discover and screen for mutants and are gaining knowledge of the structural and functional impact of mutations. In many instances, we have described the mechanism of resistance on the molecular level. We have even simulated natural evolution and predicted new resistance determinants years before they were isolated clinically. Yet, it seems that we are losing the battle against resistance. A single "mistake" at the codon level occurs at random. And evolution takes over.

Acknowledgments Research reported in this presentation was directly supported by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) under Award Numbers 5K08AI112506-02 (LKL), R01AI072219, R01AI063517, R01AI100560 (RAB), and by the Department of Veterans Affairs Research and Development under Award Number I01BX001974, VISN 10 Geriatrics Research, Education and Clinical Center (RAB).

The content is solely the responsibility of the authors and does not necessarily represent the official views of the Department of Veterans Affairs or the National Institutes of Health.

References

1. Luria SE, Delbruck M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics. 1943;28(6):491–511.
2. Davis BB, Dulbecco R, Eisen HS, Ginsberg HS. Microbiology, 4th ed. J. B. Lippincot; 1990.
3. Friedberg E, Walker GC, Siede W. DNA repair and mutagenesis. Washington, DC: ASM Press; 1995.
4. Tippin B, Pham P, Goodman MF. Error-prone replication for better or worse. Trends Microbiol. 2004;12(6):288–95.
5. Lorian V. Antibiotics in laboratory medicine. Baltimore, MD: Lippincott Williams & Wilkins; 2002.

6. LeClerc JE, Li B, Payne WL, Cebula TA. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science. 1996;274(5290):1208–11.
7. Oliver A, Baquero F, Blazquez J. The mismatch repair system (*mutS, mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. Mol Microbiol. 2002;43(6):1641–50.
8. Radman M. Enzymes of evolutionary change. Nature. 1999;401(6756):866–7. 9.
9. Cirz RT, Chin JK, Andes DR, de Crecy-Lagard V, Craig WA, Romesberg FE. Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol. 2005;3(6), e176.
10. O'Neill AJ, Chopra I. Use of mutator strains for characterization of novel antimicrobial agents. Antimicrob Agents Chemother. 2001;45(5):1599–600.
11. Ennis DG, Fisher B, Edmiston S, Mount DW. Dual role for *Escherichia coli* RecA protein in SOS mutagenesis. Proc Natl Acad Sci U S A. 1985;82(10):3325–9.
12. Yeiser B, Pepper ED, Goodman MF, Finkel SE. SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. Proc Natl Acad Sci U S A. 2002;99(13):8737–41.
13. Pham P, Rangarajan S, Woodgate R, Goodman MF. Roles of DNA polymerases V and II in SOS-induced error-prone and error-free repair in *Escherichia coli*. Proc Natl Acad Sci U S A. 2001;98(15):8350–4.
14. Tompkins JD, Nelson JL, Hazel JC, Leugers SL, Stumpf JD, Foster PL. Error-prone polymerase, DNA polymerase IV, is responsible for transient hypermutation during adaptive mutation in *Escherichia coli*. J Bacteriol. 2003;185(11):3469–72.
15. Friedberg EC, Wagner R, Radman M. Specialized DNA polymerases, cellular survival, and the genesis of mutations. Science. 2002;296(5573):1627–30.
16. Meroueh SO, Minasov G, Lee W, Shoichet BK, Mobashery S. Structural aspects for evolution of beta-lactamases from penicillin-binding proteins. J Am Chem Soc. 2003;125(32):9612–8.
17. Massova I, Mobashery S. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agents Chemother. 1998;42(1):1–17.
18. Heisig P. Actions and resistance mechanisms of beta-lactam antibiotics. Penicillin-binding proteins, beta-3-lactamases and signal proteins. Pharm Unserer Zeit. 2006;35(5):400–8.
19. Goffin C, Ghuysen JM. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol Mol Biol Rev. 1998;62(4):1079–93.
20. Hakenbeck R, Konig A, Kern I, et al. Acquisition of five high-Mr penicillin-binding protein variants during transfer of high-level beta-lactam resistance from Streptococcus mitis to *Streptococcus pneumoniae*. J Bacteriol. 1998;180(7):1831–40.
21. Dowson CG, Hutchison A, Spratt BG. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol Microbiol. 1989;3(1):95–102.
22. Hakenbeck R, Coyette J. Resistant penicillin-binding proteins. Cell Mol Life Sci. 1998;54(4):332–40.
23. Ligozzi M, Pittaluga F, Fontana R. Identification of a genetic element (*psr*) which negatively controls expression of *Enterococcus hirae* penicillin-binding protein 5. J Bacteriol. 1993;175(7):2046–51.
24. Rice LB, Bellais S, Carias LL, et al. Impact of specific *pbp5* mutations on expression of beta-lactam resistance in *Enterococcus faecium*. Antimicrob Agents Chemother. 2004;48(8):3028–32.
25. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev. 1997;61(3):377–92.
26. Hooper DC. Mechanisms of fluoroquinolone resistance. Drug Resist Updates. 1999;2(1):38–55.
27. Cole ST. *Mycobacterium tuberculosis*: drug-resistance mechanisms. Trends Microbiol. 1994;2(10):411–5.
28. Jin DJ, Gross CA. Mapping and sequencing of mutations in the *Escherichia coli* rpoB gene that lead to rifampicin resistance. J Mol Biol. 1988;202(1):45–58.
29. Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother. 2000;44(7):1771–7.
30. Poole K, Tetro K, Zhao Q, Neshat S, Heinrichs DE, Bianco N. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob Agents Chemother. 1996;40(9):2021–8.
31. Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, Plesiat P. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob Agents Chemother. 1999;43(2):287–91.
32. Schnappinger D, Hillen W. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch Microbiol. 1996;165(6):359–69.
33. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev. 2001;65(2):232–60; second page, table of contents.
34. Heuer C, Hickman RK, Curiale MS, Hillen W, Levy SB. Constitutive expression of tetracycline resistance mediated by a *Tn10*-like element in *Haemophilus parainfluenzae* results from a mutation in the repressor gene. J Bacteriol. 1987;169(3):990–4.
35. Reynolds PE. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis. 1989;8(11):943–50.
36. Arthur M, Reynolds P, Courvalin P. Glycopeptide resistance in *enterococci*. Trends Microbiol. 1996;4(10):401–7.
37. Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob Agents Chemother. 1999;43(12):2823–30.
38. Lai CJ, Dahlberg JE, Weisblum B. Structure of an inducibly methylatable nucleotide sequence in 23S ribosomal ribonucleic acid from erythromycin-resistant Staphylococcus aureus. Biochemistry. 1973;12(3):457–60.
39. Weisblum B. Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother. 1995;39(3):577–85.
40. Weisblum B, Demohn V. Erythromycin-inducible resistance in *Staphylococcus aureus*: survey of antibiotic classes involved. J Bacteriol. 1969;98(2):447–52.
41. Jacobs C, Frere JM, Normark S. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. Cell. 1997;88(6):823–32.
42. Bennett PM, Chopra I. Molecular basis of beta-lactamase induction in bacteria. Antimicrob Agents Chemother. 1993;37(2):153–8.
43. Jacobs C, Joris B, Jamin M, et al. AmpD, essential for both beta-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. Mol Microbiol. 1995;15(3):553–9.
44. Thomson CJ, Amyes SG. TRC-1: emergence of a clavulanic acid-resistant TEM beta-lactamase in a clinical strain. FEMS Microbiol Lett. 1992;70(2):113–7.
45. Ambler RP, Coulson AF, Frere JM, et al. A standard numbering scheme for the class A beta-lactamases. Biochem J. 1991;276(Pt 1):269–70.
46. Nukaga M, Mayama K, Hujer AM, Bonomo RA, Knox JR. Ultrahigh resolution structure of a class A beta-lactamase: on the mechanism and specificity of the extended-spectrum SHV-2 enzyme. J Mol Biol. 2003;328(1):289–301.
47. Zhou XY, Bordon F, Sirot D, Kitzis MD, Gutmann L. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 beta-lactamase conferring resistance to beta-lactamase inhibitors. Antimicrob Agents Chemother. 1994;38(5):1085–9.
48. Dubois V, Poirel L, Arpin C, et al. SHV-49, a novel inhibitor-resistant beta-lactamase in a clinical isolate of *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2004;48(11):4466–9.

49. Belaaouaj A, Lapoumeroulie C, Canica MM, et al. Nucleotide sequences of the genes coding for the TEM-like beta-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol Lett. 1994;120(1–2):75–80.
50. Henquell C, Chanal C, Sirot D, Labia R, Sirot J. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM beta-lactamases from clinical isolates of *Escherichia coli*. Antimicrob Agents Chemother. 1995;39(2):427–30.
51. Wang X, Minasov G, Shoichet BK. The structural bases of antibiotic resistance in the clinically derived mutant beta-lactamases TEM-30, TEM-32, and TEM-34. J Biol Chem. 2002;277(35):32149–56.
52. Robin F, Delmas J, Chanal C, Sirot D, Sirot J, Bonnet R. TEM-109 (CMT-5), a natural complex mutant of TEM-1 beta-lactamase combining the amino acid substitutions of TEM-6 and TEM-33 (IRT-5). Antimicrob Agents Chemother. 2005;49(11):4443–7.
53. Sirot D, Recule C, Chaibi EB, et al. A complex mutant of TEM-1 beta-lactamase with mutations encountered in both IRT-4 and extended-spectrum TEM-15, produced by an Escherichia coli clinical isolate. Antimicrob Agents Chemother. 1997;41(6):1322–5.
54. Babic M, Hujer AM, Bonomo RA. What's new in antibiotic resistance? Focus on beta-lactamases. Drug Resist Updat. 2006;9(3):142–56.
55. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother. 2004;48(1):1–14.
56. Huang W, Palzkill T. A natural polymorphism in beta-lactamase is a global suppressor. Proc Natl Acad Sci U S A. 1997;94(16):8801–6.
57. Farzaneh S, Chaibi EB, Peduzzi J, et al. Implication of Ile-69 and Thr-182 residues in kinetic characteristics of IRT-3 (TEM-32) beta-lactamase. Antimicrob Agents Chemother. 1996;40(10):2434–6.
58. Orencia MC, Yoon JS, Ness JE, Stemmer WP, Stevens RC. Predicting the emergence of antibiotic resistance by directed evolution and structural analysis. Nat Struct Biol. 2001;8(3):238–42.
59. Winkler ML, Bonomo RA. SHV-129: a gateway to global suppressors in the SHV beta-lactamase family? Mol Biol Evol. 2016;33(2):429–41.
60. Bradford PA. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 2001;14(4):933–51, table of contents.
61. Aubert D, Poirel L, Chevalier J, Leotard S, Pages JM, Nordmann P. Oxacillinase-mediated resistance to cefepime and susceptibility to ceftazidime in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2001;45(6):1615–20.

Target-Mediated Antibacterial Resistance

7

Liza Valdivia and Louis B. Rice

1 Introduction

Bacteria have evolved a variety of methods to avoid the lethal action of antimicrobial agents. Among the more common and diverse mechanisms of resistance are alterations of the antimicrobial targets. It is axiomatic that all antimicrobial agents have targets—proteins or other structures within the bacterial cell that are essential to the structure, growth, and/or function of the microorganism. In order to do their job effectively, antimicrobial agents must interact with these targets in a manner that precludes the normal functioning of the target molecule. Moreover, their interactions with the target must be fairly specific, otherwise the potential for interaction with human molecules, and thereby the potential for toxicity, will be increased. In a circumstance in which the antibiotic–target interaction is specific, changes in the target structure or in the environment immediately surrounding the target can have a profound impact on target–antibiotic interaction. This interaction can result in resistance, as long as the changes do not in a meaningful way impact the ability of the target molecule to serve its function in cellular structure or metabolism. The frequent incorporation of the genetic underpinnings of these modifications into mobile elements has facilitated the wide dissemination of resistance.

Target modifications resulting in antimicrobial resistance are extraordinarily common in bacteria, and quite varied in the forms they take. In some cases, target modifications represent simple point mutations in a protein molecule, usually an enzyme that catalyzes an essential cellular function. Genes encoding the target proteins can also be modified in a variegated manner through homologous recombination with foreign DNA. In some instances, bacteria import entire genes to substitute for the antimicrobial targets. In others, complex and regulated pathways are acquired that modify nonenzymatic cellular structures. Finally, proteins may be made that interact with the target in a manner that "protects" the target from interaction with the antibiotic. Examples of each of these mechanisms are listed in Table 7.1.

In this chapter, we will provide an overview of target-mediated resistance mechanisms in bacteria. We will try to draw commonalities and identify overall themes for this type of resistance. This review is meant to be illustrative, rather than exhaustive. Details of many of these mechanisms can be found in the ensuing chapters. We will not specifically address target-mediated mechanisms of resistance in fungi or viruses, although many of the same principles that we describe will apply to these other microorganisms as well.

2 Point Mutations That Create Resistance

Actively growing bacteria have many opportunities for point mutations to be introduced into critical genes. Since 1 bacterium can multiply to 10^9 bacteria in broth overnight, there is theoretically roughly that number of opportunities for mutations to be introduced. That such mutations do not emerge under nonselective conditions is often due to the presence of error-detecting genes within most bacterial genomes that recognize mismatched base pairs and repair them before they can be propagated. Mutations that do slip through the surveillance mechanisms are often less "fit" than the wild-type proteins, yielding slower or less effective replication and rapid loss due to dilution effects.

Mutants will emerge more frequently under circumstances in which the rate of mutation increases, such as defects in the mismatch repair mechanisms, and under circumstances where the mutants enjoy a selective advantage over the wild-type phenotype. The most obvious of circumstances

L. Valdivia, M.D. • L.B. Rice (✉)
Brown University and Rhode Island Hospital,
Providence, RI 02903, USA
e-mail: lvaldivia@lifespan.org; lrice@lifespan.org

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_7

Table 7.1 General target-mediated resistance mechanisms and the targets they affect

Point mutations	Mosaic genes	Target substitution	Increased target expression	Target modification or protection
Penicillin-binding proteins	Penicillin-binding proteins	Penicillin-binding proteins	Penicillin-binding proteins	Ribosomes
Topoisomerase genes	Topoisomerase genes	Dihydrofolate reductases	Dihydrofiolate reductases	Topoisomerases
Ribosomal proteins		Dihydropterate synthetases		Elongation factor-G
Ribosomal RNA				Lipopolysaccharide membrane
RNA polymerase				
Elongation factor-G				

in which point mutations confer a selective advantage are those in which the mutations confer resistance to an antimicrobial agent that is present in the environment.

It is of course important that the resistance-conferring point mutation not nullify the normal activity of the enzyme. As implied above, point mutations conferring antibiotic resistance may have a moderately deleterious effect on the activity of the target enzyme resulting in, among other things, prolonged replication times [1]. The presence of antibiotics in the environment alters the balance in that the disadvantage of prolonged replication time will be outweighed by the advantage of continued replication in the presence of antibiotic. In living systems, compensatory mutations often occur in the absence of antimicrobial selective pressure that serve to restore some degree of "fitness" while maintaining the mutation that confers resistance [1].

Since there are limits to the amount of change an enzyme can undergo while still maintaining its native activity, many point mutations modify the enzyme only slightly, and therefore only confer a modest degree of resistance. There are exceptions to this statement, such as the emergence of resistance to rifampin, in which a single point mutation in the *rpoB* RNA polymerase gene can confer extremely high levels of resistance [2]. In most instances, however, single point mutations confer only modest levels of resistance, often not even resulting in MIC increases that fall within the resistant range. Single point mutations in the "quinolone resistance-determining region" (QRDR) of topoisomerase genes *gyrA* or *parC* often confer only a modest increase in resistance to fluoroquinolones [3]. Similarly, recent work indicates that single mutations in low-affinity *pbp5* from *Enterococcus faecium* confer modest levels of resistance [4].

Single point mutations in target genes can be clinically important, however, because they are frequently combined with other mechanisms of resistance (such as efflux pump activation) in a manner that amplifies the expression of resistance [3]. The low levels of resistance conferred by a single point mutation can also increase the amount of time during a dosing interval in which the bacterium is exposed to subinhibitory levels of the selective antimicrobial agents. These periods inside the "mutant selection window" can promote the selection of further point mutations that confer higher levels of resistance [5]. Cases of clinical failure of levofloxacin in treating pneumococcal bacteremia have been reported when the initial isolate bore a single *gyrA* point mutation that conferred resistance to ciprofloxacin, but only elevated MICs (not into the resistant range) to levofloxacin [6]. Levofloxacin treatment under these conditions selected out an isolate with a second mutation, conferring high-level levofloxacin resistance.

In general, high-level resistance conferred by point mutations requires the presence of several mutations. In the case of resistance to fluoroquinolones, the most effective mutations occur in the topoisomerase that is the primary target of the specific quinolone [3]. Quinolones that target both GyrA and Topoisomerase IV require mutations in both enzymes to confer significant levels of resistance. Very high level resistance to ampicillin in *E. faecium* also requires the presence of multiple mutations [4]. We showed that none of four *pbp5* mutations alone conferred high-level resistance to *E. faecium*, but when all were present together (as is frequently the case in highly resistant *E. faecium*), high-level resistance was expressed [4].

There are instances in which a point mutation does confer a high level of resistance, but the effect of this point mutation is diluted out by the fact that there are several copies of the gene present in the microorganism. Such is the case with resistance to linezolid, which inhibits protein synthesis by interacting with the 23S subunit of the bacterial ribosome. *E. faecium* has six copies of the ribosomal RNA genes in its genome, *Staphylococcus aureus* has five, whereas *Enterococcus faecalis* has four. A single point mutation (G2567U) in the ribosomal RNA prevents linezolid binding to the ribosomal RNA. However, when only one of the six copies in *E. faecium* has the mutation, the level of resistance is very low [7]. When four or more of the copies contain the mutation, resistance is very high (128 μg/ml or more) [7]. Although it was originally thought that the need for multiple mutations would make the emergence of resistance unlikely, once linezolid was used in clinical settings resistant isolates (with multiple copies mutated) could be readily identified

[8]. It has now been shown that the first point mutation is the critical one [9]. Once that is in place, the strain can increase the percentage of mutants through recombination between resistant and susceptible copies. This recombination to confer resistance has been referred to as "gene conversion." There appears to be some fitness costs to these mutations, however, since continued passage of resistant strains in the absence of antibiotics results in a return to susceptibility, as long as one copy of the wild-type rRNA gene remains [10]. If all of the rRNA genes contain the mutation, then the resistance phenotype is much more stable [11], suggesting that gene conversion is responsible for the return to susceptibility as well as the emergence of resistance.

In general, point mutations that confer resistance in enzymes are found at the active site of the molecule. An important exception to this is the case of ceftaroline resistance. Ceftaroline, a novel antibiotic developed to compensate for the growing ubiquity of MRSA, has been shown to bind to an allosteric site of the PBP2a molecule [12]. This interaction produces a conformational change at the active site of PBP2a, effectively "opening" the site. As reduced active site binding is the main mechanism of methicillin resistance, the now open active site is left vulnerable to beta lactam binding and subsequent inhibition of cell wall cross-linking.

Since the introduction of ceftaroline, resistance has emerged in the form of point mutations at the allosteric site. Three mutations (N146K/E150K/H351N) have been described. Two of these three mutations (N146K/E150K) are found within the allosteric region [13]. These mutations have been shown to inhibit the conformational change at the active site associated with allosteric binding.

3 Mosaic Genes

In the past two decades, the genome sequences of many different species have been determined, annotated, and the results made public for detailed analysis. A consistent theme resulting from these analyses is the remarkable frequency with which gene exchange has contributed to individual variation between members of the same species. It is estimated, for example, that more than 25 % of the *E. faecalis* V583 genome has been acquired from other species [14]. Differences between uropathogenic and enteropathogenic *Escherichia coli* can be attributed to acquisition of different "pathogenicity islands" [15]. So the exchange of DNA has had profound impact on the evolution of bacterial species in many areas, including the area of antimicrobial resistance.

Most bacteria acquire exogenous DNA through the action of mobile elements that confer an ability to transfer between bacteria and an ability to integrate into the recipient genome. Examples include conjugative plasmids and conjugative transposons of many sorts [16]. A minority of bacterial species have been shown to be naturally transformable. These bacteria have the capacity, under the proper circumstances to absorb naked DNA from the environment. DNA from dead bacteria, that have no genetic material to promote entry into the recipient cell, can be taken up by these bacteria. Once inside the cell, the bacterial homologous recombination functions can integrate this acquired DNA into the genome across regions of sufficient homology. The result is mosaic genes consisting of parts derived from the parent cell and part derived from the donor DNA. If this recombination occurs in a manner that maintains the integrity of the open reading frame, a new protein may result. If the gene involved in the recombination encodes a protein that is the target for an antibiotic, and if the acquired DNA contains regions that confer a reduced susceptibility to that antibiotic, then an increase in resistance may result.

Mosaic genes have been found commonly in species that are naturally transformable. Such species include *Streptococcus pneumoniae* and species of viridans streptococci [17], as well as *Neisseria gonorrhoeae, Neisseria meningitidis,* and several non-pathogenic *Neisseria* [18, 19]. Resistance genes that have been shown to be the result of natural transformation and homologous recombination are most commonly penicillin-binding proteins [20] and topoisomerase genes [21]. Penicillin resistance in pneumococci is most commonly the result of mosaic genes, with the degree and spectrum of β-lactam resistance varying depending upon the location of the cross-over and the individual PBPs involved [22]. It has also been demonstrated that there are non-*pbp* genes involved in the development of high-level resistance in pneumococci. Alteration of one of these genes, *MurM*, has been shown to increase MICs of the bacteria from moderate to fully resistant organisms. The operon containing this gene (*murMN*) has also been shown to be naturally transformable [23].

While high-level resistance has been demonstrated in vitro, the observed level of resistance that results from these mosaic genes is usually modest. This is probably because the level of resistance that is conferred in the donor bacteria is modest as well.

The clinical impact of the low level resistance can be significant. In areas, such as the central nervous system, where it is difficult to achieve bactericidal levels of β-lactam antibiotics even with intravenous administration, low levels of penicillin or ceftriaxone resistance may require use of alternative (and often less effective) agents for successful therapy. In areas, such as the middle ear, where inhibitory levels of cephalosporins are difficult to achieve after oral administration, these low levels of resistance can result in clinical failures of this class of antibiotics.

4 Target Overproduction

Occasionally, over-expression of target molecules will be used to overcome the effects of antimicrobial agents. Increased expression of PBP4 in *S. aureus* and PBP5 in *E. faecium* and *Enterococcus hirae* have been implicated in elevated levels of penicillin resistance in these species [24–26]. Glycopeptide resistant staphylococci that emerge after prolonged exposure to vancomycin have been found to have very thick cell walls that are full of unlinked cell wall precursors [27]. These precursors are thought to serve as false targets for vancomycin, resulting in sequestration of vancomycin in the outer portions of these thick cell walls, preventing arrival at the cell membrane, where the true cell wall precursor vancomycin targets exist. Finally, promoter mutations leading to overproduction of cellular DHFR has been implicated in trimethoprim resistance in *E. coli* [28].

5 Target Substitution

When "home-grown" point mutations confer only a modest level of resistance, high-level resistance can sometimes be achieved by acquiring genes that serve the same function as the target gene, but have a much lower affinity for the antibiotic. Perhaps the most prominent example of acquisition of such a gene is the *mecA* gene of *S. aureus* and coagulase-negative staphylococci. *mecA*, which is incorporated into a mobile element designated SCC*mec*, encodes penicillin-binding protein PBP2a, which binds the anti-staphylococcal β-lactam antibiotics (semisynthetic penicillins, cephalosporins, carbapenems) with an affinity sufficiently low to result in high-level resistance to these antibiotics [29].

Historically there has been significant speculation with regard to the acquisition of *SCC mec* and subsequent methicillin resistance in staph species. Recent evidence has surfaced which has clarified the mechanism of transferrable resistance. While earlier studies suggested the importance of the presence of both a bacteriophage and penicillinase plasmid in this process, it has recently been confirmed that these components are directly involved in transfer of *SCCmec* via transduction and are required for full expression of methicillin resistance [30].

While PBP2a confers a high level of resistance, it appears to be rather specific in its requirements. For example, if the transglycosylase of *S. aureus* PBP2 is inactivated, methicillin resistance is not expressed, suggesting that it requires cooperative interaction with this transglycosylase to function [31]. Moreover, several loci have been described [designated either fem (factors essential for methicillin resistance) or aux (auxiliary)] inactivation of which abolishes the expression of methicillin resistance [29]. These factors in most cases involve the synthesis of the precursors of cell wall structures, suggesting that alterations of cell wall structures (such as alterations in the peptide bridge that PBP2a cross-links) are not tolerated by PBP2a. In this context it is interesting that in vitro data indicate that PBP2a is able to effectively cross-link precursors in vitro that are markedly different than the 5-glycine cross-bridges present in *S. aureus* [32].

E. faecium resistance to ampicillin results from a combination of point mutations in *pbp5* and the subsequent substitution of resistant *pbp5* for more susceptible genes. As noted above, high-level resistance to ampicillin in *E. faecium* results from several mutations in its native *pbp5* [4]. Increasing evidence suggests that the widespread emergence of ampicillin resistance in *E. faecium* results not from the independent mutations of *pbp5* in different strains, but more commonly from the spread of highly resistant clonal groups [33]. We have shown in vitro that resistant *pbp5* is transferable from many *E. faecium* strains [34], suggesting that gene movement contributed to the formation of the clonal groups. In contrast to *mecA* gene in *S. aureus*, in which PBP2a is expressed along with susceptible PBP2, transfer of *pbp5* between *E. faecium* strains results in replacement of the native *pbp5* (L.B. Rice, data not shown).

Acquisition of individual genes that encode alternative, low affinity target proteins has been shown to be responsible for resistance to a variety of different antimicrobial classes, including trimethoprim (through alternative dihydrofolate reductases) [28] and sulfamethoaxozole (through alternative dihydropterate synthetases) [28].

Among the more complex and intriguing examples of target substitution resulting in high levels of resistance is the emergence and spread of vancomycin resistance in enterococci and, on rare occasions, in *S. aureus* [35]. Vancomycin acts by binding to the terminal D-alanine-D-alanine of the peptidoglycan pentapeptide precursor. In so doing, it prevents access to this terminus, preventing the PBPs from performing their transpeptidase function [35]. It has also been postulated that the large size of these molecules results in steric hindrance of transglycosylation.

The transferable glycopeptide resistance operons, which likely evolved from intrinsic self-defense operons within glycopeptide-producing bacteria, produce a series of enzymes whose activity results in the substitution of normal pentapeptide precursors with those that terminate in D-alanine-D-lactate [35]. Glycopeptides bind to these precursor molecules with roughly 1000-fold lower affinity than they do normal peptidoglycan precursors. Interestingly, the enterococcal PBPs appear to have no trouble utilizing these altered precursors to form cell wall, and since the terminal amino acid is cleaved from the precursor to form the final cross-linked product, the final product is cross-linked peptidoglycan that is predicted to be identical to that observed in cells lacking the glycopeptide resistance operons.

The two operons of primary importance in conferring glycopeptide resistance in enterococci are designated *VanA* and *VanB* [36, 37]. *VanA* operons confer resistance to both vancomycin and teicoplanin, whereas *VanB* operons confer resistance to vancomycin but are not induced by the presence of teicoplanin in the media (regulatory mutants resistant to teicoplanin do emerge under teicoplanin selective pressure) [38]. Both operons have been identified within transposable elements [39, 40], facilitating their widespread dissemination within *E. faecium*. Why they have not become prevalent in *E. faecalis* and *S. aureus* remains a mystery.

6 Target Modification or Protection

Target molecules can also undergo enzymatic modification that reduces binding of an antibiotic. The most widespread example of this type of modification is in resistance to macrolides antibiotics in gram-positive bacteria. Macrolides inhibit protein synthesis by binding reversibly to the peptidyl-tRNA binding site of the 60S ribosomal subunit, inhibiting the translocation of a newly synthesized peptidyl-tRNA molecule from the acceptor site on the ribosome to the peptidyl donor site. Resistance to macrolides is commonly achieved by methylating the ribosome, thereby inhibiting macrolide binding [41]. Ribosomal methylation results in resistance to all clinically available macrolides (azithromycin, clarithromycin, erythromycin, roxithromycin), lincosamides (clindamycin), and streptograminB (quinupristin).

Several *erm* (erythromycin ribosomal methylase) genes have been characterized. In many instances, these genes are under regulatory control by a translational attenuation mechanism [42]. Macrolides induce expression of the resistance operons, whereas clindamycin does not. The presence of even the inducible variety does raise concerns about the use of clindamycin in the clinical setting, since mutations can result in constitutive expression of the *erm* genes, resulting in resistance to clindamycin.

Targets may also be protected by the expression of proteins that bind to the target in a manner that prevents interaction with the antibiotic but allows normal function of the protein. Among the best studied of these proteins is the tet(M) protein, widely prevalent in gram-positive bacteria. *Tet(M)* encodes a protein that exhibits homology to elongation factors EF-Tu and EF-G and it exhibits ribosome-dependent GTPase activity [43]. It binds to the ribosome, changing its conformation in a manner that precludes tetracycline binding. *Tet(M)* is most commonly incorporated into broad-host range conjugative transposon Tn*916* and similar elements, explaining its remarkably wide distribution in bacteria [44].

Protection proteins also have been described that confer resistance to fluoroquinolone antimicrobial agents. These proteins, referred to as QNR proteins [45], protect DNA from quinolone binding. In general, they confer only a low level of resistance. However, when combined with other mechanisms, such as QRDR mutations or efflux pumps, the level of resistance can be substantial. Additionally, acquired resistance to fusidic acid in staphylococci has been attributed to a protein that protects EF-G from fusidic acid binding [46].

Similar Mechanisms of Resistance Have Emerged in the Polymyxin Class of Antibiotics.

In light of the alarming emergence of multidrug resistant gram-negative pathogens, there has recently been renewed interest in this antimicrobial class. This group is primarily comprised of colistin (polymyxin E) and polymyxin B. These agents have demonstrated significant in vitro and in vivo activity against multidrug resistant *Klebsiella*, *Pseudomonas,* and *Acinetobacter*, thereby expanding treatment options in otherwise pan-resistant infectious disease. The mechanism of action of these agents is primarily at the level of the lipopolysaccharide outer membrane of gram-negative bacteria. The polymyxins are cationic amphiphilic compounds that are electrostatically attracted to the negatively charged LPS. The compounds displace the positively charged calcium and magnesium ions bound to the phosphate component of the lipid A moiety within the LPS, conferring a structural change in the cell membrane resulting in disruption of the LPS and cellular death [47].

Unfortunately, the more widespread use of polymyxins in recent years has compounded the historical mechanisms of resistance with novel routes of antimicrobial subversion. While some of this resistance has been observed upon reintroduction of polymyxins in treatment-experienced patients, there have been reports of resistance developing during initial courses of treatment with monotherapy as well [48]. While the exact mechanisms of resistance appear to be variable amongst bacterial species, the common end product of the resistance pathway is that of a less negatively charged lipopolysaccharide layer that has reduced affinity for the cationic polymyxins. This modification is believed to result from the addition of 4-amino-4-deoxy-l-arabinose to the phosphate group of lipid A. It has been postulated that the synthesis of the arabinose compound is regulated by a complex interplay between the polymyxin resistance operon (*pmr*) and membrane bound kinases (PhoQ, Pmr B), though the details of this interaction on an organism specific scale have yet to be fully elucidated [49].

7 Conclusion

Target modifications as a route to antimicrobial resistance are extraordinarily common in pathogenic bacteria. These modifications may result from point mutations in the genes encoding the targets, protection of the target, modification of

the target, or acquisition of a new molecule that serves the function of the susceptible target, but which is not susceptible to inhibition by the antimicrobial agent. In many cases, these modified targets are incorporated into mobile elements, facilitating their dissemination though many different species. The wide spectrum of mechanistic options available to bacteria for conferring resistance is a sobering aspect of antimicrobial development, since the spectrum of possible resistance mechanisms in nature cannot be known prior to clinical use of a new antimicrobial agent.

References

1. Bjorkman J, Nagaev I, Berg OG, Hughes D, Andersson DI. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science. 2000;287:1479–82.
2. Wehrli W. Rifampin: mechanisms of action and resistance. Rev Infect Dis. 1983;5 Suppl 3:S407–11.
3. Hooper DC. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis. 2001;7:337–41.
4. Rice LB, Bellais S, Carias LL, et al. Impact of specific pbp5 mutations on expression of beta-lactam resistance in Enterococcus faecium. Antimicrob Agents Chemother. 2004;48:3028–32.
5. Drlica K. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother. 2003;52:11–7.
6. Davidson R, Cavalcanti R, Brunton JL, et al. Resistance to levofloxacin and failure of treatment of pneumococcal pneumonia. N Engl J Med. 2002;346:747–50.
7. Marshall SH, Donskey CJ, Hutton-Thomas R, Salata RA, Rice LB. Gene dosage and linezolid resistance in Enterococcus faecium and Enterococcus faecalis. Antimicrob Agents Chemother. 2002;46:3334–6.
8. Gonzales RD, Schreckenberger PC, Graham MB, Kelkar S, DenBesten K, Quinn JP. Infections due to vancomycin-resistant Enterococcus faecium resistant to linezolid. Lancet. 2001;357:1179.
9. Lobritz M, Hutton-Thomas R, Marshall S, Rice LB. Recombination proficiency influences frequency and locus of mutational resistance to linezolid in Enterococcus faecalis. Antimicrob Agents Chemother. 2003;47:3318–20.
10. Meka VG, Gold HS, Cooke A, et al. Reversion to susceptibility in a linezolid-resistant clinical isolate of Staphylococcus aureus. J Antimicrob Chemother. 2004;54:818–20.
11. Pillai SK, Sakoulas G, Wennersten C, et al. Linezolid resistance in Staphylococcus aureus: characterization and stability of resistant phenotype. J Infect Dis. 2002;186:1603–7.
12. Fishovitz J, Rojas-Altuve A, Otero LH, et al. Disruption of allosteric response as an unprecedented mechanism of resistance to antibiotics. J Am Chem Soc. 2014;136:9814–7.
13. Otero LH, Rojas-Altuve A, Llarrull LI, et al. How allosteric control of Staphylococcus aureus penicillin binding protein 2a enables methicillin resistance and physiological function. Proc Natl Acad Sci U S A. 2013;110:16808–13.
14. Paulsen IT, Banerjei L, Myers GS, et al. Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. Science. 2003;299:2071–4.
15. Welch RA, Burland V, Plunkett 3rd G, et al. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli. Proc Natl Acad Sci U S A. 2002;99:17020–4.
16. Burrus V, Waldor MK. Shaping bacterial genomes with integrative and conjugative elements. Res Microbiol. 2004;155:376–86.
17. Dowson CG, Hutchison A, Brannigan JA, Spratt BG. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Proc Natl Acad Sci U S A. 1989;86:8842–6.
18. Spratt BG. Hybrid penicillin-binding proteins in penicillin-resistant strains of *Neisseria gonorrhoeae*. Nature (London). 1988;332:173–6.
19. Bowler LD, Zhang QY, Riou JY, Spratt BG. Interspecies recombination between the penA genes of Neisseria meningitidis and commensal Neisseria species during the emergence of penicillin resistance in N. meningitidis: natural events and laboratory simulation. J Bacteriol. 1994;176:333–7.
20. Dowson CG, Coffey TJ, Spratt BG. Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to ß-lactam antibiotics. Trends Microbiol. 1994;2:361–6.
21. Balsalobre L, Ferrandiz MJ, Linares J, Tubau F, de la Campa AG. Viridans group streptococci are donors in horizontal transfer of topoisomerase IV genes to Streptococcus pneumoniae. Antimicrob Agents Chemother. 2003;47:2072–81.
22. Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of Streptococcus pneumoniae are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob Agents Chemother. 1996;40:829–34.
23. Smith AM, Klugman KP. Alterations in MurM, a cell wall muropeptide branching enzyme, increase high-level penicillin and cephalosporin resistance in Streptococcus pneumoniae. Antimicrob Agents Chemother. 2001;45:2393–6.
24. Fontana R, Aldegheri M, Ligozzi M, Lopez H, Sucari A, Satta G. Overproduction of a low-affinity penicillin-binding protein and high-level ampicillin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother. 1994;38:1980–3.
25. Henze UU, Berger-Bachi B. Staphylococcus aureus penicillin-binding protein 4 and intrinsic beta- lactam resistance. Antimicrob Agents Chemother. 1995;39:2415–22.
26. Rice LB, Carias LL, Hutton-Thomas R, Sifaoui F, Gutmann L, Rudin SD. Penicillin-binding protein 5 and expression of ampicillin resistance in Enterococcus faecium. Antimicrob Agents Chemother. 2001;45:1480–6.
27. Cui L, Ma X, Sato K, et al. Cell wall thickening is a common feature of vancomycin resistance in Staphylococcus aureus. J Clin Microbiol. 2003;41:5–14.
28. Huovinen P. Resistance to trimethoprim-sulfamethoxazole. Clin Infect Dis. 2001;32:1608–14.
29. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 1997;10:781–91.
30. Scharn CR, Tenover FC, Goering RV. Transduction of staphylococcal cassette chromosome mec elements between strains of Staphylococcus aureus. Antimicrob Agents Chemother. 2013;57:5233–8.
31. Pinho MG, de Lencastre H, Tomasz A. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc Natl Acad Sci U S A. 2001;98:10886–91.
32. Arbeloa A, Hugonnet JE, Sentilhes AC, et al. Synthesis of mosaic peptidoglycan cross-bridges by hybrid peptidoglycan assembly pathways in gram-positive bacteria. J Biol Chem. 2004;279:41546–56.
33. Willems RJ, Top J, van Santen M, et al. Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. Emerg Infect Dis. 2005;11:821–8.
34. Rice LB, Carias LL, Rudin S, Lakticova V, Wood A, Hutton-Thomas R. Enterococcus faecium low-affinity pbp5 is a transferable determinant. Antimicrob Agents Chemother. 2005;49:5007–12.
35. Gold HS. Vancomycin-resistant enterococci: mechanisms and clinical observations. Clin Infect Dis. 2001;33:210–9.

36. Arthur M, Molinas C, Bugg TDH, Wright GD, Walsh CT, Courvalin P. Evidence for in vivo incorporation of D-lactate into peptidoglycan precursors of vancomycin-resistant enterococci. Antimicrob Agents Chemother. 1992;36:867–9.
37. Evers S, Sahm DF, Courvalin P. The *vanB* gene of vancomycin-resistant *Enterococcus faecalis* V583 is structurally-related to genes encoding D-ala:D-ala ligases and glycopeptide-resistance proteins VanA and VanC. Gene. 1993;124:143–4.
38. Evers S, Courvalin R. Regulation of VanB-type vancomycin resistance gene expression by the vanSB-VanRB two component regulatory system in *Enterococcus faecalis* V583. J Bacteriol. 1996;178:1302–9.
39. Arthur M, Molinas C, Depardieu F, Courvalin P. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in Enterococcus faecium BM4147. J Bacteriol. 1993;175:117–27.
40. Carias LL, Rudin SD, Donskey CJ, Rice LB. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J Bacteriol. 1998;180:4426–34.
41. Weisblum B. Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother. 1995;39:577–85.
42. Hahn J, Grandi G, Gryczan TJ, Dubnau D. Translational attenuation of ermC: a deletion analysis. Mol Gen Genet. 1982;186:204–16.
43. Sanchez-Pescador R, Brown JT, Roberts M, Urdea MS. Homology of the TetM with translational elongation factors: implications for potential modes of tetM-conferred tetracycline resistance. Nucleic Acids Res. 1988;16:1218.
44. Rice LB. Tn*916*-family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrob Agents Chemother. 1998;42:1871–7.
45. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis. 2006;6:629–40.
46. O'Neill AJ, Chopra I. Molecular basis of fusB-mediated resistance to fusidic acid in Staphylococcus aureus. Mol Microbiol. 2006;59:664–76.
47. Li J, Nation RL, Milne RW, Turnidge JD, Coulthard K. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. Int J Antimicrob Agents. 2005;25:11–25.
48. Ah YM, Kim AJ, Lee JY. Colistin resistance in Klebsiella pneumoniae. Int J Antimicrob Agents. 2014;44:8–15.
49. Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Updat. 2010;13:132–8.

Biochemical Logic of Antibiotic Inactivation and Modification

8

Vanessa M. D'Costa and Gerard D. Wright

1 Introduction

Bacteria have evolved a myriad of tactics to circumvent the actions of antibiotics. Bacterial resistance to antibiotics manifests itself in both general and specific protection mechanisms. Consequently, the characteristics of resistance can be paralleled to those of the mammalian immune response. Antibiotic resistance can be differentiated into: (1) nonspecific mechanisms that confer general innate immunity to a class of antibiotics (e.g., broad spectrum efflux mechanisms, target modification) and (2) highly precise responses that include selective enzyme-based mechanisms that mirror the acquired immune response with respect to target specificity and potency. Bacteria deploy both types of mechanisms in response to the presence of cytotoxic antibiotics. One of the most prevalent mechanisms of resistance involves enzymatically altering the antibiotic structure to an inactive derivative, one incapable of acting against its bacterial target. Antibiotic-inactivating enzymes can accomplish this task by one of two means: by eradicating the essential reactive center of the antibiotic or by modifying the drug in a manner that impairs target binding. By critically assessing the manner in which each antibiotic class interacts with its target and the subsequent mode of inactivation, the molecular logic of each strategy can be elucidated.

Although antibiotic resistance via target modification or efflux mechanisms results in survival of the resistant organism, the concentration of antibiotic that the bacterium is exposed to remains unaffected. Thus, other proximal susceptible organisms can still be targeted by the antimicrobial agent. In contrast, enzyme-catalyzed detoxification of antibiotics effectively (and often irreversibly) lowers the concentration of the drug, and as a result has the potential for a much broader impact on microbial growth. The presence of an antibiotic-resistant microbe can, at least in theory, promote the growth of adjacent bacteria that otherwise would be susceptible to the antibiotic by inactivating the drug in the local environment. This can occur even if it is the susceptible organism, not the resistant strain, which is the cause of infection. As a result, enzyme-catalyzed antibiotic inactivation can have a significant and broad impact on antimicrobial therapy.

Since the first reports of penicillin-inactivating strains of bacteria in the early 1940s [1], virtually all antibiotics have been shown to be modified or destroyed by a cadre of enzymes with hydrolytic, chemical group transfer, or redox ability. In Table 8.1 we itemize representative enzymes and mechanisms, differentiating between mechanisms that modify the antibiotic (e.g., acylation, phosphorylation), and those that essentially cause irreversible destruction (e.g., hydrolysis). A general observation evident from Table 8.1 is that most antibiotics that either are natural products or are based on natural product chemical scaffolds are more susceptible to some form of enzyme-based inactivation, while antibiotics of synthetic origin (e.g., fluoroquinolones) are not (however, enzyme-based inactivation of certain fluoroquinolones has been reported [2]). These relatively enzyme-impervious antibiotics are nonetheless still susceptible to resistance mechanisms, often as substrates for efflux pumps.

Walsh described the cellular impact and rationale of biochemical reactions as "molecular logic" [3] and this terminology works very well in dissecting mechanisms of antibiotic resistance. Thus enzyme-catalyzed antibiotic resistance is functionally and structurally linked to the mode of action of these agents. For example, modification of key functional groups on an antibiotic can sterically or electronically block interaction with target (see Sect. 3 below for

V.M. D'Costa, Ph.D.
Cell Biology Program, The Hospital for Sick Children,
686 Bay Street, Toronto, ON, Canada

G.D. Wright, Ph.D. (✉)
Department of Biochemistry and Biomedical Sciences,
McMaster University, Hamilton, ON, Canada

M. G. DeGroote Institute for Infectious Disease Research,
McMaster University, 1200 Main Street West,
Hamilton, ON, Canada
e-mail: wrightge@mcmaster.ca

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_8

Table 8.1 Survey of enzymatic mechanisms of antibiotic resistance

Antibiotic	Mechanisms	Enzyme
Antibiotic Destruction		
β-Lactams	Hydrolysis	β-Lactamase
Macrolides	Hydrolysis	Macrolide esterase
Type B Streptogramins	C-O-bond cleavage	Vgb lyase
Tetracyclines	Mono-oxidation	TetX
Fosfomycin	Hydrolysis	Epoxidase
	Thiol transfer	Thiol transferase
Antibiotic Modification		
Aminoglycosides	Acylation	Acetyltransferase
	Phosphorylation	Kinase
	Adenylylation	AMP-transferase
Macrolides	Phosphorylation	Kinase
	Glycosylation	UDP-glucosyl transferase
Lincosamides	Adenylylation	AMP-transferase
Rifamycins	Glycosylation	TDP-glucosyl transferase
	Phosphorylation	Kinase
Chloramphenicol	Acylation	Acetyltransferase
Type A Streptogramins	Acylation	Acetyltransferase

examples). This review describes mechanisms of antibiotic destruction and modification resulting in resistance in the context of the mode of action of the antibiotic. Our aim is not to be a comprehensive examination of the details of all known resistance mechanisms but rather to focus on selected examples to decode the molecular basis and biological impact of these inactivation strategies.

2 Destruction of Antibiotics

We classify antibiotic destruction as a mechanism that results in either ablation of a key reactive center or massive structural rearrangement that is not readily reversed under normal physiological conditions. Hydrolysis of the reactive β-lactam ring of penicillin and cephalosporin antibiotics by β-lactamases is an example of the first class, and linearization of the cyclic depsipeptide of Type B streptogramins by Vgb lyase is an example of the second. In all classes, the action of resistance enzymes tactically impacts the mode of action of the affected antibiotics to disrupt their biological activity. Examples of each class are discussed below.

2.1 β-Lactam Antibiotics

The β-lactams remain the most successful and widely used antibiotics in modern chemotherapy. These natural products and their semisynthetic derivatives act by covalently modifying so-called penicillin-binding proteins (PBPs) [4]. PBPs include membrane-associated enzymes important in bacterial peptidoglycan assembly and maintenance. Covalent modification of this subclass of PBPs by β-lactams blocks their enzymatic activity, thereby inhibiting cell wall metabolism, which results in impaired wall integrity and cell death. PBPs include the transpeptidases and DD-carboxypeptidases that act on the pentapeptide portion of the peptidoglycan repeating unit that consists of the disaccharyl unit, N-acetylglucosamine-N-acetylmuramic acid, to which a D-Ala-D-Ala terminating pentapeptide is linked through the lactyl group of N-acetylmuramic acid (Fig. 8.1). Transpeptidases and DD-carboxypeptidases use canonical Ser hydrolase chemistry to either rigidify the cell wall by synthesizing inter-strand peptidoglycan cross-links between the D-Ala-D-Ala termini of adjacent peptidoglycan strands (transpeptidases) or control cell wall strength and flexibility by cleaving the terminal D-Ala-D-Ala peptide bond of the pentapeptide (Fig. 8.1). Strominger noted almost 50 years ago that the β-lactam antibiotics sterically and electronically mimic the acyl-D-Ala-D-Ala terminus of the pentapeptide (Fig. 8.2) [5]. This model overlaps the highly strained (and thus chemically reactive) β-lactam ring over the scissile D-Ala-D-Ala peptide bond. Attack of the nucleophilic Ser hydroxyl onto the β-lactam ring carbonyl opens the cyclic structure and generates a covalent intermediate that is resistant to hydrolysis (Fig. 8.3), thereby chemically titrating PBPs into inactive complexes and shutting down cell wall synthesis.

In order to overcome the action of cytotoxic β-lactams, bacteria have evolved secreted enzymes that hydrolytically cleave the β-lactam ring of penicillins and cephalosporins [6] (Fig. 8.4). This strategy represents the most common cause of clinical resistance to β-lactam antibiotics. The molecular logic of this resistance mechanism involves the destruction of the reactive "warhead" of the β-lactam antibiotics, thereby

eliminating the essential chemical structure necessary for PBP inactivation. These hydrolytic enzymes, appropriately named β-lactamases, fall into two general classes based on enzymological inactivation strategy: Ser β-lactamases and metallo-β-lactamases (Fig. 8.4).

Serine β-lactamases are a broad family of enzymes that can be further categorized into three subclasses based on primary amino acid sequence, spectrum of substrates, and inhibition profiles [7]. Present in both Gram-positive and Gram-negative bacteria and identified both chromosomally and on mobile genetic elements, they include the well-described TEM, SHV, CTX, and OXA subfamilies [7]. Ser β-lactamases share structural homology with the DD-carboxypeptidases and operate by similar Ser hydrolase chemistry. However the hydrolytic step, which is slow in PBPs, is fast in β-lactamases, resulting in highly efficient detoxification of the antibiotics (Fig. 8.4).

Metallo-β-lactamases are a diverse class of enzymes with respect to primary sequence, and include the IMP, VIM, and NDM subfamilies associated with plasmids and integrons. These degradative enzymes have received considerable attention within the scientific community in recent years due to the identification of new NDM subfamily [8]. The first such enzyme, NDM-1, has been identified in a number of Gram-negative pathogens including *Escherichia, Klebsiella, Acinetobacter, Pseudomonas,* and *Vibrio* [8–12]. It is not only capable of hydrolyzing almost every β-lactam clinically available [8], but its location on mobile genetic elements is commonly in close proximity to resistance determinants for other antimicrobial classes. This association with resistance to classes that include macrolides, rifamycins, fluoroquinolones, aminoglycosides, and phenicols [8, 13] results in a multidrug resistance phenotype. Metallo-β-lactamases adopt a hydrolytic chemistry analogous to metallo-proteases to cleave the reactive β-lactam ring (Fig. 8.4). They contain one or two active site Zn^{2+} ions, which function in concert with available water. Hydrolysis is achieved through the activation of water by Zn^{2+}, which facilitates nucleophillic attack of the β-lactam carbonyl carbon.

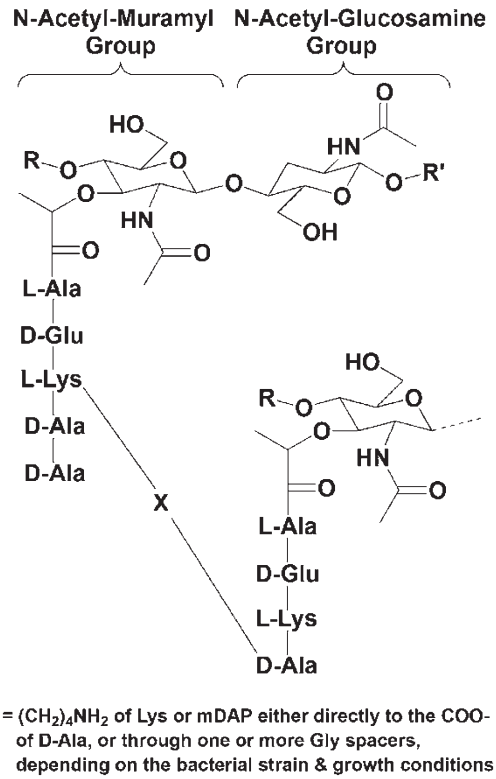


Fig. 8.1 Structure of the bacterial peptidoglycan unit. Peptidoglycan consists of repeating disaccharyl units (N-acetylglucosamine-N-acetylmuramic acid), to which a pentapeptide is linked through each N-acetylmuramic acid. Cross-linking between adjacent pentapeptides provides rigidity to the bacterial cell

2.2 Fosfomycin

Enzymes that inactivate fosfomycin also employ a mechanism that destroys a reactive chemical warhead. The key structural element of this antibiotic is a reactive epoxide that is attacked by its intracellular target, the cell wall biosynthetic enzyme MurA (Figs. 8.5 and 8.6A). This enzyme is essential for synthesis of N-acetylmuramic acid and covalent modification of a key Cys residue by fosfomycin efficiently inactivates the enzyme.

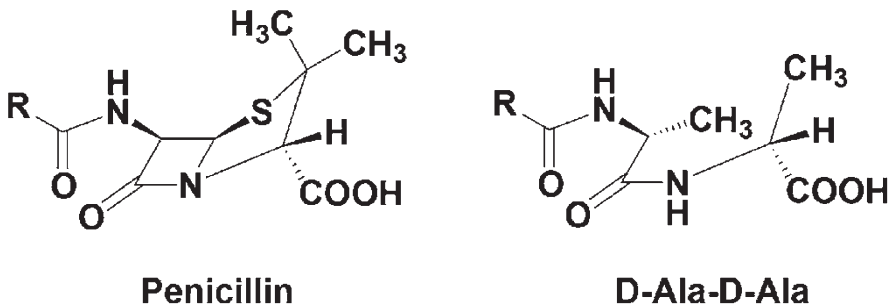


Fig. 8.2 Comparison of the β-lactam penicillin and the D-Ala-D-Ala peptidoglycan terminus

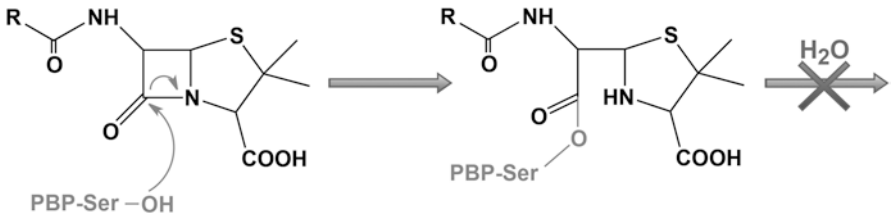


Fig. 8.3 Mechanism of action of β-lactam antibiotics on bacterial transpeptidases and DD-carboxypeptidases. Nucleophilic attack of the PBP Ser hydroxyl on the β-lactam ring carbonyl results in an opening of the β-lactam ring. The active site machinery of transpeptidase or DD-carboxypeptidases is effectively captured and as the subsequent covalent intermediate cannot be hydrolyzed

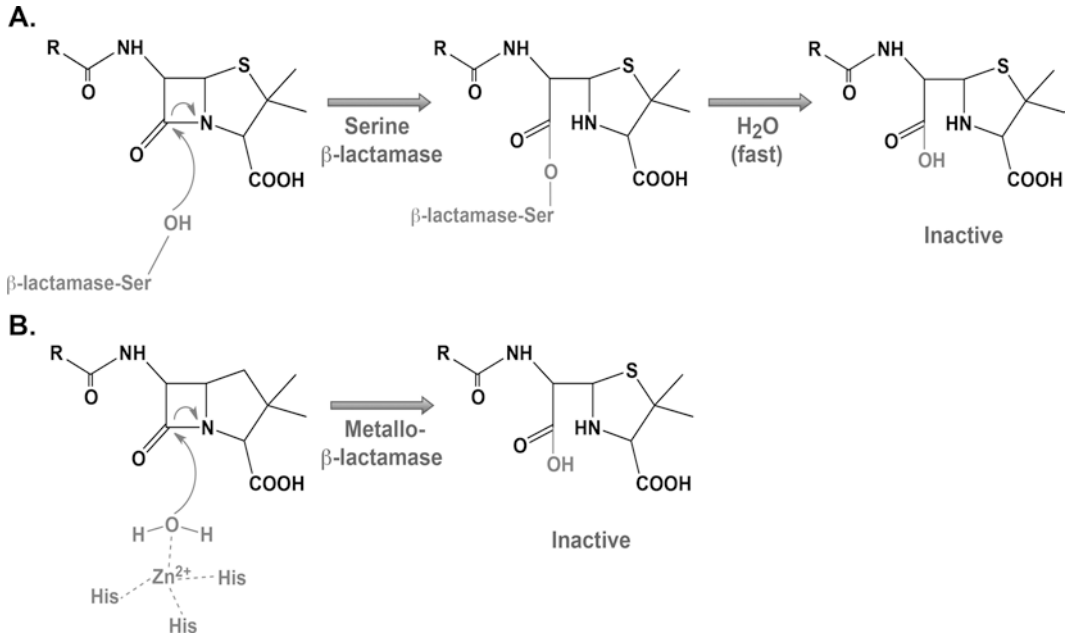


Fig. 8.4 Mechanisms of enzymatic inactivation of β-lactam antibiotics. β-Lactamases catalyze the hydrolytic cleavage of β-lactam rings. (**a**) Serine-β-lactamases form a transient enzyme-antibiotic intermediate, which is quickly hydrolyzed. (**b**) Metallo-β-lactamases utilize a bound Zn^{2+} to activate water for hydrolytic attack of the β-lactam ring

The bacterial countermeasure to inactivate this antibiotic is an epoxide ring opening reaction using metalloenzymes that employ one of two distinct chemical tactics. Both strategies result in efficient destruction of the antibiotic's reactive center, thereby blocking its action on the target MurA. The first, catalyzed by FosX, is a metal-dependent hydrolytic process that generates a vicinal diol [14] (Fig. 8.6B). FosX enzymes have been identified in the environmental bacteria *Mesorhizobium loti* and *Desulfitobacterium hafniense*, as well as in the pathogens *Listeria monocytogenes, Brucella melitensis,* and *Clostridium botulinum* [14]. The second strategy is via a thiol-dependent ring opening reaction by enzymes that use abundant intracellular thiols such as glutathione (FosA) [15] (Fig. 8.6C) and cysteine (FosB) [16] (Fig. 8.6D). FosA and FosB are approximately 48 % identical at the amino acid level. The determinant *fosA* has been detected on plasmids from several Gram-negatives including *Pseudomonas, Enterobacter,* and *Acinetobacter* [17], whereas *fosB* is associated with resistance in the Gram-positives *Staphylococcus* [18] and *Bacillus* [16]. Although no significant sequence similarity is seen between the glutathione-dependent class of Fos enzymes and canonical glutathione S-transferases, crystal structure-based studies of FosA have demonstrated homology to members of the vicinal oxygen chelate family [19].

2.3 Macrolide Antibiotics

The macrolide antibiotics include natural products such as erythromycin, and semisynthetic derivatives (e.g., clarithromycin). These antibiotics are assembled via a polyketide assembly line, cyclized to form a macrolactone ring structure, and subsequently modified by glycosylation to generate

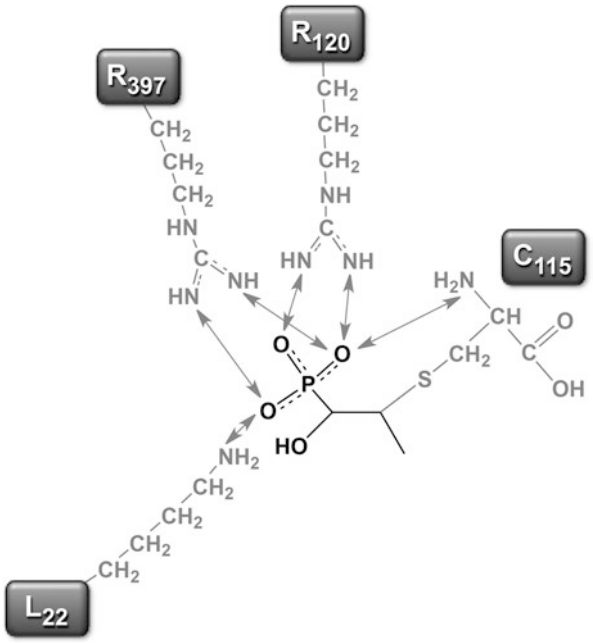


Fig. 8.5 Interactions of fosfomycin with its bacterial target, MurA. Fosfomycin forms a covalent bond with MurA's active site Cys. Additional interactions with MurA are designated as arrows. MurA residues are labeled in grey. Adapted from [103]

a mature antibiotic [20]. Macrolides inhibit bacterial translation by binding to the large ribosomal subunit in the vicinity of the peptide exit tunnel [21]. This interaction requires an intact cyclic macrolide ring and in most cases the amino sugar cladinose (Fig. 8.7).

Enzymatic resistance to macrolide antibiotics occurs either by modification of the desoamine sugar (see Sect. 3 below) or by linearization of the macrolactone ring [22] (Fig. 8.8). The latter mechanism is catalyzed by esterases that hydrolytically cleave the lactone resulting in ring opening and consequently the inability to effectively bind to the peptide exit tunnel. The erythromycin esterases EreA and EreB have been identified on integrons and R-plasmids from a variety of Gram-negatives that include *E. coli*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Salmonella,* and *Vibrio* [23–30].

2.4 Type B Streptogramins

The streptogramins are natural product inhibitors of bacterial translation that consist of two structurally distinct classes, denoted as Type A and Type B [31]. Type B streptogramins are cyclic depsipeptides that, like macrolides, bind to a

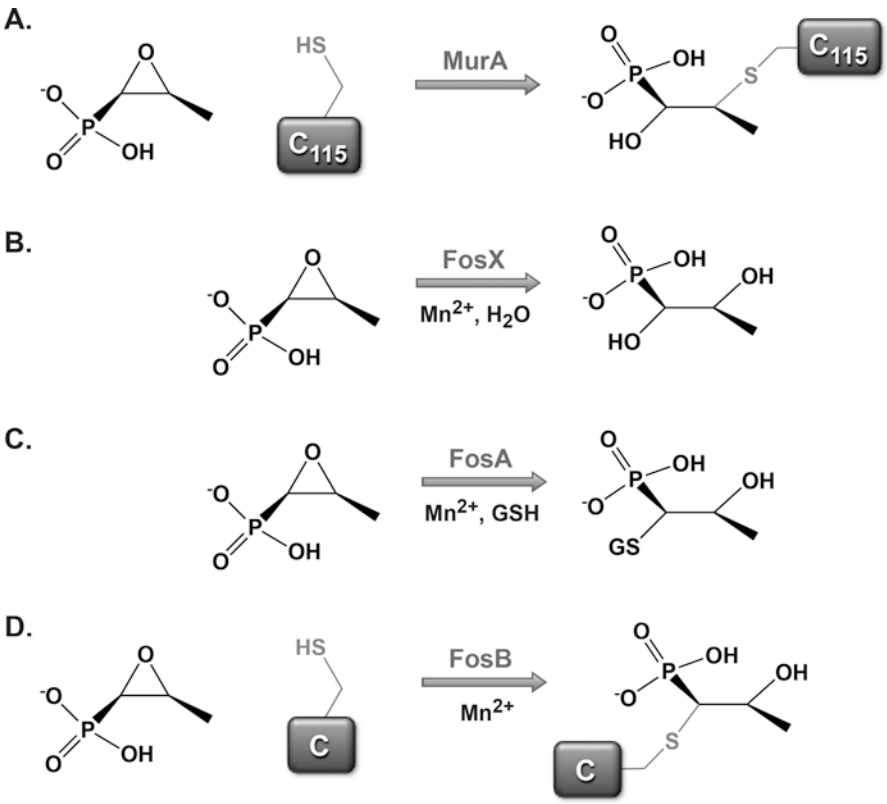


Fig. 8.6 Mechanism of fosfomycin action and inactivation. (**a**) Fosfomycin targets the active site Cys residue of MurA, forming a covalent intermediate. (**b**) FosX-mediated inactivation of fosfomycin results in the formation of a diol. (**c**) The product of FosA-mediated fosfomycin inactivation is a glutathione-fosfomycin adduct. (**d**) FosB-mediated resistance to fosfomycin results in an enzyme-antibiotic intermediate, linked by a Cys residue

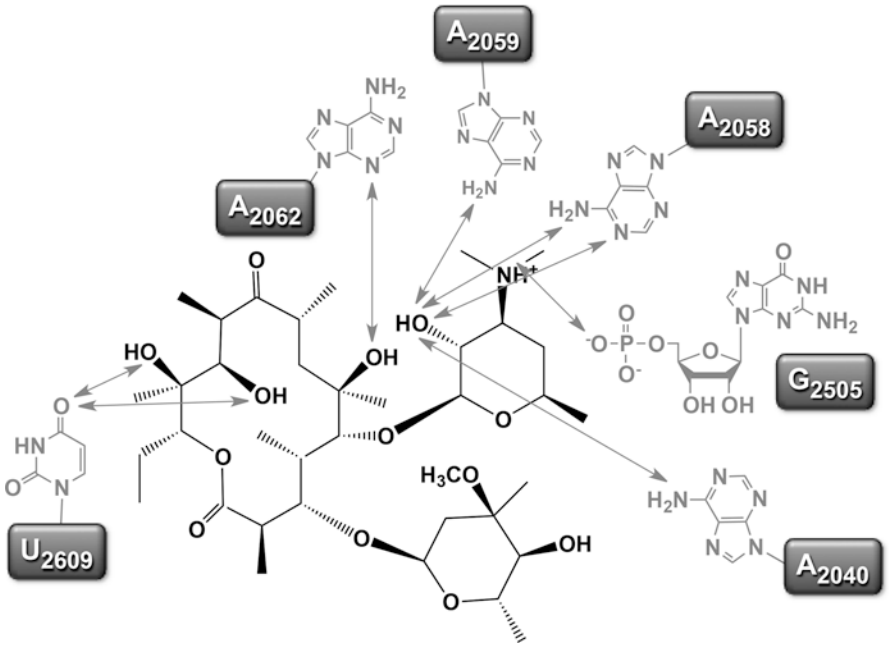


Fig. 8.7 Interactions of the macrolide erythromycin with the bacterial ribosomal RNA. Key 23S rRNA residues are shown in grey and the interactions are designated as arrows. The hydroxyl group that serves as a site of inactivation interacts with several nucleotides. Adapted from [72]

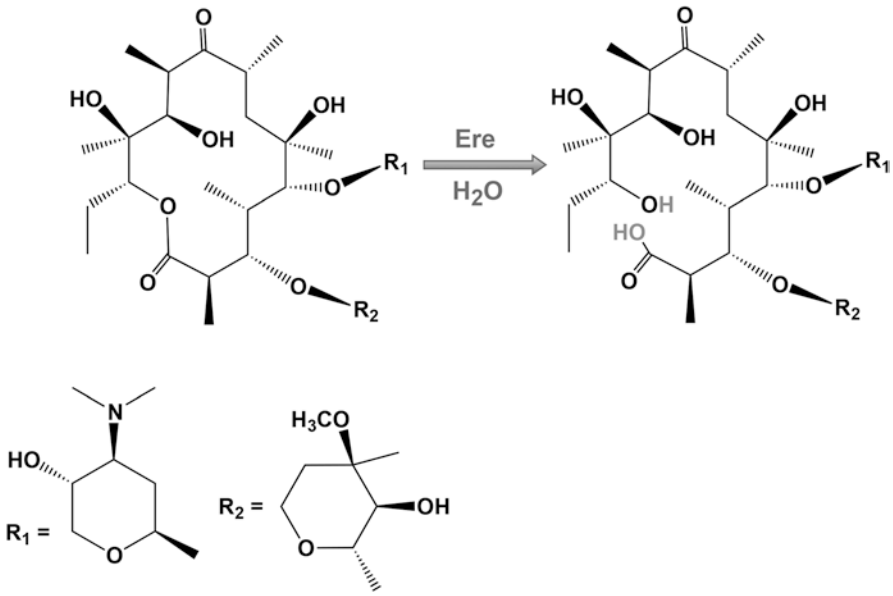


Fig. 8.8 Inactivation of the macrolide erythromycin by hydrolysis. Macrolides can be inactivated by hydrolysis of the macrolactone ring. This reaction is mediated by the esterase Ere [22]

region of the bacterial ribosome's peptide exit tunnel [32, 33] (Fig. 8.9). Type A streptogramins are hybrid peptide-polyketide antibiotics that bind to the peptidyltransferase center of the ribosome. Enzymatic resistance to Type A streptogramins occurs via an acetyltransfer mechanism, while enzymatic resistance to Type B streptogramins occurs through a ring opening reaction catalyzed by the enzyme Vgb. Vgb, originally identified in streptogramin-resistant *Staphylococcus aureus*, cleaves the cyclic peptide [34], resulting in depsipeptide linearization. The resulting structure no longer exhibits affinity for the bacterial ribosome, mirroring the biochemical logic of macrolide esterases. However, the mechanism of ring opening is quite distinct. Rather than causing a hydrolytic reaction at the thermodynamically vulnerable ester bond of Type B streptogramins, Vgb catalyzes a lyase reaction that results in a ring opening of the peptide by a C-O cleavage strategy [35] (Fig. 8.10).

2.5 Tetracycline

The tetracycline antibiotics have found extensive clinical use for almost half a century. This class of antibiotics binds divalent metals and acts by blocking bacterial translation by

binding to the small ribosomal subunit [36] (Fig. 8.11). The principle mechanisms of clinical tetracycline resistance are efflux and ribosomal protection [36, 37]. However, an enzymatic mechanism of tetracycline resistance, originally discovered in *Bacteroides* [38], has been identified that inactivates the antibiotic via an oxygen-dependent process. Purification of the enzyme that catalyzes this reaction, TetX, followed by careful analysis of the products of the reaction showed that the enzyme first facilitates mono-hydroxylation of the antibiotic at position 11a, effectively disrupting the essential metal-binding site on the molecule [39, 40] (Fig. 8.12). Furthermore, this step triggers a nonenzymatic decomposition of the antibiotic to a form of unknown structure that turned the growth media black. This enzyme is also capable of mono-hydroxylation of the latest generation of tetracycline antibiotics, the glycylcyclines, resulting in resistance, but not the subsequent nonenzymatic decomposition of the antibiotic [41].

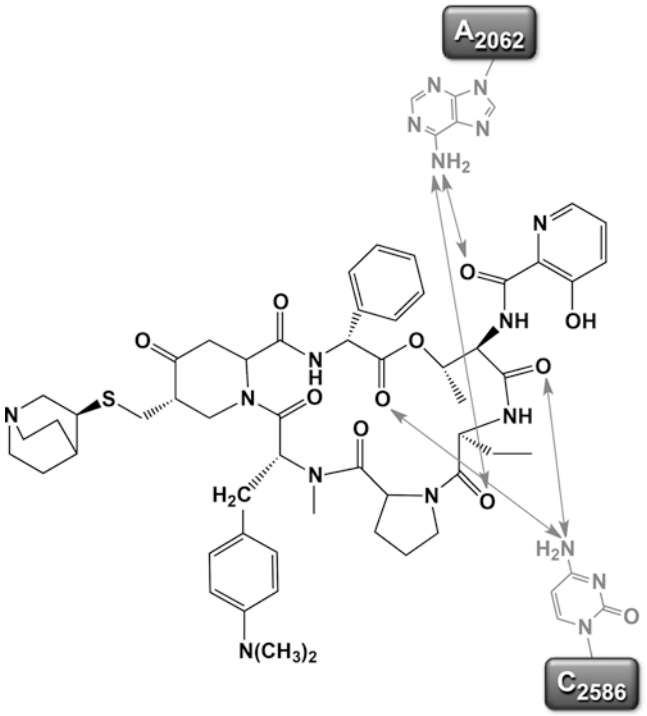


Fig. 8.9 Streptogramin B interactions with bacterial ribosome. Quinupristin, a Type B Streptogramin, binds to the bacterial ribosome's polypeptide exit tunnel. Key interactions with the 23S rRNA are designated as arrows. Adapted from [32, 33]

3 Antibiotic Modification

The most diverse class of resistance enzymes catalyzes the covalent modification of antibiotics. This strategy confers resistance by means of group transfer and includes both *O*- and *N*-acetylation, *O*-phosphorylation, *O*-nucleotidylylation, *O*-ribosylation, and *O*-glycosylation. Covalent modification of antibiotics by this class of enzymes does not destroy the essential active warheads of the compounds, as described in the previous section, but rather obstructs interaction of the antimicrobial with its target. This is accomplished by functionally derivatizing the antibiotic at structural location(s) that play an essential role in binding with the target. By doing so, key interactions (e.g., hydrogen bonding, ionic interactions, steric complementarity) are disrupted by the introduction of the modifying group, resulting in an overall decrease in affinity of the antibiotic derivative for its target in comparison to the unmodified counterpart.

This antibiotic inactivation tactic requires the presence of a co-substrate for enzyme activity, such as acetyl-CoA, ATP, or UDP-glucose. Consequently, enzyme activity is localized to the bacterial cytosol. The inactivation products are commonly stable in the cellular environment, thus the reactions are considered to be irreversible in the absence of an enzyme that counteracts the reaction. However it is conceivable that the presence of such reversing enzymes (e.g., phosphatases, acylases) can undo resistance in vivo.

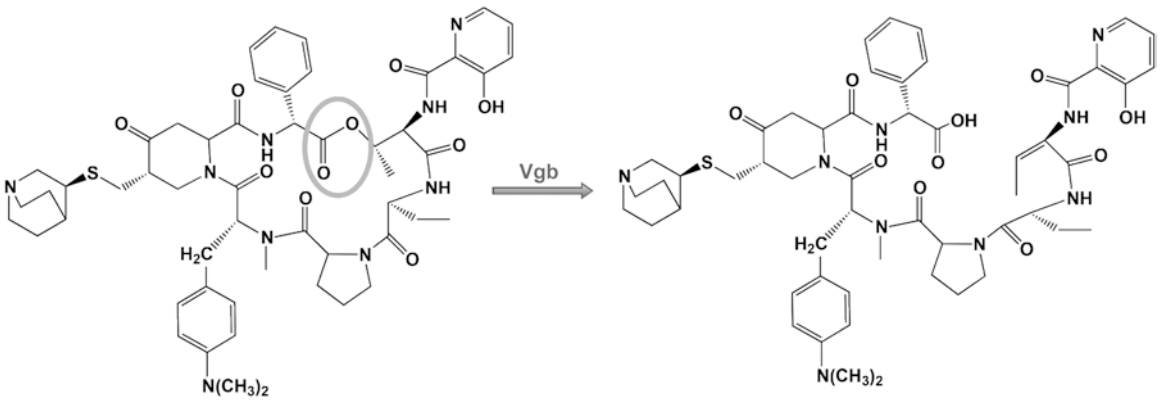


Fig. 8.10 Vgb-catalyzed inactivation of the Type B streptogramin quinupristin. Quinupristin undergoes a ring opening elimination reaction, resulting in an inactive derivative

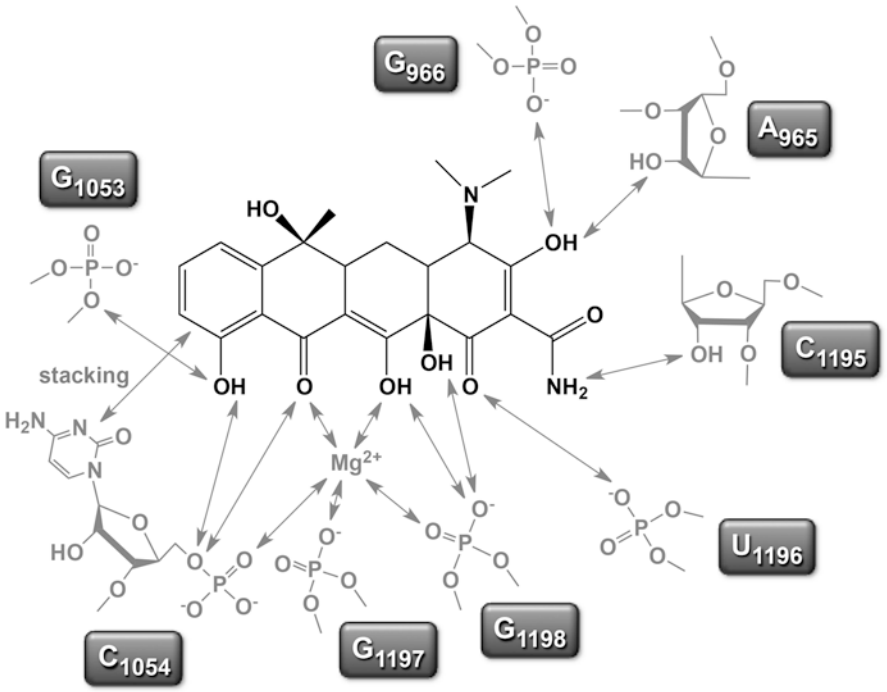


Fig. 8.11 Interactions of tetracycline with the bacterial 16S rRNA. Interactions are designated by grey arrows and key ribosomal RNA residues are indicated in grey. Adapted from [46]

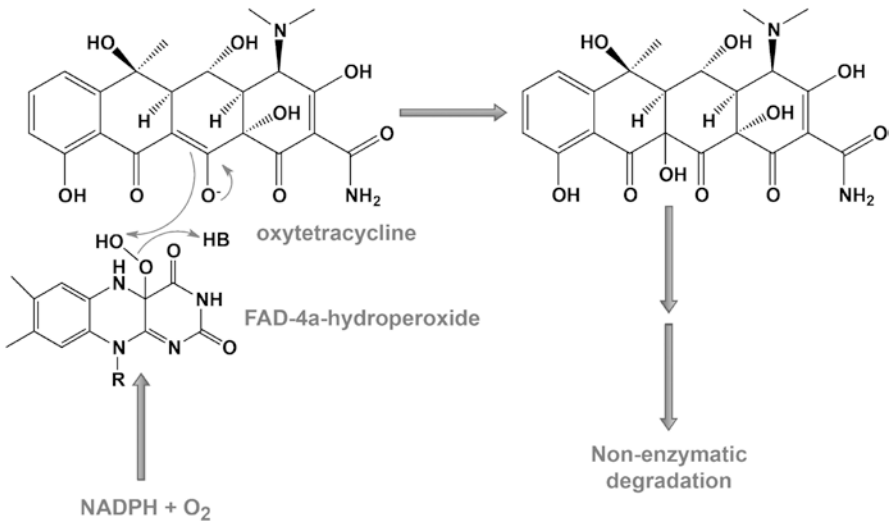


Fig. 8.12 TetX-mediated inactivation of tetracycline. TetX catalyzes the hydroxylation of the antibiotic, which interferes with the metal-binding site required for activity. Adapted from [39, 40]

3.1 Aminoglycosides

The aminoglycoside class of antibiotics is a diverse group of hydrophilic aminocyclitols modified by amino and neutral sugars that consist of both natural products and their semi-synthetic derivatives. Polycationic aminoglycoside antibiotics, as previously mentioned, act by interacting with the 16S rRNA region of the bacterial ribosome's A-site, impairing its decoding mechanism and consequently resulting in a misreading of the mRNA [42–45]. X-ray crystallographic studies of aminoglycoside antibiotics and the small ribosomal subunit or fragments of the 16S rRNA reveal that interactions between aminoglycosides and the ribosome span the entire length of the antibiotic [46–50]. The primary mode of interaction is through predicted hydrogen bonding and ionic contacts between the antibiotic amino and hydroxyl groups and the 16S rRNA (Fig. 8.13).

The most prevalent mode of clinically relevant aminoglycoside resistance is via enzymatic modification [51]. Three classes of enzymes, whose reactions differ with respect to the functional group transferred and the acceptor site, are responsible for aminoglycoside modification. Aminoglycoside acetyltransferases (AACs) modify amino groups, aminoglycoside phosphotransferases (APHs) target hydroxyl groups,

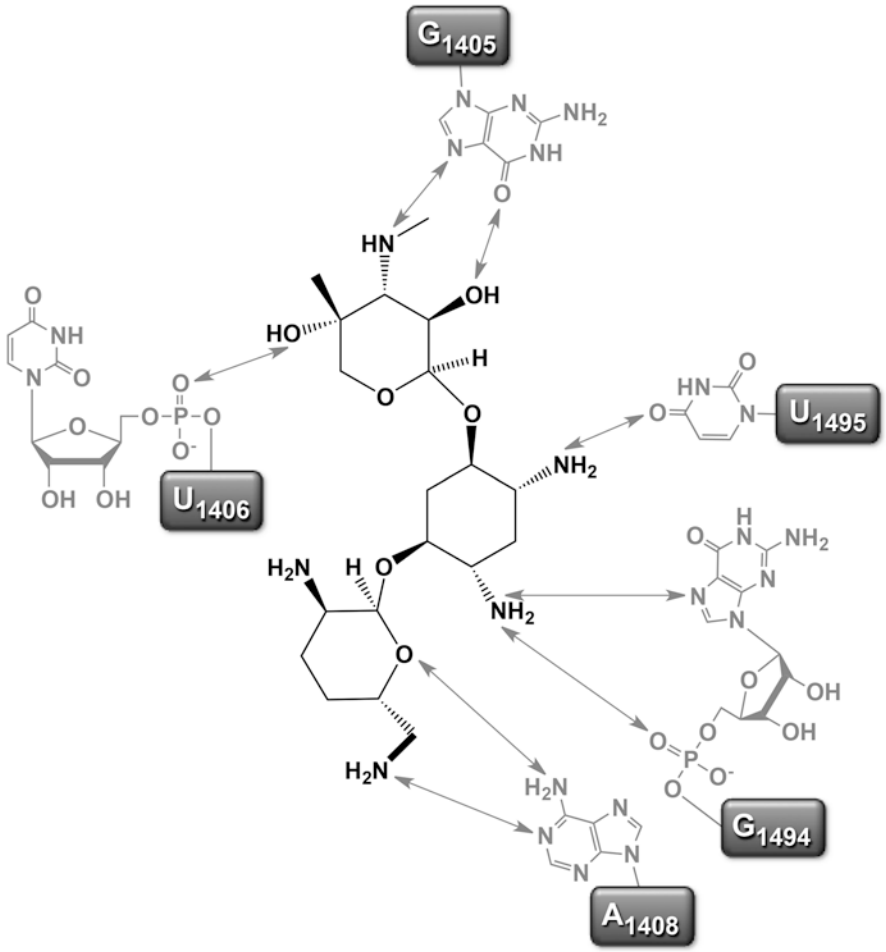


Fig. 8.13 Interactions of the aminoglycoside gentamicin C1a with the bacterial 16S rRNA. Key 16S rRNA residues are shown in grey and the interactions are designated as arrows. Adapted from [104]

and aminoglycoside nucleotidyltransferases (ANTs) modify hydroxyl groups (Fig. 8.14). There are numerous examples of each group in both Gram-positive as well as Gram-negative bacteria, and the genes encoding aminoglycoside modifying enzymes are commonly located on mobile genetic elements such as plasmids or transposons, although some have been identified within chromosomal DNA [52–54]. The action of all three classes of modifying enzyme changes the electronic properties of the antibiotic, in addition to its size and structure. These alterations result in steric and electronic clashes between the modified antibiotic and the 16S rRNA, impairing efficient binding and resulting in resistance.

3.1.1 Aminoglycoside Acetyltransferases (AAC Family)

Aminoglycoside acetyltransferases (AACs) utilize intracellular acetyl-CoA as a co-substrate, catalyzing the formation of a biologically stable amide with the aminoglycoside (Fig. 8.14B). Although AACs primarily modify amino groups (*N*-acetylation), *O*-acetylation has been documented with the acetyltransferase domain of the bifunctional enzyme AAC(6′)-APH(2″) [55] and the mycobacterial enzyme AAC(2′)-Ic [56].

AACs are members of the GCN5 superfamily of proteins [57, 58]. Although all enzymes of this class do not exhibit significant primary sequence homology or conserved catalytic residues, analysis of available X-ray crystal structures of several enzymes (AAC(6′Ii), AAC(3)-Ia, AAC(2′)-Ic, AAC(6′)-Ib, AAC(6′)-Ib-cr and AAC(6′)-Iy) indicates that the aminoglycoside-binding pocket commonly contains a highly negatively charged surface to accommodate the polycationic antibiotic [58–63].

AACs are further categorized into one of four classes based on the site of acetylation along the aminoglycoside structure: AAC(1), AAC(2′), AAC(3), and AAC(6′). By convention, the position along the amino sugar/aminocyclitol targeted is indicated in brackets, and the amino sugar/aminocyclitol modified is designated in brackets after the position of attack. For example, in Fig. 8.14, AAC(3) indicates acetylation of the 3-position of the central aminocyclitol moiety, the term (2′) suggests modification of the 2-position of the 4-substituent diaminohexose, and (2″) indicates modification of the 2-position of the 6-substituent aminohexose.

Modification of aminoglycosides by AACs results in neutralization of the positive charge on the target amino group,

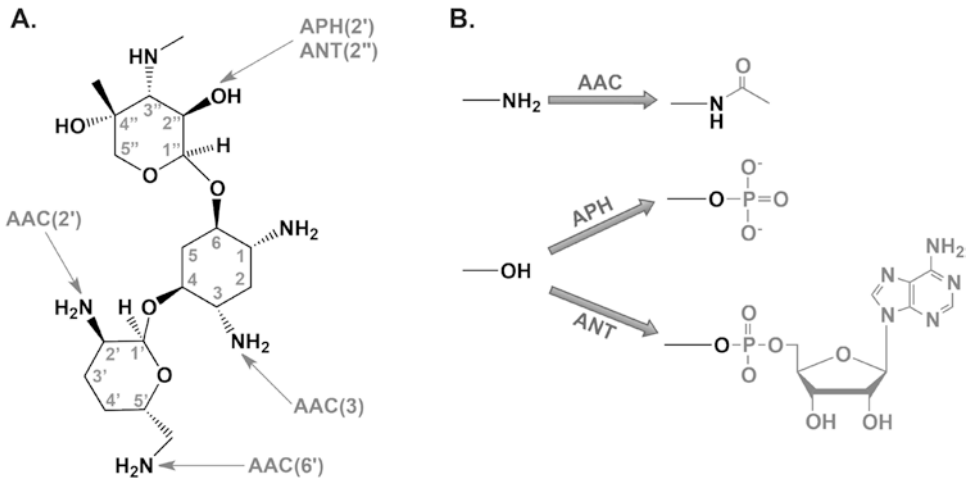


Fig. 8.14 Inactivation of the aminoglycoside gentamicin C1a by aminoglycoside modifying enzymes. Aminoglycosides can be modified by the addition of acetyl groups, phosphate groups, or AMP moieties. These enzymatic reactions are catalyzed by aminoglycoside acetyltransferases (AACs), phosphotransferases (APHs), and nucleotidyltransferases (ANTs), respectively. (**a**) Sites of aminoglycoside inactivation. Groups targeted are labeled by the corresponding resistance enzymes. (**b**) The products of aminoglycoside acetyltransferases, phosphotransferases, and nucleotidyltransferases

eliminating key ionic interactions and sterically blocking interaction with the 16S rRNA.

3.1.2 Aminoglycoside Phosphotransferases (APH Family)

Aminoglycoside phosphotransferases catalyze the phosphorylation of specific aminoglycoside hydroxyl residues (Fig. 8.14), using intracellular ATP as a phosphate donor. Classification of phosphotransferases is based on the site of action, analogous to the system described above for acetyltransferases. The APH enzymes are subdivided into seven classes, based on their site of action on the aminoglycoside: APH(2″), APH(3′), APH(3″), APH(4), APH(6), APH(7″), and APH(9). There exists very little primary sequence homology among the subclasses of APHs; however common signature sequences and residues essential for catalysis are evident [64].

The largest subclass of APHs modifies the 3′-hydroxyl of the aminoglycoside and is consequently called APH(3′) [64]. Crystal structure analysis of the enzyme APH(3′)-IIIa bound to ADP has established a remarkable similarity to known protein kinases, despite the low primary sequence similarity [65]. This may be evidence that APH(3′) and protein kinases evolved from a common ancestor, which is supported by their common sensitivity to inhibitors [66, 67].

Modification of aminoglycosides by APH-catalyzed phosphorylation results in changes in overall charge and size of the antibiotic. This results in electronic and steric clashes with the 16S rRNA and a 10^3-fold impairment of binding to the target 16S rRNA [68].

3.1.3 Aminoglycoside Nucleotidyltransferases (ANT Family)

Aminoglycoside nucleotidyltransferases utilize the co-substrate ATP to transfer an AMP moiety to selected aminoglycoside hydroxyl groups. Subdivided into five classes (ANT(2″), ANT(3″), ANT(4′), ANT(6), and ANT(9)), these inactivating enzymes has been identified in some Gram-positive bacterial isolates, as well as a broad range of Gram-negatives [53].

ANTs display very little primary sequence homology; however they exhibit a common core signature region [64]. The enzyme ANT(4′)-Ia has been crystallized and its atomic structure determined alone and in complex with the substrates kanamycin and a non-hydrolyzable ATP analogue [69, 70]. Although the primary sequence homology is only 10 %, the putative active site was determined to be structurally equivalent to that of rat DNA-polymerase β, one of the smallest and simplest of the polymerases [71], and catalyzes a similar chemical reaction.

Paralleling the strategies of the other classes of aminoglycoside modifying enzymes, the action of ANTs causes a change in antibiotic structure that results in both a steric and electronic clash between the antibiotic and its target. This theme and molecular logic finds other examples in antibiotic resistance as outlined below.

3.2 Macrolides

Macrolide antibiotics are a large class of antibiotics that include both natural products and semisynthetic derivatives. Most macrolides are derived from bacterial fermentation products, particularly from species of the actinomycete genus *Streptomyces*. Erythromycin was the first member of this class to be identified (1952), a natural product of *Streptomyces erythraeus* (now known as *Saccharopolyspora erythraea*). The name macrolide is derived from the macrolactone ring that characterizes the class, which can consist of 14–16 members and is commonly attached to one or two sugar moieties.

Macrolides have found an important role in the treatment of clinical pathogens. Since their introduction in the 1950s, efforts to expand the spectrum of activity and deal with the inevitable resistance that followed have resulted in a number of different classes of derivatives. Azalides incorporate an endocyclic nitrogen into the macrolactone ring. Azithromycin, the first azalide approved for clinical use, exhibits increased potency against a number of Gram-negative organisms, as well as a longer apparent half-life. Ketolides, which have a keto group in place of the L-cladinose in the 3-position, exhibit increased activity against a number of macrolide-resistant strains.

Macrolides, as described previously, act by binding with the 23S rRNA of the bacterial 50S ribosomal subunit adjacent to the peptide exit tunnel, blocking polymerization at the peptidyltransferase center and inducing premature peptide dissociation [33, 72]. Interactions with the ribosomal RNA occur primarily through hydrogen bonding, as shown with erythromycin in Fig. 8.7. Much of the hydrogen bonding ability of macrolides can be attributed to their hydroxyl and amino groups, which interact with the nitrogenous bases or backbone phosphate groups of the rRNA. As shown, the hydroxyl residue of the desoamine sugar plays a key role in the interaction of the macrolide with its target rRNA.

The second mode of enzymatic macrolide inactivation occurs by modification of this essential desosamine sugar (Fig. 8.15). Modification of the 2′ hydroxyl residue can occur by either phosphorylation or glycosylation. This hydroxyl group, as mentioned, plays an important role in macrolide-target binding, serving as a multiple contact site of hydrogen bonding with the 23S rRNA (Fig. 8.7). Modification of the antibiotic at this site therefore results in loss of vital structural connections with the target, and also results in steric impairment of complex formation.

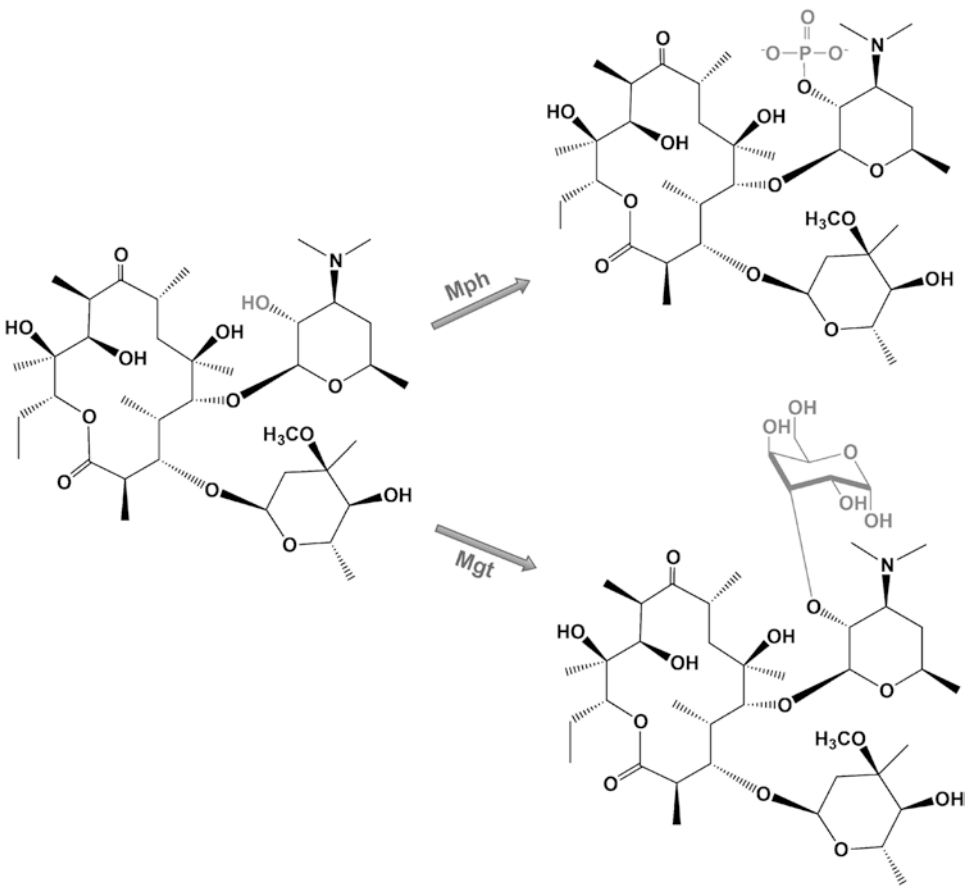


Fig. 8.15 Inactivation of the macrolide erythromycin by Mph and Mgt. Macrolides can be modified by the addition of phosphate and glucose moieties. The hydroxyl group targeted and the subsequent modifications are labeled in grey

3.2.1 Macrolide Kinases (Mph Family)

Clinical resistance to macrolides has been documented by means of phosphate transfer from GTP (and to a lesser extend ATP) by a family of macrolide-inactivating phosphotransferases, encoded by the *mph* genes (Fig. 8.15). These enzymes have been identified in both Gram-positive and Gram-negative pathogens [73–79].

Members of the Mph class of resistance enzymes appear to be extremely diverse with respect to the nucleotide sequences that encode the enzymes. The gene *mphA* exhibits a 66 % G+C content, uncharacteristically high for the organism it was originally identified in (*E. coli*, G+C content approximately 50 %) [80]. Conversely, the sequences of *mphB* and *mphC* display a G+C content of only 38 %. Another Mph subclass, encoded by *mphE*, is most closely related to MphA (approximately 38 % identical at the amino acid level) [76] and has been identified within bacterial genomes, as well as on multidrug-resistant plasmids [76–79]. The structure of these enzymes has yet to be elucidated; however they share canonical phosphate transfer residues with APHs and likely resemble these aminoglycoside resistance enzymes.

3.2.2 Macrolide Glycosyltransferases (Mgt Family)

Resistance to macrolides in antibiotic producing strains of bacteria as well as other soil-dwelling organisms is commonly accomplished by intracellular glycosylation of the antibiotic prior to export. This reaction is catalyzed by a class of enzymes called macrolide glycosyltransferases (Mgts) (Fig. 8.15). Members of this class include Mgt from the non-macrolide producing *Streptomyces lividans* [81, 82], as well as OleD [83] and GimA [84] from the macrolide producing *S. antibioticus* and *S. ambofaciens,* respectively.

Members of the Mgt family are extremely similar with respect to both DNA and primary amino acid sequences; however each enzyme appears to display a unique substrate specificity in vitro [85].

3.3 Rifamycins

The rifamycin family of antibiotics includes semisynthetic derivatives of a natural product synthesized by the actinomycete *Amycolatopsis mediterranei*. Rifampicin, first introduced in 1968 and the most widely used member of the group, has become an integral component of the multiantibiotic gold standard treatment for *Mycobacterium tuberculosis* infections.

Rifamycins target the bacterial β-subunit of RNA polymerase. The crystal structure of rifampicin bound to the RNA polymerase of *Thermus aquaticus* has been determined

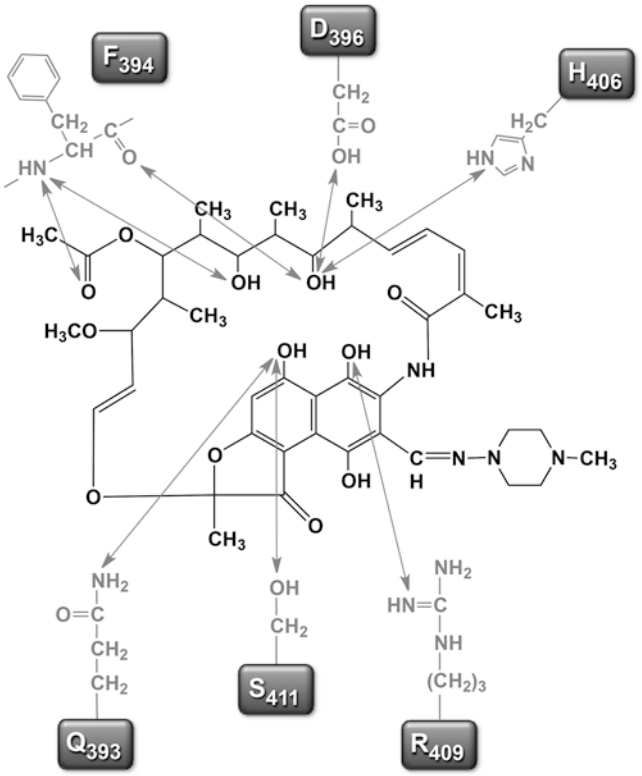


Fig. 8.16 Interactions of the rifampicin with the bacterial β-subunit of RNA polymerase. Key amino acid residues are shown in grey and the interactions are designated as arrows. Adapted from [86]

to 3.3 Å [86]. Twelve amino acid residues were shown to associate closely with rifampicin, six of which participate in hydrogen bonding, as shown in Fig. 8.16. The majority of these interactions occur at four crucial hydroxyl residues on the rifampicin molecule including a key interaction between the hydroxyl group at position 23 and the amide of Phe394.

Resistance to rifampicin commonly occurs through amino acid mutations in the RNA polymerase β-subunit. However, inactivating enzymes have also evolved to modify the antibiotic (Fig. 8.17). Group transfer can result in ADP-ribosylation, phosphate addition, and glycosylation of the rifampicin's 23-hydroxyl. By addition of a bulky functional group, rifampicin's tight binding to its target is impaired.

3.3.1 ADP-Ribosyltransferases (ARR Family)

Although both eukaryotic and prokaryotic proteins are commonly modified by means of ADP-ribosyl transfer, this mechanism of antibiotic resistance has so far only been documented for the rifamycin class. Resistance to rifampicin by means of ADP-ribosylation has been identified in numerous non-tuberculosis *Mycobacterium* strains, such as *M. smegmatis*. This modification is due to a unique ADP-ribosyltransferase known as ARR [87]. Another inactivating ribosyltransferase (ARR-2) with 55 % identity to ARR has been identified in a

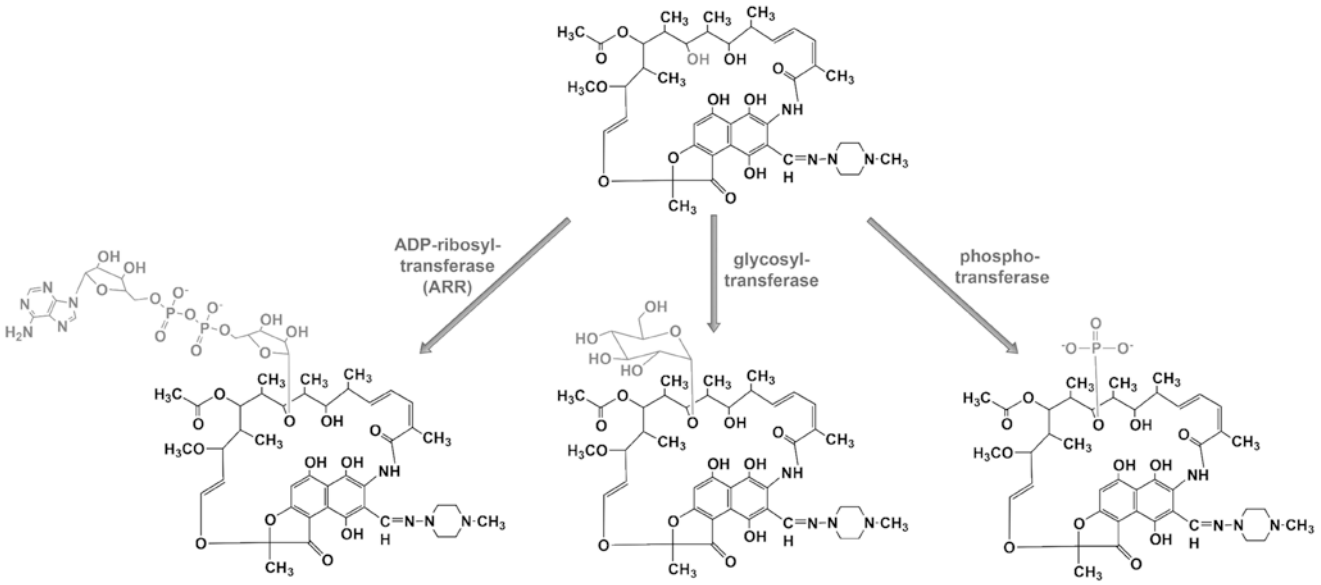


Fig. 8.17 Inactivation of rifampicin. Rifampicin can be enzymatically modified by the addition of ADP-ribose, glucose, and phosphate moieties. The hydroxyl group targeted and the subsequent modifications are labeled in grey

multidrug-resistant integron in a Gram-negative *Acinetobacter* strain [88], and orthologs have since been detected on mobile genetic elements in several other Gram-negative pathogens including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Escherichia coli* [89–91].

The ARR resistance proteins are characteristically small in size (approximately 200 amino acids), and although they do not display sequence similarity to protein ADP-ribosyltransferases, X-ray crystallographic data indicates significant structural homology between the two classes [92]. ARR resistance enzymes target the hydroxyl residue at position 23 of rifampicin (Fig. 8.17), and utilize nicotinamide adenine dinucleotide (NAD^+) as a donor for the ADP-ribosyl moiety. It has also been shown that this ADP-ribosylated antibiotic can undergo subsequent decomposition to release the ADP moiety [87, 93, 94].

3.3.2 Rifampicin Kinases

Inactivation of rifampicin by phosphorylation (Fig. 8.17) has been documented by species of *Nocardia* [95, 96], *Rhodococcus* [97], as well as *Bacillus* [98]. The kinases responsible for this inactivation have yet to be identified or studied. Phosphorylation of rifampicin's hydroxyl at position 23 logically impedes interaction with the RNA polymerase target, although little has been done to elucidate the details of this mechanism. The rifamycin phosphotransferase is unique among antibiotic kinases. Instead of sharing structure and mechanism with the protein kinase family of enzymes, its mechanism and protein organization resembles that of small molecule phosphotransferases such as PEP synthase [99].

3.3.3 Rifampicin Glycosyltransferases

Glycosylation of the 23-position of rifampicin has also been reported in *Nocardia* and *Streptomyces* species [95, 100, 101] (Fig. 8.17). Glycosylation at this position prevents hydrogen bonding with the 23-hydroxyl, hindering effective target binding to RNA polymerase β. The genes encoding the enzymes have recently been elucidated, and bear strong resemblance to the glycosyltransferases encoded within the gene clusters responsible for glycopeptide antibiotic biosynthesis [101].

4 Summary and Conclusions

Bacteria use enzymes to strategically incapacitate and neutralize antibiotics. Tactically this includes deployment of mechanisms that either destroy the essential chemical "warhead" or "active site" of the antibiotic (e.g., cleavage of the β-lactam ring by β-lactamases), or mechanisms that modify key structural elements that are essential for binding of the antibiotic to target (e.g., phosphorylation of aminoglycosides). The molecular logic of these approaches is revealed with knowledge of the interaction of the active antibiotic with its cellular target. Study of enzymatic resistance therefore

cannot only inform on molecular aspects of antibiotic–target interactions, but can serve to guide target identification where this is not yet known.

Another spin-off of the study of these mechanisms is the opportunity to develop strategies to overcome the resistance activity. For example, the observation that aminoglycosides were inactivated by phosphorylation of the hydroxyl group at position 3′ of the 6′-aminohexose ring guided the development of antibiotics such as tobramycin, which lack this hydroxyl and which were consequently resistant to this mechanism. A second approach is to develop inhibitors of resistance enzymes. This strategy has been very successful in the β-lactam arena where combinations of an antibiotic and a resistance enzyme inhibitor, such as amoxicillin and clavulanic acid, respectively (Augmentin), have emerged as billion dollar drugs.

Finally, antibiotic modifying enzymes also have the opportunity to be exploited as novel reagents in antibiotic semisynthesis as protecting agents. In some cases, antibiotic modifying proteins are employed by antibiotic producing bacteria as a means of self-protection. For example, during streptomycin biosynthesis in *Streptomyces griseus*, the enzyme StrA modifies mature antibiotic to the inactive 6-phosphoderivative. Export of this "pro-drug" is followed by unmasking of the cytotoxic agent by an extracellular phosphatase [102]. These enzymes could serve as reagents to chemically protect and deprotect sensitive structural elements in the synthesis of libraries of semisynthetic antibiotics.

Enzymatic resistance therefore provides both challenges and opportunities in new drug development. Through a combination of rigorous biochemical analysis and parallel efforts in the determination of enzyme structure and target identification, new approaches that circumvent these selective and potent agents can be developed to extend antibiotic lifetime and efficacy.

References

1. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. Nature. 1940;146:837.
2. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Hye Park C, Bush K, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12:83–8.
3. Walsh CT. Antibiotics action, origins resistance. Washington, DC: ASM Press; 2003.
4. Fisher JF, Meroueh SO, Mobashery S. Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. Chem Rev. 2005;105:395–424.
5. Tipper DJ, Strominger JL. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci U S A. 1965;54:1133–41.
6. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 1995;39:1211–33.
7. Livermore DM. beta-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev. 1995;8:557–84.
8. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother. 2009;53:5046–54.
9. Chen Y, Zhou Z, Jiang Y, Yu Y. Emergence of NDM-1-producing Acinetobacter baumannii in China. J Antimicrob Chemother. 2011;66:1255–9.
10. Jovcic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, Topisirovic L, Kojic M. Emergence of NDM-1 metallo-beta-lactamase in Pseudomonas aeruginosa clinical isolates from Serbia. Antimicrob Agents Chemother. 2011;55:3929–31.
11. Darley E, Weeks J, Jones L, Daniels V, Wootton M, MacGowan A, Walsh T. NDM-1 polymicrobial infections including Vibrio cholerae. Lancet. 2012;380:1358.
12. Decousser JW, Jansen C, Nordmann P, Emirian A, Bonnin RA, Anais L, Merle JC, Poirel L. Outbreak of NDM-1-producing Acinetobacter baumannii in France, January to May 2013. Euro Surveill. 2013;18(31):20574.
13. Janvier F, Jeannot K, Tesse S, Robert-Nicoud M, Delacour H, Rapp C, Merens A. Molecular characterization of blaNDM-1 in a sequence type 235 Pseudomonas aeruginosa isolate from France. Antimicrob Agents Chemother. 2013;57:3408–11.
14. Fillgrove KL, Pakhomova S, Newcomer ME, Armstrong RN. Mechanistic diversity of fosfomycin resistance in pathogenic microorganisms. J Am Chem Soc. 2003;125:15730–1.
15. Bernat BA, Laughlin LT, Armstrong RN. Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. Biochemistry. 1997;36:3050–5.
16. Cao M, Bernat BA, Wang Z, Armstrong RN, Helmann JD. FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in *Bacillus subtilis*. J Bacteriol. 2001;183:2380–3.
17. Teran FJ, Suarez JE, Hardisson C, Mendoza MC. Molecular epidemiology of plasmid mediated resistance to fosfomycin among bacteria isolated from different environments. FEMS Microbiol Lett. 1988;55:213.
18. Etienne J, Gerbaud G, Courvalin P, Fleurette J. Plasmid-mediated resistance to fosfomycin in Staphylococcus epidermidis. FEMS Microbiol Lett. 1989;52:133–7.
19. Rife CL, Pharris RE, Newcomer ME, Armstrong RN. Crystal structure of a genomically encoded fosfomycin resistance protein (FosA) at 1.19 A resolution by MAD phasing off the L-III edge of Tl(+). J Am Chem Soc. 2002;124:11001–3.
20. Katz L, McDaniel R. Novel macrolides through genetic engineering. Med Res Rev. 1999;19:543–58.
21. Schlunzen F, Zarivach R, Harms J, Bashan A, Tocilj A, Albrecht R, Yonath A, Franceschi F. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. Nature. 2001;413:814–21.
22. Morar M, Pengelly K, Koteva K, Wright GD. Mechanism and diversity of the erythromycin esterase family of enzymes. Biochemistry. 2012;51:1740–51.
23. Arthur M, Autissier D, Courvalin P. Analysis of the nucleotide sequence of the *ereB* gene encoding the erythromycin esterase type II. Nucleic Acids Res. 1986;14:4987–99.
24. Biskri L, Mazel D. Erythromycin esterase gene *ere(A)* is located in a functional gene cassette in an unusual class 2 integron. Antimicrob Agents Chemother. 2003;47:3326–31.
25. Ounissi H, Courvalin P. Nucleotide sequence of the gene ereA encoding the erythromycin esterase in *Escherichia coli*. Gene. 1985;35:271–8.

26. Plante I, Centron D, Roy PH. An integron cassette encoding erythromycin esterase, *ere(A)*, from *Providencia stuartii*. J Antimicrob Chemother. 2003;51:787–90.
27. Arthur M, Andremont A, Courvalin P. Distribution of erythromycin esterase and rRNA methylase genes in members of the family Enterobacteriaceae highly resistant to erythromycin. Antimicrob Agents Chemother. 1987;31:404–9.
28. Nakamura A, Nakazawa K, Miyakozawa I, Mizukoshi S, Tsurubuchi K, Nakagawa M, O'Hara K, Sawai T. Macrolide esterase-producing *Escherichia coli* clinically isolated in Japan. J Antibiot (Tokyo). 2000;53:516–24.
29. Murphy BP, O'Mahony R, Buckley JF, Shine P, Boyd EF, Gilroy D, Fanning S. Investigation of a global collection of nontyphoidal *Salmonella* of various serotypes cultured between 1953 and 2004 for the presence of class 1 integrons. FEMS Microbiol Lett. 2007;266:170–6.
30. Thungapathra M, Sinha KK, Chaudhuri SR, Garg P, Ramamurthy T, Nair GB, Ghosh A. Occurrence of antibiotic resistance gene cassettes *aac(6′)-Ib, dfrA5, dfrA12*, and *ereA2* in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. Antimicrob Agents Chemother. 2002;46:2948–55.
31. Mukhtar TA, Wright GD. Streptogramins, oxazolidinones, and other inhibitors of bacterial protein synthesis. Chem Rev. 2005; 105:529–42.
32. Harms JM, Schlunzen F, Fucini P, Bartels H, Yonath A. Alterations at the peptidyl transferase centre of the ribosome induced by the synergistic action of the streptogramins dalfopristin and quinupristin. BMC Biol. 2004;2:4.
33. Tu D, Blaha G, Moore PB, Steitz TA. Structures of MLSBK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell. 2005;121:257–70.
34. Allignet J, Loncle V, Mazodier P, el Solh N. Nucleotide sequence of a staphylococcal plasmid gene, *vgb*, encoding a hydrolase inactivating the B components of virginiamycin-like antibiotics. Plasmid. 1988;20:271–5.
35. Mukhtar TA, Koteva KP, Hughes DW, Wright GD. Vgb from *Staphylococcus aureus* inactivates streptogramin B antibiotics by an elimination mechanism not hydrolysis. Biochemistry. 2001; 40:8877–86.
36. Chopra I, Hawkey PM, Hinton M. Tetracyclines, molecular and clinical aspects. J Antimicrob Chemother. 1992;29:245–77.
37. Poole K. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother. 2005;56:20–51.
38. Speer BS, Salyers AA. Characterization of a novel tetracyline resistance that functions only in aerobically grown *Escherichia coli*. J Bacteriol. 1988;170:1423–9.
39. Yang W, Moore IF, Koteva KP, Bareich DC, Hughes DW, Wright GD. TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. J Biol Chem. 2004;279: 52346–52.
40. Volkers G, Palm GJ, Weiss MS, Wright GD, Hinrichs W. Structural basis for a new tetracycline resistance mechanism relying on the TetX monooxygenase. FEBS Lett. 2011;585:1061–6.
41. Moore IF, Hughes DW, Wright GD. Tigecycline is modified by the flavin-dependent monooxygenase TetX. Biochemistry. 2005;44: 11829–35.
42. Davies J, Davis BD. Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. The effect of drug concentration. J Biol Chem. 1968;243:3312–6.
43. Edelmann P, Gallant J. Mistranslation in E coli. Cell. 1977;10: 131–7.
44. Moazed D, Noller HF. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature. 1987;327:389–94.
45. Woodcock J, Moazed D, Cannon M, Davies J, Noller HF. Interaction of antibiotics with A- and P-site-specific bases in 16S ribosomal RNA. Embo J. 1991;10:3099–103.
46. Brodersen DE, Clemons Jr WM, Carter AP, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. Cell. 2000;103:1143–54.
47. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature. 2000;407:340–8.
48. Ogle JM, Brodersen DE, Clemons Jr WM, Tarry MJ, Carter AP, Ramakrishnan V. Recognition of cognate transfer RNA by the 30S ribosomal subunit. Science. 2001;292:897–902.
49. Vicens Q, Westhof E. Crystal structure of a complex between the aminoglycoside tobramycin and an oligonucleotide containing the ribosomal decoding a site. Chem Biol. 2002;9:747–55.
50. Vicens Q, Westhof E. Crystal structure of geneticin bound to a bacterial 16S ribosomal RNA A site oligonucleotide. J Mol Biol. 2003;326:1175–88.
51. Wright GD. Aminoglycoside-modifying enzymes. Curr Opin Microbiol. 1999;2:499–503.
52. Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. Chem Rev. 2005;105:477–98.
53. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev. 1993;57: 138–63.
54. Wright GD, Berghuis AM, Mobashery S. Aminoglycoside antibiotics. Structures, functions, and resistance. Adv Exp Med Biol. 1998;456:27–69.
55. Daigle DM, Hughes DW, Wright GD. Prodigious substrate specificity of AAC(6′)-APH(2″), an aminoglycoside antibiotic resistance determinant in enterococci and staphylococci. Chem Biol. 1999;6:99–110.
56. Hegde SS, Javid-Majd F, Blanchard JS. Overexpression and mechanistic analysis of chromosomally encoded aminoglycoside 2'-N-acetyltransferase (AAC(2')-Ic) from *Mycobacterium tuberculosis*. J Biol Chem. 2001;276:45876–81.
57. Vetting MW, de Carvalho LPS, Yu M, Hegde SS, Magnet S, Roderick SL, Blanchard JS. Structure and functions of the GNAT superfamily of acetyltransferases. Arch Biochem Biophys. 2005;433:212–26.
58. Wybenga-Groot LE, Draker K, Wright GD, Berghuis AM. Crystal structure of an aminoglycoside 6'-N-acetyltransferase: defining the GCN5-related N-acetyltransferase superfamily fold. Struct Fold Des. 1999;7:497–507.
59. Vetting MW, Hegde SS, Javid-Majd F, Blanchard JS, Roderick SL. Aminoglycoside 2′-N-acetyltransferase from *Mycobacterium tuberculosis* in complex with coenzyme A and aminoglycoside substrates. Nat Struct Biol. 2002;9:653–8.
60. Vetting MW, Magnet S, Nieves E, Roderick SL, Blanchard JS. A bacterial acetyltransferase capable of regioselective N-acetylation of antibiotics and histones. Chem Biol. 2004;11:565–73.
61. Wolf E, Vassilev A, Makino Y, Sali A, Nakatani Y, Burley SK. Crystal structure of a GCN5-related N-acetyltransferase: *Serratia marcescens* aminoglycoside 3-N-acetyltransferase. Cell. 1998;94:439–49.
62. Maurice F, Broutin I, Podglajen I, Benas P, Collatz E, Dardel F. Enzyme structural plasticity and the emergence of broad-spectrum antibiotic resistance. EMBO Rep. 2008;9:344–9.
63. Vetting MW, Park CH, Hegde SS, Jacoby GA, Hooper DC, Blanchard JS. Mechanistic and structural analysis of aminoglycoside N-acetyltransferase AAC(6')-Ib and its bifunctional, fluoroquinolone-active AAC(6')-Ib-cr variant. Biochemistry. 2008;47:9825–35.
64. Wright GD, Thompson PR. Aminoglycoside phosphotransferases: proteins, structure, and mechanism. Front Biosci. 1999; 4:D9–21.

65. Hon WC, McKay GA, Thompson PR, Sweet RM, Yang DS, Wright GD, Berghuis AM. Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell. 1997;89:887–95.
66. Shakya T, Stogios PJ, Waglechner N, Evdokimova E, Ejim L, Blanchard JE, McArthur AG, Savchenko A, Wright GD. A small molecule discrimination map of the antibiotic resistance kinome. Chem Biol. 2011;18:1591–601.
67. Stogios PJ, Spanogiannopoulos P, Evdokimova E, Egorova O, Shakya T, Todorovic N, Capretta A, Wright GD, Savchenko A. Structure-guided optimization of protein kinase inhibitors reverses aminoglycoside antibiotic resistance. Biochem J. 2013;454: 191–200.
68. Llano-Sotelo B, Azucena Jr EF, Kotra LP, Mobashery S, Chow CS. Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. Chem Biol. 2002;9:455–63.
69. Pedersen LC, Benning MM, Holden HM. Structural investigation of the antibiotic and ATP-binding sites in kanamycin nucleotidyltransferase. Biochemistry. 1995;34:13305–11.
70. Sakon J, Liao HH, Kanikula AM, Benning MM, Rayment I, Holden HM. Molecular structure of kanamycin nucleotidyltransferase determined to 3.0-A resolution. Biochemistry. 1993;32: 11977–84.
71. Holm L, Sander C. DNA polymerase beta belongs to an ancient nucleotidyltransferase superfamily. Trends Biochem Sci. 1995; 20:345–7.
72. Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, Steitz TA. The structures of four macrolide antibiotics bound to the large ribosomal subunit. Mol Cell. 2002;10:117–28.
73. Matsuoka M, Endou K, Kobayashi H, Inoue M, Nakajima Y. A plasmid that encodes three genes for resistance to macrolide antibiotics in *Staphylococcus aureus*. FEMS Microbiol Lett. 1998;167:221–7.
74. Noguchi N, Katayama J, O'Hara K. Cloning and nucleotide sequence of the mphB gene for macrolide 2'-phosphotransferase II in *Escherichia coli*. FEMS Microbiol Lett. 1996;144:197–202.
75. O'Hara K, Kanda T, Ohmiya K, Ebisu T, Kono M. Purification and characterization of macrolide 2'-phosphotransferase from a strain of *Escherichia coli* that is highly resistant to erythromycin. Antimicrob Agents Chemother. 1989;33:1354–7.
76. Kadlec K, Brenner Michael G, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, Watts JL, Schwarz S. Molecular basis of macrolide, triamilide, and lincosamide resistance in *Pasteurella multocida* from bovine respiratory disease. Antimicrob Agents Chemother. 2011;55:2475–7.
77. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE. 2012;7, e34953.
78. Shen P, Wei Z, Jiang Y, Du X, Ji S, Yu Y, Li L. Novel genetic environment of the carbapenem-hydrolyzing beta-lactamase KPC-2 among Enterobacteriaceae in China. Antimicrob Agents Chemother. 2009;53:4333–8.
79. Poirel L, Mansour W, Bouallegue O, Nordmann P. Carbapenem-resistant *Acinetobacter baumannii* isolates from Tunisia producing the OXA-58-like carbapenem-hydrolyzing oxacillinase OXA-97. Antimicrob Agents Chemother. 2008;52:1613–7.
80. Noguchi N, Emura A, Matsuyama H, O'Hara K, Sasatsu M, Kono M. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2'-phosphotransferase I in *Escherichia coli*. Antimicrob Agents Chemother. 1995;39:2359–63.
81. Jenkins G, Cundliffe E. Cloning and characterization of two genes from *Streptomyces lividans* that confer inducible resistance to lincomycin and macrolide antibiotics. Gene. 1991;108:55–62.
82. Cundliffe E. Glycosylation of macrolide antibiotics in extracts of *Streptomyces lividans*. Antimicrob Agents Chemother. 1992;36: 348–52.
83. Quiros LM, Aguirrezabalaga I, Olano C, Mendez C, Salas JA. Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. Mol Microbiol. 1998;28:1177–85.
84. Gourmelen A, Blondelet-Rouault MH, Pernodet JL. Characterization of a glycosyl transferase inactivating macrolides, encoded by *gimA* from *Streptomyces ambofaciens*. Antimicrob Agents Chemother. 1998;42:2612–9.
85. Yang M, Proctor MR, Bolam DN, Errey JC, Field RA, Gilbert HJ, Davis BG. Probing the breadth of macrolide glycosyltransferases: in vitro remodeling of a polyketide antibiotic creates active bacterial uptake and enhances potency. J Am Chem Soc. 2005;127: 9336–7.
86. Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. Cell. 2001;104:901–12.
87. Quan S, Venter H, Dabbs ER. Ribosylative inactivation of rifampin by *Mycobacterium smegmatis* is a principal contributor to its low susceptibility to this antibiotic. Antimicrob Agents Chemother. 1997;41:2456–60.
88. Houang ET, Chu YW, Lo WS, Chu KY, Cheng AF. Epidemiology of rifampin ADP-ribosyltransferase (*arr-2*) and metallo-beta-lactamase (*blaIMP-4*) gene cassettes in class 1 integrons in Acinetobacter strains isolated from blood cultures in 1997 to 2000. Antimicrob Agents Chemother. 2003;47:1382–90.
89. Tribuddharat C, Fennewald M. Integron-mediated rifampin resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1999;43:960–2.
90. Naas T, Mikami Y, Imai T, Poirel L, Nordmann P. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. J Bacteriol. 2001;183:235–49.
91. Arlet G, Nadjar D, Herrmann JL, Donay JL, Lagrange PH, Philippon A. Plasmid-mediated rifampin resistance encoded by an *arr-2*-like gene cassette in K*lebsiella pneumoniae* producing an ACC-1 class C beta-lactamase. Antimicrob Agents Chemother. 2001;45:2971–2.
92. Baysarowich J, Koteva K, Hughes DW, Ejim L, Griffiths E, Zhang K, Junop M, Wright GD. Rifamycin antibiotic resistance by ADP-ribosylation: Structure and diversity of Arr. Proc Natl Acad Sci U S A. 2008;105:4886–91.
93. Morisaki N, Hashimoto Y, Furihata K, Imai T, Watanabe K, Mikami Y, Yazawa K, Ando A, Nagata Y, Dabbs ER. Structures of ADP-ribosylated rifampicin and its metabolite: intermediates of rifampicin-ribosylation by *Mycobacterium smegmatis* DSM43756. J Antibiot (Tokyo). 2000;53:269–75.
94. Morisaki N, Kobayashi H, Iwasaki S, Furihata K, Dabbs ER, Yazawa K, Mikami Y. Structure determination of ribosylated rifampicin and its derivative: new inactivated metabolites of rifampicin by mycobacterial strains. J Antibiot (Tokyo). 1995;48: 1299–303.
95. Morisaki N, Iwasaki S, Yazawa K, Mikami Y, Maeda A. Inactivated products of rifampicin by pathogenic *Nocardia spp*. structures of glycosylated and phosphorylated metabolites of rifampicin and 3-formylrifamycin SV. J Antibiot (Tokyo). 1993;46:1605–10.

96. Yazawa K, Mikami Y, Maeda A, Morisaki N, Iwasaki S. Phosphorylative inactivation of rifampicin by *Nocardia otitidis-caviarum*. J Antimicrob Chemother. 1994;33:1127–35.
97. Tanaka Y, Yazawa K, Dabbs ER, Nishikawa K, Komaki H, Mikami Y, Miyaji M, Morisaki N, Iwasaki S. Different rifampicin inactivation mechanisms in *Nocardia* and related taxa. Microbiol Immunol. 1996;40:1–4.
98. Dabbs ER, Yazawa K, Mikami Y, Miyaji M, Morisaki N, Iwasaki S, Furihata K. Ribosylation by mycobacterial strains as a new mechanism of rifampin inactivation. Antimicrob Agents Chemother. 1995;39:1007–9.
99. Spanogiannopoulos P, Waglechner N, Koteva K, Wright GD. A rifamycin inactivating phosphotransferase family shared by environmental and pathogenic bacteria. Proc Natl Acad Sci U S A. 2014;111:7102–7.
100. Yazawa K, Mikami Y, Maeda A, Akao M, Morisaki N, Iwasaki S. Inactivation of rifampin by *Nocardia brasiliensis*. Antimicrob Agents Chemother. 1993;37:1313–7.
101. Spanogiannopoulos P, Thaker M, Koteva K, Waglechner N, Wright GD. Characterization of a rifampin-inactivating glycosyltransferase from a screen of environmental actinomycetes. Antimicrob Agents Chemother. 2012;56:5061–9.
102. Piepersberg W. Molecular biology. Biochemistry, and fermentation of aminoglycoside antibiotics. In: Strohl W, editor. Biotechnology of industrial antibiotics. 2nd ed. New York: Marcel Dekker; 1997. p. 81–163.
103. Skarzynski T, Mistry A, Wonacott A, Hutchinson SE, Kelly VA, Duncan K. Structure of UDP-N-acetylglucosamine enolpyruvyl transferase, an enzyme essential for the synthesis of bacterial peptidoglycan, complexed with substrate UDP-N-acetylglucosamine and the drug fosfomycin. Structure. 1996;4:1465–74.
104. Hobbie SN, Pfister P, Brull C, Westhof E, Bottger EC. Analysis of the contribution of individual substituents in 4,6-aminoglycoside-ribosome interaction. Antimicrob Agents Chemother. 2005;49:5112–8.

Antibiotic Resistance due to Reduced Uptake

9

Lucía Fernández, Joseph B. McPhee, Sandeep Tamber, Michelle D. Brazas, Shawn Lewenza, and Robert E.W. Hancock

1 Introduction

The introduction of antibiotic therapy for the treatment of bacterial infections has led to a much longer human life span compared to that in the pre-antibiotic era. However, a disturbing trend has also been noted in that, within a very short period of time following the introduction of a new antibiotic, resistance to that antibiotic begins to emerge– a factor that is becoming increasingly meaningful as the discovery of new antibiotics wanes [1–3]. There are a number of mechanisms by which a bacterium may become resistant to a particular antibiotic. Generally these include, but are not limited to, modification of the drug to render it inactive, modification of the drug target, such that it is incapable of interacting with the drug and decreased uptake of the antibiotic into the cell, due to reduced transport and/or increased efflux. Recent functional genomic studies have also implied that antibiotics may have more complex mechanisms of action than first thought and we are beginning to appreciate that in addition to the mutation of primary targets, subtle mutations in secondary targets are likely to be influential [4, 5]. Moreover, a growing body of evidence suggests that the temporary changes in susceptibility associated with the phenomenon of adaptive resistance may also be important for the global rise in bacterial resistance to antimicrobial compounds [6]. This chapter will focus on the contribution of a decreased antibiotic uptake to an increase in antibacterial resistance.

L. Fernández
Instituto de Productos Lacteos de Asturias (IPLA), Consejo Superior de Investigaciones Cientificas (CSIC), Villaviciosa, Asturias, Spain

J.B. McPhee, Ph.D.
Department of Chemistry and Biology, Ryerson University, 350 Victoria Street, Toronto, ON, Canada, M5B 2K3

S. Tamber, Ph.D.
Bureau of Microbial Hazards, Health Canada, 251 Sir Frederick Banting Driveway PL 2204E, Ottawa, ON, Canada, K1A 0K9

M.D. Brazas, Ph.D.
Ontario Institute for Cancer Research, MaRS Centre, 661 University Avenue, Suite 510, Toronto, ON, Canada, M5G 0A3

S. Lewenza, Ph.D.
Faculty of Science and Technology, Athabasca University, 1 University Drive, Athabasca, AB, Canada, T9S 3A3

R.E.W. Hancock, Ph.D. (✉)
Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada

Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, BC, Canada
e-mail: bob@hancocklab.com

2 Envelope Structure

2.1 Cytoplasmic Membrane

The cytoplasmic membrane is common to all bacterial species. For Gram-positive bacteria it is the primary barrier to antibiotic penetration, while an outer membrane further protects Gram-negative bacteria [7]. In both cases, the cytoplasmic membrane is the site of essential functions such as nutrient transport, energy generation, the enzymatic assembly of lipid-linked monomers of cell envelope macromolecules (e.g. the peptidoglycan or lipopolysaccharide), and protein secretion. The cytoplasmic membrane is a phospholipids bilayer that acts as a hydrophobic barrier controlling the movement of solutes into the cell and enclosing the cytoplasmic contents of bacteria. This bilayer is studded with integral membrane proteins that carry out essential membrane functions. The density of cytoplasmic membrane proteins is high enough such that proteins are separated from each other by only three or four phospholipid molecules [8].

Phospholipids generally contain a glycerol 3-phosphate backbone attached to a hydrophilic head group and hydrophobic fatty acids. The lipids often have a positive charge to balance the negative charge on the phosphate and are termed zwitterionic, or have no charge on the headgroup giving the phospho-

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_9

lipid a net negative charge. Although the type and proportion of phospholipids produced will vary under different environmental conditions, a typical membrane composition for *Escherichia coli* is 75% zwitterionic phosphatidylethanolamine (PE), 20% anionic phosphatidylglycerol (PG), and 5% anionic cardiolipin (CL, or diphosphatidyl glycerol) [9]. Membrane lipids are amphipathic and given an appropriate balance of headgroups, will spontaneously form bilayers to create a hydrophobic core that contains the fatty acyl chains separating the polar head groups on both sides of the bilayer. The fatty acyl chains are usually either saturated or contain a single double bond and are termed unsaturated, while the acyl chain may comprise 14–22 carbons. For example, the predominant fatty acids in the cytoplasmic membrane lipids of *E. coli* are saturated palmitic acid (16:0), the unsaturated species palmitoleic acid (cis-ω9,10−16:1) and cis-vaccenic acid (cis-ω11,12−18:1) [8].

The fluid mosaic model describes the properties of a membrane whereby both phospholipids and proteins diffuse laterally along the plane of a membrane, although proteins diffuse at a slower rate than lipids [8]. Generally speaking, phospholipids do not readily flip from one leaflet in the bilayer to the other, since it is thermodynamically unfavourable for the polar head group to pass through the hydrophobic core. When bacterial cells are grown at increasing temperatures, there is generally an increased production of rigid, saturated fatty acids and a decreased production of flexible, unsaturated fatty acids in order to maintain membrane fluidity at a physiologically appropriate level.

2.2 Periplasm/Peptidoglycan

Located between the cytoplasmic membrane and outer membrane of Gram-negative bacteria is the periplasm (Fig. 9.1a). Based on thin section transmission electron microscopy, the periplasm is estimated to be between 13 and 25 nm in width [10–12], depending on the sample preparation method used, and this can be compared to the width of membranes that are about 7–10 nm for the inner membrane and 10–30 for the outer membrane (NB, the membrane bilayer of the outer membrane is only slightly larger than that of the cytoplasmic but the long sugar chains of lipolysaccharide, LPS, can thicken the outer membrane adding a capsule-like aspect to the surface of the outer membrane [13]. The peptidoglycan layer is located within the periplasmic region. Given its position, the periplasm plays an important role in buffering the cell from changes in both the intracellular and extracellular environments. To facilitate this function, the periplasm contains anionic sugar polymers termed membrane-derived oligosaccharides as well as many proteins including (1) specific solute or ion binding proteins for the uptake of sugars, amino acids, peptides, vitamins and ions; (2) catabolic enzymes for the degradation of complex molecules into simpler ones that can be transported across the inner membrane; (3) detoxifying enzymes, like β-lactamases and aminoglycoside-modifying enzymes, for the degradation or modification of potential cell inhibitors; (4) hydrolytic enzymes, like nucleases and alkaline phosphatases and (5) proteins which aid in the assembly or translocation of major envelope proteins, peptidoglycan, LPS or capsules [14].

Despite some disparity in measurements of the size of the periplasmic space, the physiological state of the periplasm is thought to be gel-like. Hobot et al. [10] proposed that the periplasm is organized in a gradient of increasing peptidoglycan polymerization from the cytoplasmic membrane to the outer membrane. This peptidoglycan framework is filled with an aqueous solution containing periplasmic proteins, oligosaccharides and other small molecules. More recently, this model has been refined to propose that periplasmic proteins rather than peptidoglycan polymers account for the gel-like state of the periplasm [15]. Measurements of periplasmic protein mobility are consistent with this modification of the model [16]. Whatever the physiological state, the periplasm is a dynamic rather than a static environment, and is often underestimated for its significant role in cellular homeostasis.

The term peptidoglycan was first introduced by Weidel and Pelzer [17] to describe a "rigid bag of the volume and shape of the cell". Peptidoglycan is the polymer that encompasses the bacterial cell providing both strength and structure to the cell and is sometimes called the cell wall or murein sacculus. Due to the high metabolic activity and correspondingly high solute concentration within the cell, bacteria must contain an osmotic pressure that is between 5 and 20 atmospheres and thus greater than that of the surrounding medium. The peptidoglycan layer is the structure that facilitates maintenance of this pressure difference and is therefore absolutely essential to cell survival. Nevertheless, the peptidoglycan layer has sufficient plasticity to allow for both cell growth and division and specific enzymes that can remodel the peptidoglycan locally to permit these essential functions, with which peptidoglycan is intimately involved.

Although it is conserved in all eubacteria, differences exist in the peptidoglycan layer between Gram-positive and Gram-negative bacteria. In Gram-positive organisms, the peptidoglycan layer is multilayered and relatively thick (5–25 nm) [18, 19]. Various acidic and/or neutral polymers like teichoic acid or teichuronic acid are covalently attached to the peptidoglycan layer (Fig. 9.1b). In Gram-negative organisms, the peptidoglycan layer is located between the cytoplasmic and outer membranes and tends to be only a few layers [20] and 1.5–6 nm thick [21], although recent studies suggest that the peptidoglycan chains may be at least partially oriented perpendicularly to the surface of the cytoplasmic membrane [22]. Lipoproteins embedded in the outer membrane and peptidoglycan-associated proteins (covalent and non-covalent) anchor the peptidoglycan layer to the outer membrane.

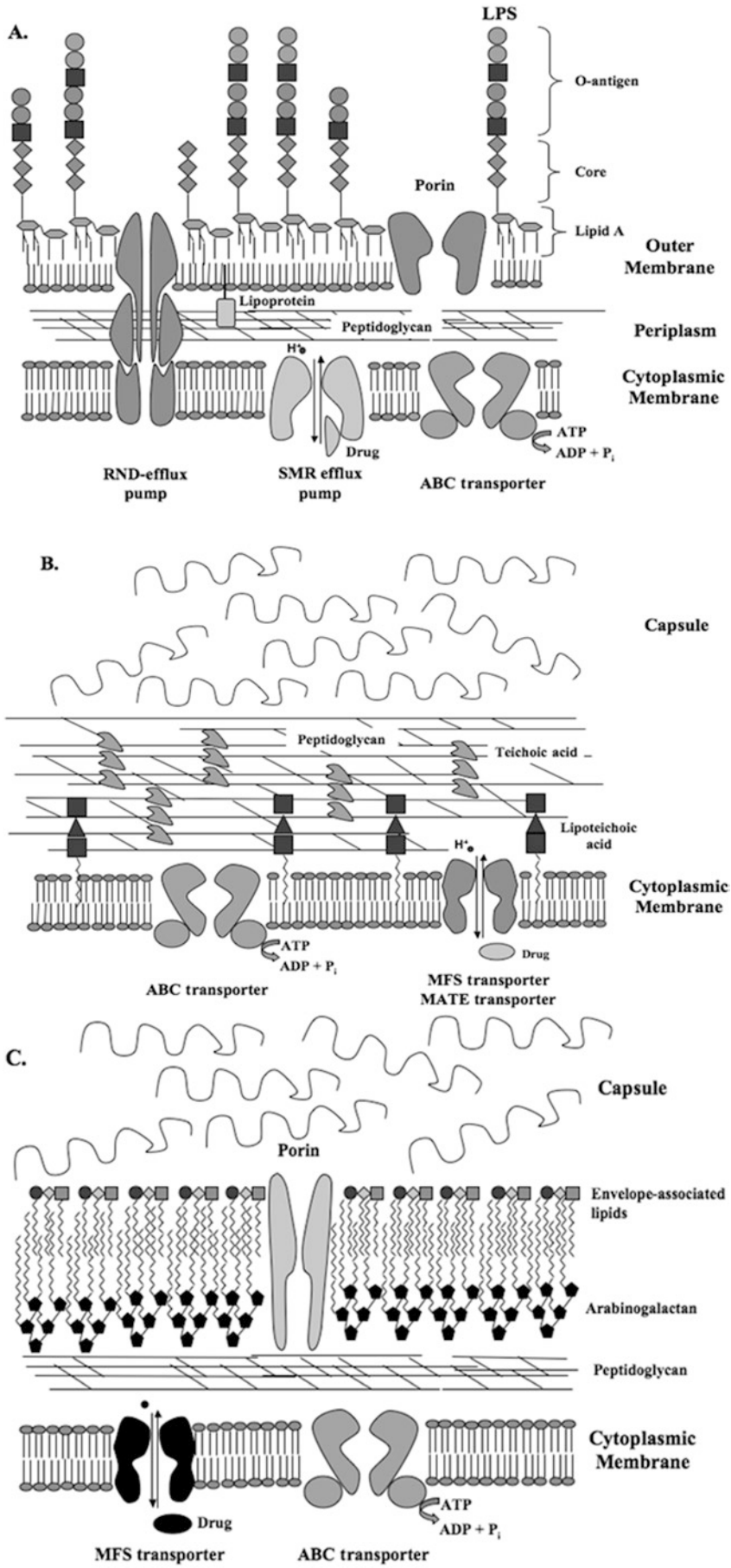


Fig. 9.1 The structure and arrangement of the cell envelope components of (**a**) Gram-negative bacteria, (**b**) Gram-positive bacteria and (**c**) mycobacteria. Note that although representations of example clinically relevant efflux system are shown, each type of bacterium may contain members of other classes of efflux systems, in addition to those displayed

Peptidoglycan is composed of a polysaccharide backbone made up of β, 1–4-linked alternating residues of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), cross-linked through peptide side chains. A short peptide of four amino acids is attached to the carboxyl group of NAM of mature peptidoglycan. Variability in the peptidoglycan structure is largely due to differences in the short peptide, although differences in the glycan backbone and nature of the cross-link are also observed [23]. In *E. coli*, for example, the mature stem peptide is composed of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and D-alanine, whereas in *Staphylococcus aureus meso*-diaminopimelic acid is replaced by L-lysine.

The average glycan strand is about 30 muropeptide units in length [24, 25]. Individual strands are cross-linked to each other either directly or indirectly through peptide side chains, and these covalent peptide cross-links provide the strength required to resist the internal osmotic pressure. In Gram-negative bacteria, for example, *E. coli*, direct cross-linking occurs between the carboxyl group of the D-alanine in position 4 of one stem peptide and the free amino group of *meso*-diaminopimelic acid in the adjacent strand. Cross-linking in Gram-positive bacteria is indirect, and occurs through an inter-peptide bridge of five glycines in *S. aureus,* for example. The degree of cross-linking and cross-linking position also differs between species of bacteria [26, 27], with Gram-positive organisms having a higher degree of cross-linking than Gram-negative organisms, which have the added protection of the outer membrane.

2.3 Outer Membrane

The outer membrane is an unusual bilayer membrane found only in Gram-negative bacteria (Fig. 9.1a) [7]. What makes this structure unique is the asymmetric nature of the bilayer. The composition of the inner leaflet is similar to that of the cytoplasmic (inner) membrane, with phosphatidylethanolamine being the predominant phospholipid and minor amounts of other phospholipids, e.g. phosphatidylglycerol and cardiolipin (diphosphatidylglycerol). As with the cytoplasmic membrane, the lipid composition of the outer membrane is not static; it varies with the environmental conditions in which the bacteria are found.

There is some dispute as to whether phospholipids are also found in the outer leaflet of the outer membrane; however, the most predominant lipidic species of the outer leaflet is a long polymeric glycolipid termed LPS. LPS has a tripartite structure consisting of a Lipid A moiety, a core oligosaccharide and a longer *O*-polysaccharide.

The Lipid A (or endotoxin) backbone usually consists of a diglucosamine residue that is phosphorylated at its C1 and C4′ positions. The disaccharide is covalently *N*- or *O*-linked to anywhere from 4 to 7 fatty acids that anchor it into the membrane. These fatty acids tend to be saturated and hydroxylated at the C3 position. This 3–OH group may have another fatty acid as a substituent, producing an acyl-oxyacyl structure that is a characteristic feature of Lipid A.

The diglucosamine backbone of Lipid A is conserved amongst most Gram-negative bacteria. The fatty acid composition, however, is quite variable from species to species. Different environmental conditions can also induce changes in the fatty acid profile. How these differences in fatty acid composition influence their packing behaviour and thus membrane fluidity and transport are discussed below. In some bacteria, under particular conditions (e.g. low concentrations of divalent cations in the growth medium), the phosphate groups of the diglucosamine moiety can be substituted with the positively charged sugar arabinosamine, whereas phosphatidylethanolamine substitutions can also occur. These changes increase the resistance of the bacteria to certain cationic antibiotics and are discussed in detail in Sect. 4.2.

The core oligosaccharide of LPS is covalently attached to the Lipid A via the unique sugar molecule 2-keto-3-deoxyoctulosonic acid (KDO). In addition to KDO, this region also includes a variety of other sugar molecules such as L-glycero-D-manno-heptose and its optical isomers, glucose, galactose, rhamnose, etc. Some of these sugars may be modified by the addition of phosphate, pyrophosphate, phospholipids (e.g. phophatidylethanolamine, phosphatidylcholine) or amino acids (e.g. alanine). The overall structure of the core oligosaccharide is relatively conserved within a given bacterial genus but may vary somewhat with respect to sugar composition, substitution and/or connectivity [28].

Between approximately 10 and 25 % of the core oligosaccharides are covalently linked to the *O*-polysaccharide (or *O*-antigen), a string of sugar repeat units, that vary substantially even within a species. This diversity is proposed to be driven by selective pressure (e.g. from the immune response or from phage susceptibility) that arises from being exposed to the external environment [29]. The basic structure of the *O*-polysaccharide consists of a mono- to octa-saccharide repeat. Over 60 different sugars from different Gram-negative bacteria have been identified as being components of an *O*-polysaccharide. Some examples of these sugars include glucose, mannose, ribose, rhamnose, glucosamine, fucosamine and amino hexuronic acids such as quinovosamine.

The number of *O*-repeats varies from 0 to 50 units and this produces a characteristic ladder pattern when LPS is resolved on an acrylamide gel. Some mucosal pathogens, such as *Bordetella pertussis*, completely lack an *O*-antigen and are thus said to possess LOS (lipooligosaccharide) rather than LPS [30]. Other organisms, such as *Pseudomonas aeruginosa*, can have *O*-antigens that extend more than 40 nm from the surface of the cell [13].

In addition to LPS, the outer membrane contains a moderate number of proteins present in high copy number. These proteins are involved in a variety of cellular processes that include selective permeation, cell shape and membrane stabilization, motility, adherence, transport and interaction with the immune system, bacteriophages and other bacteria [7, 31].

An abundant class of outer membrane proteins is the lipoproteins. These are relatively small proteins that are present in high copy number (~7×10^5/cell). They are modified at an *N*-terminal cysteine with an *N*-acyl diacyl glyceride residue that non-covalently inserts into the outer membrane to anchor the proteins. Lipoproteins are thought to stabilize the cell wall by associating either covalently or non-covalently with the peptidoglycan depending on the organism. In *Pseudomonas* species, for example, the lipoproteins examined to date are all non-covalently associated with the peptidoglycan. In *E. coli*, however, a third of the major lipoprotein molecules are covalently linked to the diaminopimelate groups of the peptidoglycan via their C-terminal lysine or arginine residues.

Outer membrane transporters are involved in both the uptake (porins) and efflux (efflux channels) of compounds into and out of the cell. Both of these protein classes adopt a β-barrel structure in the outer membrane although their architecture is very different with the porins containing one water-filled channel per monomer (or often three per trimer) and the efflux channels containing one channel made from three monomers. Efflux channels have an additional α-helical periplasmic domain, which is discussed in a later chapter and elsewhere [32]. Amino acids with non-polar side chains form the outer surface of the barrel and interact with membrane lipids, thus stabilizing the structure. Hydrophilic amino acids line the interior of the channels, providing a polar environment for hydrophilic compounds to travel through.

2.4 Mycobacterial Cell Envelope

Although phylogenetically classified as Gram-positive bacteria, the mycobacteria have a uniquely organized cell envelope (Fig. 9.1c). As with other bacteria, the cytoplasmic membrane forms an inner barrier between the cytoplasm and the environment, and its lipid composition is similar to that of other bacteria. This is surrounded by a layer of peptidoglycan, with a structure similar to that of Gram-negative bacteria (i.e. relatively thin). External to this is the arabinogalactan layer, consisting of a complex branched network of polysaccharide. Each arabinogalactan residue consists of a polymer of galactofuranose, many of which possess five or six covalently attached arabinose moieties (Fig. 9.1c). Each of the arabinose groups in these terminal groups are ester-linked via the 1′-hydroxyl moiety to lipidic mycolic acids which extend to the bacterial surface. The mycolates attached to the arabinogalactan are very long (60–90 carbons) and may contain unusual cyclopropane moieties within their acyl chains [33]. Due to the length of these fatty chains, they are found in the gel state with phase transition temperatures as high as 60–70 °C [34]. The composition of the membrane varies due to regulation by temperature and/or environment, analogous to lipid compositional changes in other types of bacteria. There is some evidence for the presence of another glycolipid monolayer consisting of trehalose dimycolates, sulfolipids, phythiocerol dimycocerosate and phenolic glycolipids external to the mycolate residues of the arabinogalactan. The approximate thickness of the mycolate bilayer is ~37–90 nm, substantially larger than that of a Gram-negative outer membrane [35, 36]. Like the Gram-negative bacterial outer membrane there are porin-like molecules that traverse the mycolic acid layer but they have a rather unique structure [36, 37]. In some senses, the envelope of mycobacteria resembles the outer membrane of Gram-negative bacteria, and due to the presence of this thickened highly hydrophobic envelope, mycobacteria are characterized by their extremely low permeability to most hydrophilic antibiotics.

2.5 Capsule

Many bacteria in their natural habitats produce extracellular polysaccharide capsules. Capsular polysaccharides are either homo- or hetero-polymers of repeating sugar units, connected by glycosidic bonds to form the capsule structure. Because of the broad range of monosaccharide units and glycosidic bond configurations possible, bacterial capsules are extremely diverse. Initially capsules were divided into groups (referenced to *E. coli*) based on the presence of common monosaccharides [38], but more recently capsule classification has been based solely on genetic and biosynthetic criteria to divide *E. coli* capsules into four distinct groups [39]. This updated classification scheme (again referenced to *E. coli*) accounts for the observation that not all capsules are composed of polysaccharide K antigens; previous classifications were based on the biochemical division of K antigens, which all form capsules.

Capsule layers are highly hydrated, containing over 95 % water [40], and as such may function to protect the organism from desiccation. Consistent with this suggestion, mucoid isolates are more resistant to drying than their non-mucoid isogenic counterparts [41], and changes in extracellular osmolarity are known to induce expression of capsule molecules [42, 43]. Polysaccharide capsules also function as adherence factors. Capsules facilitate both biofilm formation and niche colonization [44, 45] by promoting the adherence of bacteria to each other and to surfaces. This ability of bacteria to attach to surfaces and establish a biofilm plays an

important role in initiating and maintaining infection [46, 47]. For example, *P. aeruginosa* infections of the cystic fibrosis lung are often characterized by overexpression of alginate and biofilm formation [48], which probably helps to protect the bacteria from opsonization and killing by neutrophils and macrophages in the lung.

Infections are further maintained through the ability of the capsule to resist both the non-specific and specific immune responses of the host. Polysaccharide capsules are poor activators of the alternative complement pathway [49–51] and furthermore mask underlying cell surface structures, which do typically activate this pathway [52, 53]. This reduced ability to activate opsonic fragments of complement (e.g. C3b), and the net negative charge of the capsule surface works to inhibit phagocytosis [54, 55]. Capsular polysaccharides also confer resistance to the host's specific immune response, by mimicking the structure of polysaccharides found in the host, and consequently are usually poor immunogens [56–58]. The presence of capsule in some species also appears to reduce killing by cationic antimicrobial peptides [59, 60]. For instance, a non-capsulated *Klebsiella pneumoniae* mutant strain was more readily killed by different antimicrobial peptides, including the human neutrophil defensin 1, β-defensin 1, and the bacterial lipopeptide polymyxin B. The mechanism behind this decreased susceptibility seems to be related to the ability of anionic capsule polysaccharide to bind the positively charged peptides, thereby making the outer membrane less accessible and limiting self-promoted uptake of the peptide [59]. Interestingly, exposure to certain antimicrobial peptides in *K. pneumoniae* promotes the production of capsule polysaccharides and their release into the extracellular milieu [60].

2.6 S-Layer

Protein surface layers, generally known as S-layers, are part of the cell envelope of most Archaea as well as many bacteria, both Gram-negative and Gram-positive, including the pathogens *Clostridium difficile*, *Bacillus anthracis*, *Campylobacter* sp. and some Enterobacteriaceae. In Gram-negative bacteria, the S-layer is bound to the cell surface through interactions with LPS. In contrast, in Archaea and Gram-positive bacteria the S-layer is attached by non-covalent bonds to other cell wall components like peptidoglycan, pseudomurein or secondary cell wall polymers [61]. Generally, these paracrystalline pronteinaceous lattices consist of a single protein or glycoprotein. However, some microorganisms can alter the specific protein that constitutes the S-layer depending on the environmental conditions. Additionally, examples of complex multiprotein S-layers have been observed in certain species [62].

Significantly, the S-layer protein tends to be the most abundant protein in the cell, comprising approximately 7–30 % of the total protein content [63]. This suggests that the role of this envelope component may be very important for the survival of the cell. Despite this, no clear general role for S-layers has been defined to date. It is thought that these structures would have originally had a structural role, providing the cell with mechanical, thermal and osmotic stability and even determining the cell shape [64]. In Archaea, this may indeed be the main role of the S-layer in the absence of other cell wall components. In bacteria, however, the presence of other structures suggests that the S-layer might exert additional secondary functions, such as protecting the cell from potential threats from the extracellular milieu [64]. In this sense, it is worth noting a recent study investigating the role of the RsaA-protein S-layer of the bacterium *Caulobacter crescentus*. It was shown [65] that *C. crescentus* strains possessing an S-layer showed increased resistance to cationic antimicrobial peptides, but not to the anionic peptide daptomycin. The authors hypothesized that this is likely due to interactions between negatively charged amino acids in RsaA and the positively charged peptides. This finding is very significant considering that antimicrobial peptides are widely present in nature, as they are produced by practically all living organisms.

3 Intrinsic Resistance

3.1 Restricted Permeability

3.1.1 Gram-Negative Bacteria

The outer membrane of Gram-negative bacteria is a semipermeable barrier to the uptake of most hydrophilic molecules larger than a certain size exclusion limit. An analogy is often drawn to this membrane constituting a molecular sieve although this is only really true for negatively charged or neutral polar molecules, as both positively charged and hydrophobic molecules can pass across the outer membrane by other routes. For the former molecules, uptake is limited by the size of the water-filled channels of β-barrel proteins termed porins [66]. The total surface area of the outer membrane that is occupied by such channels has been estimated as approximately 0.6 % in *E. coli*, and this together with limited diffusion imposed by frictional interactions between molecules passing through the channel and the amino acids lining the channel wall severely restricts uptake of hydrophilic molecules, especially those like β-lactams, trisaccharides, and tetrapeptides that have sizes that are not much smaller than the restricting diameters of these channels in, e.g. *E. coli*. Other bacteria, e.g. *P. aeruginosa*, have a much smaller number of channels leading to an overall outer

membrane permeability that is only 1–8 % that of the *E. coli* outer membrane, even though *P. aeruginosa* has larger-sized channels and a larger exclusion limit. Restricted permeability through the outer membrane clearly contributes therefore to the observation that Gram-negative bacteria tend to have higher intrinsic resistance to most antibiotics than their Gram-positive counterparts, a factor that is a major contributor to the drastic dearth of discovery of new Gram-negative selective antibiotics.

It is worth considering the nature of the "fabric" of the outer membrane molecular sieve. As mentioned above, the outer membrane surface largely contains, as its major lipidic molecule, the highly anionic glycolipid LPS, which is partly neutralized, cross-bridged and thus stabilized by divalent cations, predominantly Mg^{2+} and Ca^{2+}. This surface thus tends to repel neutral and anionic polar molecules, but as described below can actually serve to permit self-promoted uptake of cationic molecules. Further evidence that the outer membrane is a barrier to uptake of hydrophilic antibiotics is seen in the fact that increasing outer membrane permeability by cloning in large, abundant porins leads to increased antibiotic susceptibility in *P. aeruginosa* [67], while disrupting the fabric of the outer membrane by removal of divalent cations with chelators like EDTA has a similar effect [68, 69].

3.2 Mycobacteria

Based upon the low susceptibility of mycobacteria to most antimicrobials, it is clear that the cell wall of this organism forms a significant antimicrobial barrier. Indeed, early studies examining the permeability of *Mycobacterium chelonae* showed that it was approximately tenfold less permeable to hydrophilic β-lactam antibiotics than was *P. aeruginosa* [70] (i.e. 100- to 1000-fold less permeable than the *E. coli* outer membrane).

In contrast to the trimeric general porins of Gram-negative bacteria that have a single pore per monomer, MspA is an octamer of small subunits that assemble to form a single central channel [36], and channel numbers tend to be relatively low. In addition, the MspA pore is much longer than for the general porins, presumably due to the thickness of the mycobacterial cell wall. Therefore, substrate interactions with the channel interior may be more pronounced in mycobacteria and might hinder solute diffusion. Indeed, this appears to be the case as the deletion of MspA from *Mycobacterium smegmatis* results in both increased resistance to hydrophilic antibiotics and decreased growth due to lowered permeability to nutrients [70, 71].

3.3 Efflux

Intrinsic antibiotic resistance in Gram-negative bacteria is due to the synergy between low outer membrane permeability that restricts the rate of exposure of the interior of the cell to antibiotics, and the presence of additional resistance mechanisms such as drug modification (e.g. β-lactamases) and multidrug efflux systems. Cytoplasmic membrane-localized efflux pumps are widespread among bacteria and are divided into five major classes on the basis of bioenergetic and structural criteria [72], and it is worth noting that in addition to contributing to antibiotic efflux, many of these pumps also have roles in normal cell physiology [73].

The ATP-binding cassette (ABC) superfamily is an ATP-driven efflux system found in Gram-negative and Gram-positive bacteria, as well as in mycobacteria. The major facilitator superfamily (MFS) is another ancient efflux system that uses chemiosmotic energy and functions as a drug-ion antiporter. The resistance/nodulation/cell division (RND) family and the small multidrug resistance (SMR) family are both proton-driven pumps although the former comprises multi-subunit complexes. The fifth system is the multidrug and toxic compound extrusion family (MATE) and also utilizes the chemiosmotic gradient across the cytoplasmic membrane to energize transport. Gram-positive bacteria often employ MFS efflux pumps such as NorA in *S. aureus* [74] which provide resistance to fluoroquinolones.

In Gram-negative bacteria, the RND (resistance-nodulation-division) family of pump proteins are the predominant class [75] involved in intrinsic resistance. RND transporters are tripartite systems consisting of an outer membrane channel-tunnel, an inner membrane pump and a peripheral cytoplasmic membrane/periplasmic linker protein. A broad range of structurally unrelated substrates are known to be pumped out of bacterial cells including most types of antibiotics, biocides, heavy metals, organic solvents, dyes and detergents [76]. Given the ubiquitous distribution of efflux systems in bacteria, there is much interest in determining the natural and intended substrates of these efflux systems [73]. In *E. coli,* for example, efflux pumps are capable of shuttling toxic fatty acids and bile salts out of the cell and thus it has been suggested that normal metabolic intermediates and noxious compounds that *E. coli* encounters in the gut during infection may be natural substrates [76]. Also interestingly, the MexEF-OprN efflux pump of *P. aeruginosa* has been shown to export a precursor of the *Pseudomonas* quinolone signal (PQS), which is part of the intricate quorum sensing network in this pathogen [77]. Another finding that could help to understand the natural function of efflux pumps is the regulation of the expression of different efflux systems in *P. aeruginosa* under conditions of stress [78–80].

In many bacteria, the expression of efflux system genes is tightly controlled. This is not surprising as there is evidence indicating that overexpression of efflux systems is, in many cases, associated with a loss of fitness and virulence [81, 82]. Although antibiotic efflux is typically described as an intrinsic resistance mechanism, there are a number of mutational events that can lead to increased expression of efflux systems, and therefore increased resistance. Also, efflux mechanisms may be subject to adaptive resistance and can be induced by specific environmental cues, including various stresses and exposure to antibiotics [83]. For example, *tetR*, the negative regulator of the MFS tetracycline efflux pump, is ordinarily bound to the operator sequence upstream of the efflux genes, preventing expression under normal conditions [84]. In the presence of its substrate (i.e. tetracycline) the TetR protein is released from the operator and transcription of the gene(s) involved (*tetK*, *tetL* and/or *tetB*) proceeds. Thus the bacteria do not become resistant to tetracycline unless tetracycline is actually present.

A similar general principle exists for many RND efflux systems in wild-type bacteria in that expression of efflux pumps is tightly regulated, although some pumps are always expressed at basal levels. However, unlike the situation with the TetR protein described above, the actual efflux genes are often not induced by the known substrates of the particular efflux pump. Rather, what often occurs is that a mutation appears in the regulator of the efflux system following antimicrobial therapy such that the genes encoding for the pump components are expressed constitutively at higher levels leading to increased resistance to all substrates that the pump can efflux. The mutations are often stable point mutations that reduce the DNA binding affinity of particular repressors for their target regulatory regions within promoters and lead to constitutive expression of efflux components [85]. Many clinical isolates of the cystic fibrosis pathogen *P. aeruginosa* have multidrug resistance phenotypes due to regulatory mutations that are probably selected for in the lungs of CF patients who are often on chronic antimicrobial therapy [76].

Interestingly, a recent study described heterogeneity regarding the expression of efflux pumps and porins within the same bacterial population of *Salmonella enterica* as determined by single-cell gene expression analysis [86].

4 Antibiotic Penetration and Resistance Mechanisms

4.1 Porin Pathway

Porins permit the diffusion of a variety of compounds into the periplasm. There are three classes of porins: general, specific and gated (Fig. 9.2). Uptake through general porins is considered passive, as it involves passive diffusion through the aqueous channels of the porin and is dependent only on the physicochemical properties of the solute (i.e. size, charge, polarity and the magnitude of the concentration gradient across the membrane) relative to the side chains of the amino acids lining the pore and especially those side chains found at the most constricted part of the channel. The crystal structures of several general porins have been solved and reveal that they are trimers of 16 stranded anti-parallel β-barrels that enclose a pore lined predominantly with hydrophilic amino acids [87–89]. These β-strands tend to be connected by short (3–4 amino acid) turn regions on the periplasmic

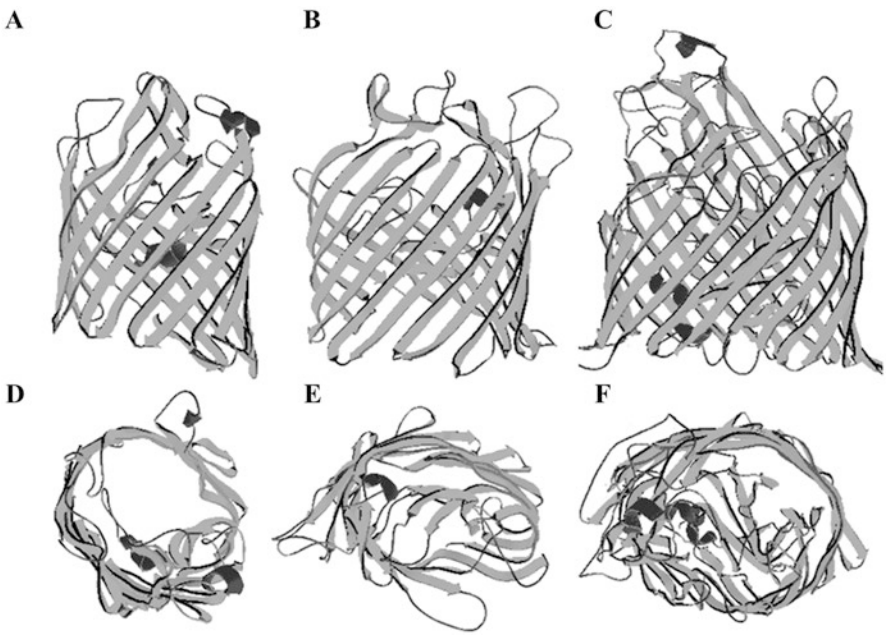


Fig. 9.2 Representative structures of the porin molecules of Escherichia coli. Side (**a**) and top (**d**) view of the OmpF general porin [86]. Side (**b**) and top (**e**) view of the maltodextrin-specific channel, LamB [91]. Side (**c**) and top (**f**) view of the gated porin FhuA [101]. Note the varying degrees of channel constriction imparted in each porin type by the inward folding of various extracellular loops or domains (see text for complete description)

side of the porin and much longer loops of amino acids on the external side of the outer membrane. The cross section of the channel interior somewhat resembles an hourglass and can be conceptually divided into three zones: the external mouth, the constriction zone or eyelet, and the exit. The mouth of the general porin pore acts as a crude filter. This region is rich in charged amino acids and may be somewhat restricted by one or more extracellular loops that fold into it. The purpose of these two features is to constrict the opening, both physically and electrostatically such that large, hydrophobic and/or highly charged compounds cannot enter the cell. The eyelet is the narrowest part of the channel, usually formed by a single loop 3 that folds from the external surface back into the porin channel. The size of this eyelet determines the maximum size, i.e. the exclusion limit of molecules that can pass through the channel. For the prototypic bacterium, *E. coli*, the exclusion limit determined by the major porins OmpF and OmpC is around 600 Da (equivalent to a trisaccharide or tetrapeptide), although there are subtle differences in channel size for these two proteins. Therefore, for this and other enterobacteriaceae, it is presumed that small, hydrophilic antibiotics such as chloramphenicol, tetracycline, fluoroquinolones and β-lactams (including cephalosporins and carbapenems) might utilize these channels as entry points. This fact has been confirmed by the isolation of mutants, both in the clinic and in vitro, that are resistant to the above-mentioned antibiotics due to either a complete loss of or diminished porin expression [90–92].

Specific porins are similar to general porins with one major exception; they have stereospecific binding sites for their substrates, which are located in part in the eyelet. This specificity narrows the structural range of molecules that can pass through these channels. The crystal structure of the LamB channel of *E. coli* has been solved and shows that this porin is highly specialized for the uptake of maltodextrins [93]. The eyelet of this porin is more constrained than in general porins due to the folding of two additional loops into the mouth of the channel. Six contiguous aromatic amino acids (the greasy slide) form a path through the channel, down which the sugar molecules travel. In addition, the channel interior is lined with polar amino acids (the polar track) that stabilize the hydroxyl groups of the sugars [94]. An analogous design is found for the phosphate-specific porin OprP of *P. aeruginosa* [95]. The channel interior of this trimeric porin is quite constricted and reveals a phosphate-binding "arginine ladder" comprising eight arginine residues that span from the extracellular surface down to a constriction zone where phosphate is coordinated. Lysine residues also coat the inner periplasmic surface of this channel creating an "electropositive-sink" that pulls the phosphates through the eyelet and into the cell.

Due to their specialized nature, the only antibiotics that should be able to penetrate specific porins are those that mimic the channel's natural substrates. Indeed, this is true for the Tsx channel of *E. coli*. Specific for nucleosides, this porin also takes up the structurally related antibiotic albicidin [96, 97]. Similarly, the OprD porin of *P. aeruginosa* is specific for the uptake of the basic amino acids arginine and lysine and basic dipeptides, as well as the structurally analogous carbapenem antibiotics imipenem and meropenem [98, 99]. Recently we also demonstrated that the tricarboxylate-inducible porin OpdH, a homolog of OprD, appeared to be involved in the uptake of the bulky cephalosporin ceftazidime [100]. It should also be noted, that low levels of structurally unrelated compounds can also diffuse through specific porins. This is especially the case for non-fermentative organisms, like *Pseudomonas*, which lack classic general porins. For example, the OprD porin in addition to taking up basic amino acids is the major facilitator involved in the diffusion across the outer membrane of compounds up to 200 Da in mass [67].

Gated porins, also known as TonB-dependent receptors, are monomeric proteins consisting of 22-stranded β-barrels, and permit the specific entry of larger compounds such as iron-siderophore complexes into the cell. The mouth of these channels is blocked by a globular domain termed the plug [99, 102]. Uptake is initiated once a substrate docks onto a gating porin. This binding, in conjunction with energy input from the TonB energy transducing protein, results in a series of conformational changes in the plug domain that culminate in both the release of the substrate and the revelation of a translocation pathway [103].

Due to their large channel sizes, gated porins may seem like the ideal conduits for antibiotic uptake; however, this use is generally limited by the specificity of substrate docking. It is known that there are certain gated receptors that have somewhat lower selectivity, e.g. Cir and FhuA. However although providing antibiotics with iron binding groups (e.g. catechol or heme groups) can improve uptake across the outer membrane, and consequently lower MICs, none of these substituted drugs have been clinically successful and this may reflect mechanisms of toxicity and/or interference with iron metabolism in the host. Specific antibiotics that can be taken up by ferric-siderophore receptors include albomycin, a structural analogue of ferrichrome, which is taken up by the FhuA gated-porin receptor. Interestingly, rifamycin CGP 4832 (a rifampin derivative), a structurally unrelated antibiotic, is also taken up by FhuA [104]. The crystal structures of FhuA in complexes with both of these antibiotics indicate that despite differences in structure, both antibiotics bind to the same residues of the porin [105], indicating that gated porins tolerate some structural flexibility.

As described above, mycobacterial envelopes contain a class of porins that although structurally unrelated to Gram-negative porins, serve as the major pathway for hydrophilic antibiotics. There are two types of mycobacterial porins represented by OmpATb, which is not well studied, and MspA, which has been crystallized [37]. MspA from *M. smegmatis*,

the best-characterized mycobacterial porin, is the major route of entry for hydrophilic compounds into this organism [37]. However, the medically important mycobacteria, *M. tuberculosis*, and *M. bovis* BCG seem to lack MspA-type porins, and depend exclusively on OmpATb-type porins, an observation that may explain the intrinsically lower susceptibility of these organisms to hydrophilic antibiotics compared to *M. smegmatis*. The diameter of the MspA channel from *M. smegmatis* is apparently larger than that of the OmpATb porin from *M. tuberculosis*, which is not well characterized, and cloning of the *M. smegmatis* MspA protein into *M. tuberculosis* increases the sensitivity of *M. tuberculosis* to β-lactams by up to 16-fold [106]. Additionally, the growth rate of *M. tuberculosis* expressing *M. smegmatis* MspA is increased, suggesting that nutrient uptake in this species is also limited by the small pore size of OmpATb. Regardless of which porin proteins a particular strain expresses, the porin pathway seems to be involved in the uptake of pyrazinamide [107] and β-lactams [108].

4.2 Self-Promoted Uptake and Regulatory Mutants

The self-promoted uptake pathway is limited to Gram-negative bacteria and generally pertains to the passage of cationic amphipathic molecules across the outer membrane. Self-promoted uptake involves the interaction of polycations with sites on the surface of the outer membrane at which divalent cations cross-bridge adjacent LPS molecules. Displacement of these divalent cations leads to local distortion of outer membrane structure and this provides sites for uptake of other polycationic antibiotic molecules; thus these polycations promote their own uptake rather than diffusing across the outer membrane through water-filled channels.

Recently, it has become clear that self-promoted uptake is quite effective in many species of bacteria, including *E. coli*, *P. aeruginosa*, *Salmonella enterica* and *Yersinia* sp., which all seem to have the potential to be killed by antibiotics that access the self-promoted uptake pathway [69]. Other species such as *Burkholderia cenocepacia* and *Helicobacter pylori* show a significantly lower rate of killing by antibiotics that would normally enter via this pathway [109, 110]. For species that are normally sensitive to killing via the self-promoted uptake pathway, the organism in question generally maintains a level of control over the effectiveness and/or accessibility of this pathway [111–114], as discussed in more detail below.

The characteristics of the LPS of a particular bacterial strain primarily determine whether or not a particular bacterium possesses an effective self-promoted uptake pathway. As described in Sect. 3.2, the structure of bacterial LPS is complex and species-specific. The LPS of many bacteria is characterized by a large number [3–12] of negatively charged phosphate groups and anionic sugars (e.g. KDO) in the core oligosaccharide and usually two additional phosphates attached to the Lipid A moieties of the LPS [29]. These negatively charged groups are ordinarily bridged by divalent cations, which serve to stabilize the outer membrane by preventing the LPS molecules from repelling one another. Studies carried out with chelators of divalent cations, such as EDTA, have shown that when the cell is rapidly depleted of the divalent cations bound to the LPS, there is a massive disruption in outer membrane integrity, with a concomitant loss of ~50 % of the LPS [69]. Thus, these divalent cations are an integral component required for maintenance of outer membrane structure.

Cationic antibiotics and the cationic antimicrobial peptides can also disrupt the bacterial outer membrane. The cationic peptides are ubiquitous in nature and form an important component of the human innate immune system [115]. Basically, these are small peptides that have a net positive charge due to the presence of a number of lysine or arginine residues in their sequence. Soil-dwelling bacteria, lactic acid bacteria, plants, insects, fish, birds, amphibians and other animals also produce cationic peptides. Studies with the cationic lipopeptide antibiotic polymyxin B showed that when bacteria are exposed to this antibiotic the integrity of the bacterial outer membrane is rapidly destroyed, indicating that the outer membrane might be a primary determinant by which these compounds gained access to Gram-negative cells [116, 117]. Cationic antimicrobial peptides have a number of physical properties that are important for their activity. As suggested by their name, the cationic nature of the molecule is very important and substituting uncharged for the charged amino acids severely impairs their antimicrobial ability. Additionally these peptides usually contain up to 50 % hydrophobic amino acids and consequently can insert into membranes while folding into an amphipathic structure that contains both a highly polar face and a hydrophobic face.

Regulation of self-promoted uptake has been studied in a number of organisms, including *E. coli*, *S. enterica* and *P. aeruginosa*. The genetics of resistance are perhaps best understood in *E. coli* and *S. enterica* and these systems will serve as the model for the remainder of this discussion, with important exceptions being highlighted where applicable. Early work in *S. enterica* showed that there were two loci responsible for increased resistance to polymyxin B and other cationic antimicrobial peptides and that these mapped to two systems named *pmrAB* (polymyxin resistance gene A and B) and *phoPQ* as reviewed elsewhere [118]. Both of these systems are two-component regulatory systems that normally turn on genes in response to a given environmental condition, limiting concentrations of divalent cations for the *phoPQ* system [119], and high concentrations of ferric iron in the case of the *pmrAB* system of *S. enterica* [120].

S. enterica are intracellular pathogens that encounter limiting divalent cation concentrations and high concentrations of antimicrobial peptides when engulfed by the host cell. Thus the bacterium senses the limiting divalent cation concentration and responds in a way that makes it more resistant to cationic peptides. Alternatively it was recently demonstrated that cationic peptides can bind directly to PhoQ and regulate their own resistance [121]. Although the precise mechanism underlying signalling by cationic peptides is not completely defined, it appears to involve interaction with a cytoplasmic-membrane-facing polyanionic domain of PhoQ. Clearly, direct regulation by a host molecule would appear to provide a distinct advantage to the bacterium in a host at a site where Mg^{2+} is not limiting and where the concentration of antimicrobial peptides is very high, such as, for example, the granules of cells or the lumen of the lung. When these systems are turned on by any of the mentioned conditions, the expression of a number of genes is modified, including those that affect susceptibility to cationic peptides that are taken up by self-promoted uptake.

To decrease susceptibility to agents taken up by self-promoted uptake, bacteria regulate gene sets, through PhoPQ or PmrAB or both, that alter their LPS in a number of important ways. The most important is reduction of the requirement for divalent cation cross-bridging of the LPS. Bacteria accomplish this by masking the negatively charged groups via the synthesis and addition of N_4-aminoarabinose and phosphoethanolamine to the Lipid A phosphates [122]. In addition to this modification, activation of the *phoPQ* system leads to increased expression of the *pagP* gene. The PagP protein catalyses the addition of an extra acyl chain to the hydrophobic portion of Lipid A [123]. The addition of this extra fatty acid increases the amphipathicity of the Lipid A, thereby making the outer leaflet more stable in the presence of bulky cationic peptide molecules. Both of these additions lead to substantially increased resistance to molecules that utilize the self-promoted uptake pathway. The PhoPQ system in *Salmonella* also regulates the production of an outer membrane protease, PgtE [124]. When this protein is expressed, it is capable of degrading certain cationic peptides that access the cell via the self-promoted uptake pathway, thus providing another way of reducing influx of the antibiotic.

Although the system described above is essentially conserved for Enterobacteriaceae, there are major differences in other organisms. In *P. aeruginosa,* for example, LPS modification genes responsible for the addition of N_4-aminoarabinose are also regulated by sub-inhibitory concentrations of cationic antimicrobial peptides, but this regulation is independent of either the PmrAB or the PhoPQ systems [125]. Recent studies have identified two different two-component regulatory systems, namely ParRS and CprRS, that participate in peptide-mediated induction of Lipid A modification in *P. aeruginosa* [126, 127]. Different peptides regulated one or the other or both pumps [127]. It is worth noting that the activation of ParRS has also been found to regulate resistance to other antimicrobial classes by modulating the expression of efflux pumps and porins [128]. This highlights the synergy that exists between different mechanisms involved in the control of antimicrobial uptake in bacterial cells. Additionally, in *Pseudomonas* the PmrAB system is regulated by the presence of limiting divalent cation concentrations, similar to PhoPQ [125], in contrast to *E. coli*, *Salmonella* and *Erwinia* where it is regulated by high concentrations of Fe^{3+}. Although the precise mechanism by which this signalling takes place is ill-defined, it would appear to provide a distinct advantage to the bacterium in the CF lung, where Mg^{2+} is not limiting and where the concentration of antimicrobial peptides is very high. Overall these systems seem to be arranged in such a way as to limit bacterial susceptibility to self-promoted uptake in environments where the bacterium is likely to encounter cationic antimicrobial peptides or limiting divalent cation concentrations. Interestingly, in another CF pathogen *B. cenocepacia*, a microorganism that exhibits a high intrinsic resistance to antimicrobial peptides, aminoarabinose modification of the Lipid A is constitutive [129, 130]. Moreover, it appears that this modification is a prerequisite for cell viability in this species.

As Gram-positive bacteria do not possess outer membranes, they utilize other mechanisms for decreasing uptake into the cell and consequently have different resistance mechanisms for cationic peptides. These include the modification of peptidoglycan or lysinylation of phosphatidylglycerol in *S. aureus* [131]. The general principle appears to be the same however, in that by decreasing the affinity of envelope components for catonic peptides, resistance is promoted.

4.3 Hydrophobic Pathway

As suggested by the name, the hydrophobic pathway involves the passage of antimicrobial compounds through the hydrophobic interior of the lipid bilayer. The hydrophobic pathway of antimicrobial uptake tends to be more important in Gram-positive bacteria than it is in Gram-negative bacteria, since slowed hydrophobic passage through the Gram-negative outer membrane can be counteracted by active efflux through RND efflux systems. In contrast, the peptidoglycan layer of Gram-positive bacteria has a diffusion limit of approximately 50 kDa and decreased uptake very seldom contributes to resistance. The hydrophobic pathway is especially important for molecules that are active on intracellular targets, but that do not access a specific transporter. In Gram-positive bacteria, this includes many commonly used antibiotics including fluoroquinolones (which can be present at low concentration in an uncharged form), and macrolides.

As mentioned above, bacterial outer membranes have somewhat diminished hydrophobic uptake through the outer membrane bilayer primarily due to the reduced fluidity of the LPS monolayer compared to the cytoplasmic membrane. However, certain mutants that affect LPS core biosynthesis, e.g. lpxA and lpxD, exhibit up to 1000-fold increased sensitivity to hydrophobic antimicrobials [132], largely by increasing uptake to an extent where it overwhelms efflux systems. In addition, a study with a series of isogenic LPS mutant strains of *E. coli* and *Salmonella enterica* demonstrated that the susceptibility of each mutant to hydrophobic antibiotics increased as the length of the LPS decreased [133]. This study further supports the role of the LPS of Gram-negative bacteria as major determinant of reduced permeation of hydrophobic antibiotics.

Although the porin-mediated pathway described above is somewhat important in mycobacteria, it is believed that many clinically relevant antibiotics used for anti-mycobacterial therapy access the cytoplasm via the hydrophobic pathway. The general rate of diffusion across the mycobacterial envelope is slower due to the high rigidity of the mycolate bilayer, but does not seem to be reinforced by a broad spectrum efflux system that pumps out hydrophobic compounds as in Gram-negatives. Consequently, rifampin, isoniazid and hydrophobic fluoroquinolones are thought to access the cell via the hydrophobic pathway [134].

4.4 Inner Membrane Transporters

A small number of antibiotics use specific membrane transporters to get across the cytoplasmic membrane, leading generally to a requirement that cells be energized for uptake. Usually this involves structural features that are conserved between the antibiotic and the normal substrate for the transporter. Thus, the antibiotic D-cycloserine is transported across the bacterial cytoplasmic membrane via the D-alanine transport system in a manner that is dependent upon the proton motive force [135]. Fosfomycin, an antibiotic that inhibits the biosynthesis of peptidoglycan, crosses the cytoplasmic membrane using the glycerol-3-phosphate or hexose phosphate transporters [135]. The antibiotic streptozotocin is also taken across the inner membrane via an active transport process involving the phosphoenol-pyruvate phosphotransferase system.

Aminoglycoside antibiotic uptake is still fairly poorly characterized. The drugs are taken up in a three-step process whereby the first step involves electrostatic LPS interactions on the surface followed by two energy-dependent phases of uptake (EDP I and EDP II) [136]. EDP I is believed to represent the initial stages of aminoglycoside passage across the cytoplasmic membrane and binding to the ribosome. It is thought that some aspect of electron transport drives the vectorial transport of aminoglycosides across the cytoplasmic membrane during EDP I, possibly the shuttling of ubiquinones across the membrane [137]. At this point the aminoglycoside triggers an event that initiates cell death and at the same time promotes an acceleration of energy dependent aminoglycoside uptake in the EDP II. Many aminoglycoside resistant mutants are altered in the energization of uptake, while a very common mechanism known as impermeability type resistance has been associated with disregulation of RND efflux pumps in *P. aeruginosa* [138].

5 Synergy

Synergy between antimicrobials is a common theme that is clinically utilized in the treatment of complicated infections. Often this is stated to be because one antibiotic assists the uptake of another. In many instances there is little direct evidence for this. However it should be noted that it has been well established that those molecules that access self-promoted uptake and act by increasing outer membrane permeability also have the capability to increase permeability to other antibiotics. Deacylated polymyxin B is the prototype for such molecules [139] and it has also been shown that cationic peptides have this property as do other polycations and divalent cation chelators [69].

6 Conclusions

It is now well established that decreased outer membrane permeability is a common mechanism leading to clinical resistance. Because in Gram-negative bacteria this often involves uptake pathways of broad significance, these mutants tend to be cross-resistant to several antibiotic classes. While we still have exploitable mechanisms (e.g. self-promoted uptake) that can be manipulated to increase uptake in poorly susceptible bacteria, a recent meta-analysis has described an increase in the rates of resistance to polymyxin B in MDR isolates of *P. aeruginosa*, *Acinetobacter baumanii* and *K. pneumoniae*, suggesting that even these drugs of last resort may become decreasingly effective as their use becomes more widespread [140]. Only through continued research will we be able to overcome these setbacks and effectively exploit the uptake systems described in this review.

Acknowledgements Financial assistance from the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research is gratefully acknowledged. REWH holds a Canada Research Chair.

References

1. Spellberg B, Powers JH, Brass EP, Miller LG, Edwards Jr JE. Trends in antimicrobial drug development: implications for the future. Clin Infect Dis. 2004;38(9):1279–86.
2. Theuretzbacher U, Toney JH. Nature's clarion call of antibacterial resistance: are we listening? Curr Opin Investig Drugs. 2006;7(2): 158–66.
3. Hancock REW. The end of an era? Nat Rev Drug Discov. 2006;6(28):28.
4. Brazas MD, Hancock REW. Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance. Drug Discov Today. 2005;10(18):1245–52.
5. El'garch F, Jeannot K, Hocquet D, Llanes-Barakat C, Plesiat P. Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob Agents Chemother. 2007;51(3):1016–21.
6. Fernández L, Breidenstein EB, Hancock REW. Creeping baselines and adaptive resistance to antibiotics. Drug Resist Updat. 2011;14(1):1–21.
7. Hancock REW, Egli C, Karunaratne N. Molecular organization and structural role of outer membrane macromolecules. In: Ghuysen JM, Hakenbeck R, editors. Bacterial cell envelope. Amsterdam: Elsevier Science Publishers BV; 1994. p. 263–79.
8. Kadner RJ. Cytoplasmic membrane. In: Neidhardt FC, editor. *Escherichia coli* and *Salmonella*. 2nd ed. Washington, DC: ASM Press; 1996. p. 58–87.
9. Cronan JE. Bacterial membrane lipids: where do we stand? Annu Rev Microbiol. 2003;57:203–24.
10. Hobot JA, Carlemalm E, Villiger W, Kellenberger E. Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J Bacteriol. 1984; 160(1):143–52.
11. Leduc M, Frehel C, Siegel E, Van Heijenoort J. Multilayered distribution of peptidoglycan in the periplasmic space of *Escherichia coli*. J Gen Microbiol. 1989;135(Pt 5):1243–54.
12. Dubochet J, McDowall AW, Menge B, Schmid EN, Lickfeld KG. Electron microscopy of frozen-hydrated bacteria. J Bacteriol. 1983;155(1):381–90.
13. Lam JS, Lam MY, MacDonald LA, Hancock RE. Visualization of *Pseudomonas aeruginosa* O antigens by using a protein A-dextran-colloidal gold conjugate with both immunoglobulin G and immunoglobulin M monoclonal antibodies. J Bacteriol. 1987;169(8): 3531–8.
14. Oliver DB. Periplasm. In: Neidhardt FC, editor. *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. Washington, DC: ASM Press; 1996. p. 88–103.
15. Van Wielink JE, Duine JA. How big is the periplasmic space? Trends Biochem Sci. 1990;15(4):136–7.
16. Brass JM, Higgins CF, Foley M, Rugman PA, Birmingham J, Garland PB. Lateral diffusion of proteins in the periplasm of *Escherichia coli*. J Bacteriol. 1986;165(3):787–95.
17. Weidel W, Pelzer H. Bagshaped macromolecules—a new outlook on bacterial cell walls. Adv Enzymol Relat Areas Mol Biol. 1964;26:193–232.
18. Takade A, Umeda A, Matsuoka M, Yoshida S, Nakamura M, Amako K. Comparative studies of the cell structures of *Mycobacterium leprae* and *M. tuberculosis* using the electron microscopy freeze-substitution technique. Microbiol Immunol. 2003; 47(4):265–70.
19. Paul TR, Beveridge TJ. Reevaluation of envelope profiles and cytoplasmic ultrastructure of mycobacteria processed by conventional embedding and freeze-substitution protocols. J Bacteriol. 1992;174(20):6508–17.
20. Labischinski H, Goodell EW, Goodell A, Hochberg ML. Direct proof of a "more-than-single-layered" peptidoglycan architecture of *Escherichia coli* W7: a neutron small-angle scattering study. J Bacteriol. 1991;173(2):751–6.
21. Yao X, Jericho M, Pink D, Beveridge T. Thickness and elasticity of gram-negative murein sacculi measured by atomic force microscopy. J Bacteriol. 1999;181(22):6865–75.
22. Dmitriev BA, Ehlers S, Rietschel ET. Layered murein revisited: a fundamentally new concept of bacterial cell wall structure, biogenesis and function. Med Microbiol Immunol. 1999;187(3): 173–81.
23. van Heijenoort J, Gutmann L. Correlation between the structure of the bacterial peptidoglycan monomer unit, the specificity of transpeptidation, and susceptibility to beta-lactams. Proc Natl Acad Sci U S A. 2000;97(10):5028–30.
24. Glauner B, Holtje JV, Schwarz U. The composition of the murein of *Escherichia coli*. J Biol Chem. 1988;263(21):10088–95.
25. Gmeiner J, Essig P, Martin HH. Characterization of minor fragments after digestion of Escherichia coli murein with endo- N, O-diacetylmuramidase from Chalaropsis, and determination of glycan chain length. FEBS Lett. 1982;138(1):109–12.
26. Labischinski H, Barnickel G, Naumann D, Keller P. Conformational and topological aspects of the three-dimensional architecture of bacterial peptidoglycan. Ann Inst Pasteur Microbiol. 1985;136A(1): 45–50.
27. Naumann D, Barnickel G, Bradaczek H, Labischinski H, Giesbrecht P. Infrared spectroscopy, a tool for probing bacterial peptidoglycan. Potentialities of infrared spectroscopy for cell wall analytical studies and rejection of models based on crystalline chitin. Eur J Biochem. 1982;125(3):505–15.
28. Raetz CR. Biochemistry of endotoxins. Annu Rev Biochem. 1990;59:129–70.
29. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem. 2002;71:635–700.
30. Preston A, Mandrell RE, Gibson BW, Apicella MA. The lipooligosaccharides of pathogenic Gram-negative bacteria. Crit Rev Microbiol. 1996;22(3):139–80.
31. Hancock REW, Brinkman FS. Function of pseudomonas porins in uptake and efflux. Annu Rev Microbiol. 2002;56:17–38.
32. Koronakis V. TolC—the bacterial exit duct for proteins and drugs. FEBS Lett. 2003;555(1):66–71.
33. Draper P. The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci. 1998;3:D1253–61.
34. Liu J, Barry III CE, Besra GS, Nikaido H. Mycolic acid structure determines the fluidity of the mycobacterial cell wall. J Biol Chem. 1996;271(47):29545–51.
35. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem. 1995;64:29–63.
36. Faller M, Niederweis M, Schulz GE. The structure of a mycobacterial outer-membrane channel. Science. 2004;303(5661): 1189–92.
37. Niederweis M. Mycobacterial porins—new channel proteins in unique outer membranes. Mol Microbiol. 2003;49(5):1167–77.
38. Jann B, Jann K. Capsules of *Escherichia coli*. In: Sussman M, editor. Mechanisms of virulence. Cambridge: Cambridge University Press; 1997.
39. Whitfield C, Roberts IS. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol. 1999;31(5):1307–19.
40. Costerton JW, Irvin RT, Cheng KJ. The bacterial glycocalyx in nature and disease. Annu Rev Microbiol. 1981;35:299–324.
41. Ophir T, Gutnick DL. A role for exopolysaccharides required in the protection of microorganisms from dessication. Appl Environ Microbiol. 1994;60:740–5.

42. Berry A, DeVault JD, Chakrabarty AM. High osmolarity is a signal for enhanced algD transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. J Bacteriol. 1989;171(5): 2312–7.
43. Pickard D, Li J, Roberts M, et al. Characterization of defined *ompR* mutants of *Salmonella* typhi: *ompR* is involved in the regulation of Vi polysaccharide expression. Infect Immun. 1994;62(9): 3984–93.
44. Sauer K. The genomics and proteomics of biofilm formation. Genome Biol. 2003;4(6):219.
45. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2(2):95–108.
46. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol. 2002;292(2):107–13.
47. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002; 15(2):167–93.
48. Govan J. Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis. In: Griffiths E, Donachie W, Stephen J, editors. Bacterial infections of the respiratory and gastrointestinal mucosae. Oxford: IRL Press; 1988. p. 67–96.
49. Michalek MT, Mold C, Bremer EG. Inhibition of the alternative pathway of human complement by structural analogues of sialic acid. J Immunol. 1988;140(5):1588–94.
50. Platt MW, Correa Jr N, Mold C. Growth of group B streptococci in human serum leads to increased cell surface sialic acid and decreased activation of the alternative complement pathway. Can J Microbiol. 1994;40(2):99–105.
51. Edwards MS, Kasper DL, Jennings HJ, Baker CJ, Nicholson-Weller A. Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. J Immunol. 1982;128(3):1278–83.
52. Marques MB, Kasper DL, Pangburn MK, Wessels MR. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect Immun. 1992; 60(10):3986–93.
53. Bortolussi R, Ferrieri P, Bjorksten B, Quie PG. Capsular K1 polysaccharide of *Escherichia coli*: relationship to virulence in newborn rats and resistance to phagocytosis. Infect Immun. 1979; 25(1):293–8.
54. Nosanchuk JD, Casadevall A. Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. Infect Immun. 1997; 65(5):1836–41.
55. Wibawan IW, Lammler C. Influence of capsular neuraminic acid on properties of streptococci of serological group B. J Gen Microbiol. 1991;137(Pt 12):2721–5.
56. Mandrell RE, McLaughlin R, Aba Kwaik Y, et al. Lipooligosaccharides (LOS) of some *Haemophilus* species mimic human glycosphingolipids, and some LOS are sialylated. Infect Immun. 1992;60(4):1322–8.
57. Mandrell RE, Griffiss JM, Macher BA. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. J Exp Med. 1988; 168(1):107–26.
58. Vann WF, Schmidt MA, Jann B, Jann K. The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective *Escherichia coli* 010:K5:H4. A polymer similar to desulfoheparin. Eur J Biochem. 1981;116(2):359–64.
59. Campos MA, Vargas MA, Regueiro V, Llompart CM, Albertí S, Bengoechea JA. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun. 2004;72(12): 7107–14.
60. Llobet E, Tomás JM, Bengoechea JA. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology. 2008;154(Pt 12):3877–86.
61. Pavkov-Keller T, Howorka S, Keller W. The structure of bacterial S-layer proteins. Prog Mol Biol Transl Sci. 2011;103:73–130.
62. Fagan RP, Fairweather NF. Biogenesis and functions of bacterial S-layers. Nat Rev Microbiol. 2014;12(3):211–22.
63. Lau JH, Nomellini JF, Smit J. Analysis of high-level S-layer protein secretion in *Caulobacter crescentus*. Can J Microbiol. 2010;56:501–14.
64. Engelhardt H. Are S-layers exoskeletons? The basic function of protein surface layers revisited. J Struct Biol. 2007;160(2): 115–24.
65. de la Fuente-Núñez C, Mertens J, Smit J, Hancock RE. The bacterial surface layer provides protection against antimicrobial peptides. Appl Environ Microbiol. 2012;78(15):5452–6.
66. Tamber S, Hancock REW. On the mechanism of solute uptake in *Pseudomonas*. Front Biosci. 2003;8:s472–83.
67. Huang H, Hancock REW. Genetic definition of the substrate selectivity of outer membrane porin protein OprD of *Pseudomonas aeruginosa*. J Bacteriol. 1993;175(24):7793–800.
68. Vaara M. Increased outer membrane resistance to ethylenediaminetetraacetate and cations in novel lipid A mutants. J Bacteriol. 1981;148(2):426–34.
69. Hancock REW, Wong PG. Compounds which increase the permeability of the *Pseudomonas aeruginosa* outer membrane. Antimicrob Agents Chemother. 1984;26(1):48–52.
70. Stephan J, Mailaender C, Etienne G, Daffe M, Niederweis M. Multidrug resistance of a porin deletion mutant of *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 2004;48(11):4163–70.
71. Stephan J, Bender J, Wolschendorf F, et al. The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. Mol Microbiol. 2005;58(3):714–30.
72. Paulsen IT. Multidrug efflux pumps and resistance: regulation and evolution. Curr Opin Microbiol. 2003;6(5):446–51.
73. Piddock LJ. Multidrug-resistance efflux pumps—not just for resistance. Nat Rev Microbiol. 2006;4(8):629–36.
74. Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J Bacteriol. 1990;172(12):6942–9.
75. Poole K. Multidrug resistance in Gram-negative bacteria. Curr Opin Microbiol. 2001;4(5):500–8.
76. Poole K. Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect. 2004;10(1):12–26.
77. Lamarche MG, Déziel E. MexEF-OprN efflux pump exports the Pseudomonas quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). PLoS ONE. 2011;6(9), e24310.
78. Fetar H, Gilmour C, Klinoski R, Daigle DM, Dean CR, Poole K. mexEF-oprN multidrug efflux operon of Pseudomonas aeruginosa: regulation by the MexT activator in response to nitrosative stress and chloramphenicol. Antimicrob Agents Chemother. 2011;55(2):508–14.
79. Fraud S, Campigotto AJ, Chen Z, Poole K. MexCD-OprJ multidrug efflux system of Pseudomonas aeruginosa: involvement in chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response sigma factor. Antimicrob Agents Chemother. 2011;55(2):508–14.
80. Fraud S, Poole K. Oxidative stress induction of the MexXY multidrug efflux genes and promotion of aminoglycoside resistance development in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2011;55(3):1068–74.
81. Abdelraouf K, Kabbara S, Ledesma KR, Poole K, Tam VH. Effect of multidrug resistance-conferring mutations on the fitness and

virulence of Pseudomonas aeruginosa. J Antimicrob Chemother. 2011;66(6):1311–7.
82. Sánchez P, Linares JF, Ruiz-Díez B, Campanario E, Navas A, Baquero F, Martínez JL. Fitness of in vitro selected Pseudomonas aeruginosa nalB and nfxB multidrug resistant mutants. J Antimicrob Chemother. 2002;50(5):657–64.
83. Fernández L, Hancock REW. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev. 2012;25(4):661–81.
84. Ramos JL, Martinez-Bueno M, Molina-Henares AJ, et al. The TetR family of transcriptional repressors. Microbiol Mol Biol Rev. 2005;69(2):326–56.
85. Schweizer HP. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res. 2003;2(1):48–62.
86. Sánchez-Romero MA, Casadesús J. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. Proc Natl Acad Sci U S A. 2014;111(1):355–60.
87. Cowan SW, Garavito RM, Jansonius JN, et al. The structure of OmpF porin in a tetragonal crystal form. Structure. 1995;3(10): 1041–50.
88. Cowan SW, Schirmer T, Rummel G, et al. Crystal structures explain functional properties of two *Escherichia coli* porins. Nature. 1992;358(6389):727–33.
89. Kreusch A, Neubuser A, Schiltz E, Weckesser J, Schulz GE. Structure of the membrane channel porin from *Rhodopseudomonas blastica* at 2.0 A resolution. Protein Sci. 1994;3(1):58–63.
90. Hernandez-Alles S, Conejo M, Pascual A, Tomas JM, Benedi VJ, Martinez-Martinez L. Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. J Antimicrob Chemother. 2000;46(2):273–7.
91. Hernandez-Alles S, Benedi VJ, Martinez-Martinez L, et al. Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. Antimicrob Agents Chemother. 1999;43(4):937–9.
92. Pragai Z, Nagy E. In-vitro selection of porin-deficient mutants of two strains of *Klebsiella pneumoniae* with reduced susceptibilities to meropenem, but not to imipenem. J Antimicrob Chemother. 1998;42(6):821–4.
93. Schirmer T, Keller TA, Wang YF, Rosenbusch JP. Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. Science. 1995;267(5197):512–4.
94. Van Gelder P, Dumas F, Bartoldus I, et al. Sugar transport through maltoporin of *Escherichia coli*: role of the greasy slide. J Bacteriol. 2002;184(11):2994–9.
95. Moraes TF, Bains M, Hancock RE, Strynadka NC. An arginine ladder in OprP mediates phosphate-specific transfer across the outer membrane. Nat Struct Mol Biol. 2007;14(1):85–7.
96. Fsihi H, Kottwitz B, Bremer E. Single amino acid substitutions affecting the substrate specificity of the *Escherichia coli* K-12 nucleoside-specific Tsx channel. J Biol Chem. 1993;268(23): 17495–503.
97. Benz R, Schmid A, Maier C, Bremer E. Characterization of the nucleoside-binding site inside the Tsx channel of *Escherichia coli* outer membrane. Reconstitution experiments with lipid bilayer membranes. Eur J Biochem. 1988;176(3):699–705.
98. Trias J, Nikaido H. Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. J Biol Chem. 1990;265(26):15680–4.
99. Trias J, Nikaido H. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1990;34(1):52–7.
100. Tamber S, Maier E, Benz R, Hancock REW. Characterization of OpdH, a *Pseudomonas aeruginosa* porin involved in the uptake of tricarboxylates. J Bacteriol. 2007;189(3):929–39.
101. Koebnik R, Locher KP, Van Gelder P. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol. 2000;37(2):239–53.
102. Ferguson AD, Hofmann E, Coulton JW, Diederichs K, Welte W. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science. 1998;282(5397):2215–20.
103. Locher KP, Rees B, Koebnik R, et al. Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell. 1998;95(6):771–8.
104. Ferguson AD, Kodding J, Walker G, et al. Active transport of an antibiotic rifamycin derivative by the outer-membrane protein FhuA. Structure (Camb). 2001;9(8):707–16.
105. Ferguson AD, Braun V, Fiedler HP, Coulton JW, Diederichs K, Welte W. Crystal structure of the antibiotic albomycin in complex with the outer membrane transporter FhuA. Protein Sci. 2000;9(5):956–63.
106. Mailaender C, Reiling N, Engelhardt H, Bossmann S, Ehlers S, Niederweis M. The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. Microbiology. 2004;150(Pt 4): 853–64.
107. Raynaud C, Laneelle MA, Senaratne RH, Draper P, Laneelle G, Daffe M. Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity. Microbiology. 1999;145(Pt 6):1359–67.
108. Jarlier V, Nikaido H. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J Bacteriol. 1990;172(3):1418–23.
109. Moore RA, Bates NC, Hancock REW. Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. Antimicrob Agents Chemother. 1986;29(3):496–500.
110. Bina J, Alm RA, Uria-Nickelsen M, Thoimas SR, Trust TJ, Hancock REW. *Helicobacter pylori* uptake and efflux: basis for intrinsic susceptibility to antibiotics in vitro. Antimicrob Agents Chemother. 2000;44(2):248–54.
111. Macfarlane EL, Kwasnicka A, Ochs MM, Hancock REW. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol Microbiol. 1999;34(2):305–16.
112. Peterson AA, Hancock REW, McGroarty EJ. Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. J Bacteriol. 1985;164(3):1256–61.
113. Peterson AA, Fesik SW, McGroarty EJ. Decreased binding of antibiotics to lipopolysaccharides from polymyxin-resistant strains of *Escherichia coli* and *Salmonella* typhimurium. Antimicrob Agents Chemother. 1987;31(2):230–7.
114. Skurnik M, Venho R, Bengoechea JA, Moriyon I. The lipopolysaccharide outer core of *Yersinia enterocolitica* serotype O:3 is required for virulence and plays a role in outer membrane integrity. Mol Microbiol. 1999;31(5):1443–62.
115. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 2006;24(12):1551–7.
116. Gilleland Jr HE, Murray RG. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. J Bacteriol. 1976;125(1):267–81.
117. Lounatmaa K, Nanninga N. Effect of polymyxin on the outer membrane of *Salmonella typhimurium*: freeze-fracture studies. J Bacteriol. 1976;128(2):665–7.
118. Kato A, Latifi T, Groisman EA. Closing the loop: the PmrA/PmrB two-component system negatively controls expression of its post-transcriptional activator PmrD. Proc Natl Acad Sci U S A. 2003;100(8):4706–11.
119. Garcia Vescovi E, Soncini FC, Groisman EA. Mg2+ as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell. 1996;84(1):165–74.

120. Wosten MM, Kox LF, Chamnongpol S, Soncini FC, Groisman EA. A signal transduction system that responds to extracellular iron. Cell. 2000;103(1):113–25.
121. Bader MW, Sanowar S, Daley ME, et al. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell. 2005;122(3): 461–72.
122. Nummila K, Kilpelainen I, Zahringer U, Vaara M, Helander IM. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. Mol Microbiol. 1995;16(2):271–8.
123. Guo L, Lim KB, Poduje CM, et al. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell. 1998;95(2):189–98.
124. Guina T, Yi EC, Wang H, Hackett M, Miller SI. A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar Typhimurium promotes resistance to alpha-helical antimicrobial peptides. J Bacteriol. 2000;182(14):4077–86.
125. McPhee JB, Lewenza S, Hancock REW. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol Microbiol. 2003;50(1):205–17.
126. Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock REW. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in Pseudomonas aeruginosa is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother. 2010;54(8):3372–82.
127. Fernández L, Jenssen H, Bains M, Wiegand I, Gooderham WJ, Hancock REW. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in Pseudomonas aeruginosa independently of ParRS. Antimicrob Agents Chemother. 2012;56(12):6212–22.
128. Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2011;55(3):1211–21.
129. Hamad MA, Di Lorenzo F, Molinaro A, Valvano MA. Aminoarabinose is essential for lipopolysaccharide export and intrinsic antimicrobial peptide resistance in Burkholderia cenocepacia. Mol Microbiol. 2012;85(5):962–74.
130. Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, Campopiano DJ, et al. A putative gene cluster for aminoarabinose biosynthesis is essential for Burkholderia cenocepacia viability. J Bacteriol. 2007;189:3639–44.
131. Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol. 2006;4(7):529–36.
132. Vaara M, Nurminen M. Outer membrane permeability barrier in *Escherichia coli* mutants that are defective in the late acyltransferases of lipid A biosynthesis. Antimicrob Agents Chemother. 1999;43(6):1459–62.
133. Snyder DS, McIntosh TJ. The lipopolysaccharide barrier: correlation of antibiotic susceptibility with antibiotic permeability and fluorescent probe binding kinetics. Biochemistry. 2000;39(38): 11777–87.
134. Lambert PA. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. Symp Ser Soc Appl Microbiol. 2002;31:46S–54.
135. Chopra I, Ball P. Transport of antibiotics into bacteria. Adv Microb Physiol. 1982;23:183–240.
136. Hancock RE. Aminoglycoside uptake and mode of action-with special reference to streptomycin and gentamicin. II. Effects of aminoglycosides on cells. J Antimicrob Chemother. 1981;8(6): 429–45.
137. Bryan LE, Kwan S. Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of streptomycin and gentamicin. Antimicrob Agents Chemother. 1983;23(6):835–45.
138. Westbrock-Wadman S, Sherman DR, Hickey MJ, et al. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrob Agents Chemother. 1999;43(12):2975–83.
139. Vaara M, Vaara T. Sensitization of Gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide. Nature. 1983;303(5917):526–8.
140. Falagas ME, Bliziotis IA. Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? Int J Antimicrob Agents; 2007.

Active Efflux as a Mechanism of Resistance to Antimicrobial Drugs

10

Xian-Zhi Li

1 Introduction

Active drug extrusion mediated by efflux transporters is a phenomenon widely observed in both prokaryotic and eukaryotic cells including bacteria, fungi, parasites, and mammalian cells. This energy-dependent active process, as a cause of resistance to antimicrobial drugs, makes a great contribution to intrinsic and acquired resistance in bacteria, fungi, and parasites—working against a broad range of antimicrobial agents and can further interplay with other resistance mechanisms. Initially revealed in the 1970s in mammalian cancer cells with P-glycoprotein for its role in resistance to diverse cytotoxic agents [1], drug efflux was subsequently (in 1970s and 1980s) identified in bacteria (i.e., *Escherichia coli* for tetracycline-specific resistance) [2, 3], in fungi (e.g., for resistance to fenarimol in *Aspergillus nidulans* and resistance to benomyl and methotrexate in *Candida albicans*) [4–6], and in parasites (*Plasmodium falciparum* for chloroquine resistance) [7, 8]. To date, a growing list of drug efflux pumps has been described, and these membrane-associated efflux machineries can provide either multidrug or drug-specific resistance. Drug transporters also function beyond resistance, such as those involved in stress response and virulence. There is an ever-growing understanding of drug transporters with respect to their classification, structure, transport mechanisms, regulation, and inhibition. Indeed, the importance of efflux transporters as a mechanism of drug resistance of clinical significance cannot be overestimated since this mechanism mediates both intrinsic and acquire resistance and poses a major challenge for chemotherapy and drug development [9–12].

This chapter provides an overview of active efflux mechanisms as an important determinant of clinically relevant drug resistance in microbes and parasites with a focus on bacterial multidrug resistance (MDR) efflux pumps of the resistance-nodulation-cell division (RND) superfamily in Gram-negative bacteria. More examples of efflux-mediated drug resistance can be found in various chapters of this book dealing with the pathogen-specific resistance. In addition, drug efflux pumps also possess functions that go beyond resistance and may be related to their physiological role for survival in diverse environments in regard to stress response and pathogenicity [10, 11]. The development of clinically suitable efflux pump inhibitors and the pump-circumventing agents continues to be a challenging task, but will likely play an important role in combating efflux-mediated antimicrobial resistance. Furthermore, the clinical significance of drug transporters highlights the importance of prudent antimicrobial use for minimizing the emergence and spread of resistance.

2 Classification and Transport Mechanisms of Drug Resistance Transporters

Transport (uptake and efflux) systems with various functions exist in all domains of living cells and consist of integral membrane proteins generally encoded by a chromosome. The wide presence of transporters has been confirmed by the genomic data available to date. Numerous of these transporters have been involved in the active export of drugs and/or other toxic molecules including clinically used chemotherapeutic agents such as antibacterial, antifungal, and antiparasitic agents (these are together referred as antimicrobial agents here). Several examined bacterial species indicate that the number of MDR pumps appear to be quite proportional to the total transporter numbers and genome sizes [13].

Through the functional and phylogenic classification of the membrane transport proteins, the Milton Saier group has established a transporter classification system database which groups transporters (primarily via the protein sequences) into the class, subclass, family, and subfamily (*http://www.tcdb.org*) [14, 15]. There are currently over 800

X.-Z. Li, M.D., Ph.D. (✉)
Human Safety Division, Veterinary Drugs Directorate, Health Products and Food Branch, Health Canada, Ottawa, ON, Canada
e-mail: xianzhi.li@hc-sc.gc.ca

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_10

transporter families including various superfamilies. Efflux transporters involved in antimicrobial resistance largely occur in five superfamilies: (1) ATP-Binding Cassette (ABC) Superfamily; (2) Drug/Metabolite Transporter (DMT) Superfamily (to which the Small Multidrug Resistance [SMR] family belongs); (3) Major Facilitator Superfamily (MFS); (4) Multidrug/Oligosaccharidyle-lipid/Polysaccharide (MOP) export Superfamily (to which the drug resistance-related Multidrug And Toxic compound Extrusion [MATE] family belongs); and (5) RND Superfamily. A recent study reports a new Proteobacterial Antimicrobial Compound Efflux (PACE) family based on the homologs of the *Acinetobacter* chlorhexidine efflux (AceI) protein; many homologs of this PACE family provide resistance to biocides when expressed from a plasmid in *E. coli* [16]. Except for the primary active transporters of the ABC superfamily which couple ATP binding and hydrolysis with substrate translocation, the transporters of the other four superfamilies and the PACE family are secondary transporters and require a proton-motive force (e.g., they are H^+ or Na^+/drug antiporters).

Drug efflux pumps within these superfamilies or families can also be categorized into either single-component (singlet) efflux pumps or multicomponent efflux pumps on the basis of the composition of the transporters (Fig. 10.1). Such categorization is particularly useful for the consideration of pump effectiveness in conferring resistance in bacteria [12, 17], where Gram-positive bacteria (including mycobacteria) and Gram-negative bacteria each show unique structures of their cell envelope barrier [18, 19]. Singlet transporters, which include the ABC, MFS, MATE, and SMR transporters, occur in all species and function via the pumps themselves (with either homo or heteromeric subunits). Multicomponent transporters typically operate in Gram-negative bacteria through complex efflux machinery that often include the pump, accessory adaptor proteins (also called membrane fusion proteins), and outer membrane channel proteins. This tripartite structure provides an effective mechanism to directly pump substrates out of the cell (i.e., across the outer membrane permeability barrier). The multicomponent transporters are typically seen with the RND pumps and are also observed with certain ABC and MFS pumps [12]. The structural and mechanical characteristics of the five aforementioned drug resistance superfamilies are described below.

2.1 ABC Superfamily

The transporters in the ABC superfamily comprise both importers and exporters and are involved in the translocation across membranes of diverse substrates including amino acids, proteins, polysaccharides, sugars, drugs, and ion complexes. A number of ABC exporters are involved in drug resistance in bacteria, fungi, parasites, and cancer cells. P-glycoprotein (Pgp, or MDR1) is the prototypical ABC drug exporter in eukaryotes involved in the efflux of a broad range of cytotoxic agents [1]. The bacterial ABC transporter, LmrA of *Lactococcus lactis*, can functionally complement Pgp, showing the conservation of MDR pumps from bacteria to humans [20]. The crystal structures of a dozen ABC transporters have been widely available, and thus have provided insight on the transport mechanisms including the substrate promiscuity of many drug transporters [21–23]. ABC transporters consist of two integral transmembrane domains (TMDs) and two cytosolic nucleotide-binding domains (NBDs) (Fig. 10.1). The number and arrangement of the TMDs and NBDs may vary based on the specific transporters in a given species. The TBDs confer drug substrate specificity. ABC exporters in bacteria have a TMD fused with an NBD to form a half-transporter and the two homo- or hetero-half-transporters then dimerize to produce a functional full transporter. For example, the staphylococcal Sav1866 ABC drug exporter possesses the homodimeric protein with 12 transmembrane helices. The ATP-bound state displays an outward-facing conformation with the two NBDs in close contact and the two TBDs forming a central cavity. The latter is considered as the drug translocation pathway and is shielded from the cytoplasm, but exposed to the extracellular space [21].

Several models were proposed to elucidate the transport mechanisms of ABC pumps. The switch model suggests the dimerization of the NBDs upon binding of two ATP molecules and disassociation upon ATP hydrolysis [24]. The constant contact model shows that the NBDs do not fully dissociate and ATP hydrolysis occurs alternately at one of the active sites [25]. A newer model termed the "reciprocating twin-channel" model considers the characteristics of the earlier models and establishes the sequential process of ATB binding, hydrolysis, translocation, and release in one of the two functionally separate substrate translocation pathways. These pathways operate in a reciprocating manner in the TMDs, coupled to an alternating cycle of ATP hydrolysis in the NBDs [26]. The clinical significance of ABC drug exporters is dependent on the species and is considered to be relatively more important in eukaryotic cells [11, 27] as further described in later sections.

2.2 DMT Superfamily

This superfamily includes more than 30 recognized families with a multitude of functions in both prokaryotic and eukaryotic cells. Each of these families displays a distinct topology of varied (e.g., 4, 5, or 8–10) putative transmembrane segments (TMSs) [14, 28]. Of this superfamily, the most characterized is the primordial SMR family, which typically occurs in prokaryotes and can be encoded by either chromosome or plasmids.

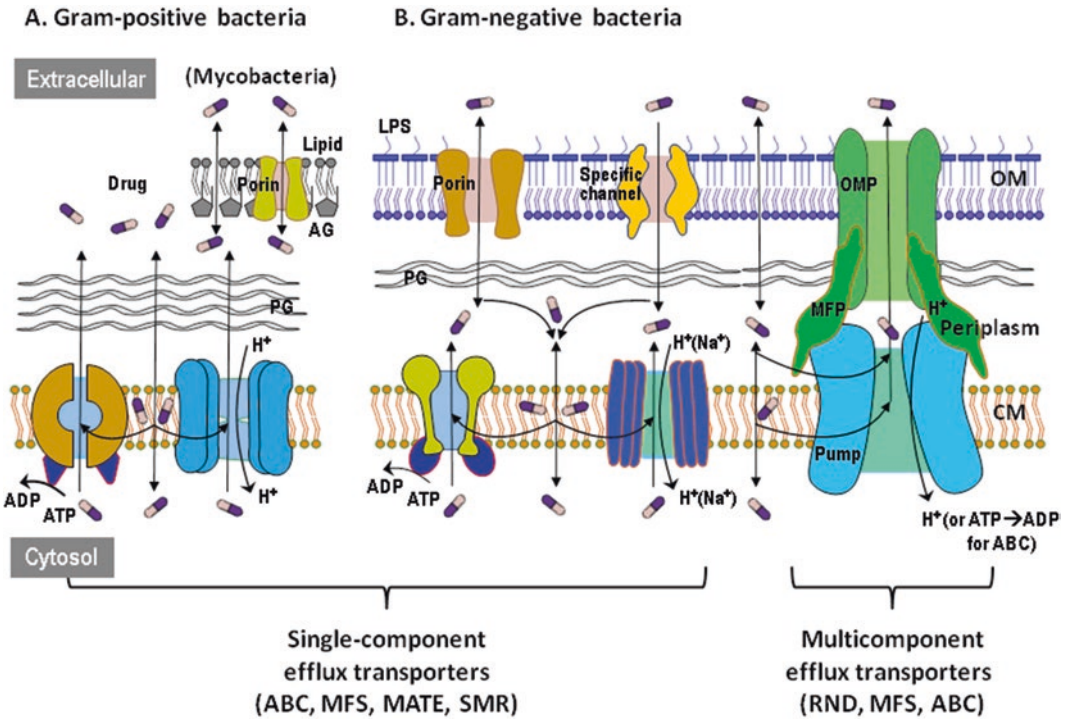


Fig. 10.1 Schematic representation of drug influx and efflux across the cell envelope barrier in bacteria. **(a)** In Gram-positive bacteria, the drugs enter the cell via the penetration of the peptidoglycan (PG) and the cytoplasmic membrane (CM) (in mycobacteria, the drug also needs to pass via diffusion an additional outer barrier comprising porins and mycolyl arabinogalactan [AG] that forms a barrier complex with PG). The efflux pumps belonging either ABC transporters or proton-dependent secondary transporters are single-component exporters that may incorporate the drug from the CM and/or the cytosol. **(b)** In Gram-negative bacteria, the unique asymmetric lipopolysaccharide (LPS)-containing outer membrane (OM) functions as a strong permeability barrier. The drug may enter the periplasm via three possible pathways, porin (e.g., small hydrophilic molecules), specific channel (e.g., carbapenem antibiotics), or lipid bilayer (e.g., lipophilic molecules). The efflux pumps can be either the single-component pumps or multicomponent pumps that take the drug from the CM or cytosol. However, available studies support that multicomponent RND pumps incorporate drug from the periplasm or CM (not from the cytosol) and directly extrude the substrate out of the cell (see also Fig. 10.2). A tripartite efflux complex typically contains a pump, OM channel protein (OMP) and an accessory membrane fusion protein (MFP). A single-component pump may function with porins or other types of protein channels to make the efflux process effective. The competition between the influx and efflux processes ultimately determines the steady state of drug molecules in the cell

The encoding genes may also be linked to integrons, a feature that facilitates the spread of SMR-encoding genes. The SMR efflux proteins consist of homodimeric (e.g., EmrE of *E. coli* [29]) or heterodimeric (e.g., the paired EbrAB of *Bacillus subtilis* [30]) subunits of about 100–120 amino acid residues and 4 TMSs [10]. Representative examples of SMR exporters include the Smr protein of *Staphylococcus aureus* [31] and the EmrE protein of *E. coli* [29]. The substrate profile of SMR is generally cation-specific, including lipophilic, monocationic or dicationic antiseptics and disinfectants such as tetraphenylphosphonium and paraquat, and thus the underlined transport mechanism typically involves the exchange of incoming H^+ with the extrusion of cationic substrates. Interestingly, an outer membrane protein termed OmpW is reported to participate in the efflux of cationic agents by EmrE [32], but OmpW appears to be a narrow channel protein [12], warranting further investigation. It is important to note that EmrE is among the limited drug exporters whose inactivation confers *E. coli* hypersusceptible to multiple agents in the presence of the predominant RND-type AcrAB pump. When expressed from a plasmid, an SMR pump of *E. coli*, YdgEF (MdtIJ), also provides resistance to an anionic agent, sodium dodecyl sulfate [33]. The clinical significance of SMR pumps in intrinsic drug resistance has been so far demonstrated not only in *E. coli* (with EmrE) but also in *Klebsiella pneumoniae* (with KpnEF) [34], *Acinetobacter baumannii* (with AbeS) [35], and *Pseudomonas aeruginosa* [36], because their inactivation confer the increased susceptibility to several clinically relevant antimicrobial agents [12]. Additionally, the chloramphenicol-sensitivity protein (RarD) family with 10 TMSs is also included in this superfamily [28]. Another member of the DMT family with putative 10 TMSs is the chloroquine resistance transporter in *P. falciparum* (PfCRT) whose mutation (e.g., Lys67Thr) is widely observed in chloroquine-resistant *P. falciparum* strains [37]; the resistant form of PfCRT is able to transport chloroquine [38].

2.3 MFS

So far, the MFS has the largest number of families (over 10,000 sequenced members within 85 families as of 2016) including uniporters, symporters, and antiporters involved in the translocation of diverse substrates (http://www.tcdb.org) [39, 40]. The MFS transporters are ubiquitously distributed in all domains of living organisms. Those exporters involved in conferring drug resistance belong to a limited number of families and comprise 12- or 14-TMSs [39]. In bacteria and fungi, MFS exporters mostly appear to function as single-component pumps (represented by the NorA pump of *S. aureus*, EmrD and MdfA pumps of *E. coli*, AfuMdr3p of *Aspergillus fumigatus,* and CaMdr1p of *Candida albicans*; see below their substrate profiles) [10, 11]. Yet, in Gram-negative bacteria, certain MFS pumps function as multicomponent pumps (such as those represented by the EmrAB-TolC and EmrKY-TolC efflux systems of *E. coli*) that contain not only cytoplasmic membrane transporters (e.g., EmrB and EmrY), but also periplasmic adaptor proteins (e.g., EmrA and EmrK) and outer membrane channel proteins (e.g., TolC) (Fig. 10.1) [10]. These aforementioned pumps are encoded by the chromosome. Other pumps such as the firstly described bacterial, single-component Tet pumps are usually encoded by plasmids of various bacterial hosts including *S. aureus* and *E. coli* [3, 41]. The substrate specificity of MFS drug exporters are often drug-specific or at least have a narrower substrate profile in comparison with that of the RND exporters.

2.4 MOP Superfamily

This superfamily currently contains a dozen of distantly related exporter families (*http://www.tcdb.org*), which are distributed in both prokaryotes and eukaryotes and are functionally involved in the export of drugs, polysaccharides, oligosaccharidyl-lipids, lipopolysaccharide precursors, and virulence proteins [42]. One of the families, the MATE family, widespread in bacteria and represented by the well-characterized NorM of *Vibrio parahaemolyticus*, displays 12 TMSs and functions as Na^+ (or H^+)-drug antiporters [43, 44]. The structure of NorM from *Vibrio cholerae* shows an outward-facing conformation with two portals open to the outer leaflet of the cytoplasmic membrane [45]. Cation-binding sites were identified in NorM, consistent with the role for MATE pumps in extruding cationic dyes, aminoglycosides, and fluoroquinolones. MepA of *S. aureus* also confers resistance to monovalent and divalent biocides, fluoroquinolones, and tigecycline [46, 47]. To date, at least two dozens of bacterial MATE pumps have been characterized in terms of their substrate profiles [9, 10]. Yet, most of these pumps have been studied through plasmid-based overexpression in a hypersusceptible *E. coli* host, and thus their clinical significance remains to be further determined. Certain mammalian MATE pumps also contribute to resistance against anticancer agents [48].

2.5 RND Superfamily

This superfamily is also ubiquitously distributed in bacteria, archaea, and eukaryotes and is currently grouped into nine phylogenetic families (*http://www.tcdb.org*) [49]. In particular, three primary families including the heavy metal efflux (HME) family, the hydrophobe/amphiphile efflux-1 (HAE-1) family, and the nodulation factor exporter (NFE) family are generally limited to Gram-negative bacteria and are involved in export of heavy metal salts, multiple drugs, and lipooligosaccharides (nodulation factors). The mostly studied RND systems are the AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa* belonging to HAE-1 family. They were discovered in the early 1990s [50–53] and have served as the archetypal RND pumps for our in-depth understanding of the structure, transport mechanism, regulation, and inhibition of the RND systems.

RND pumps are H^+-substrate antiporters and function as tripartite complexes that typically comprise three components: an RND pump (e.g., AcrB and MexB) located in the cytoplasmic membrane (i.e., inner membrane), a periplasmic adaptor protein (e.g., AcrA and MexA), and an outer membrane channel protein (TolC and OprM) (Figs. 10.1 and 10.2) [54]. Even for AcrD, an RND pump encoded by an individual gene, its function requires AcrA and TolC to form a multicomponent complex [12]. Generally encoded by chromosomes, the three components may be encoded either by the same operons or by genes not clustered physically. An outer membrane component such as TolC or OprM functions in multiple efflux complexes. The RND pumps play a predominant role in Gram-negative bacteria in conferring high-level intrinsic and acquired resistance to a broad range of antimicrobial agents and toxic compounds and also function beyond drug resistance in stress response and pathogenicity [12]. The contribution of RND pumps to resistance become particularly effective when the outer membrane permeability to the pump substrates is limited. RND pumps can also collaborate with the single-component pumps (e.g., the MFS or SMR pumps) and increase their effectiveness [17]. There are often multiple RND systems in a given species. For example, *E. coli* has 6 RND efflux systems of the HAE-1 family and 1 RND system of the HME family. The latter efflux system, CusCBA tripartite complex, provides resistance to Ag(I) and Cu(I) ions [55]. In *P. aeruginosa*, there are 17 RND-type transporters as shown in two widely stuided strains, PAO1 and UCBPP-PA14. To date, 12 RND systems have been characterized for their substrate profiles with MexAB-OprM and MexXY-OprM/OprA as the major pumps of clinical significance [12, 56].

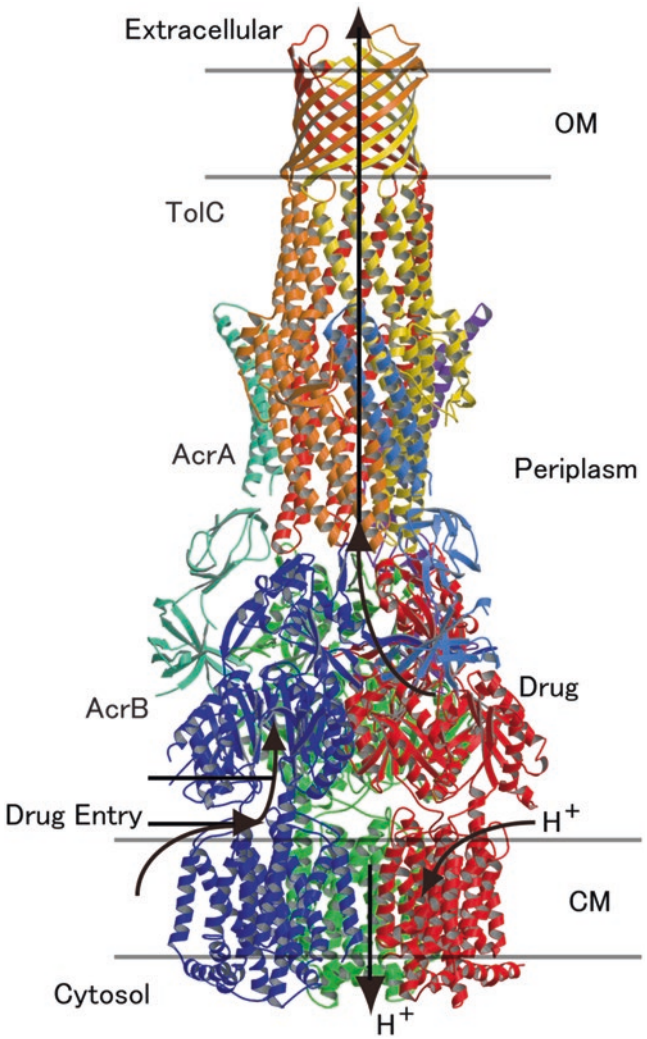


Fig. 10.2 Structural model of the *E. coli* AcrAB-TolC efflux pump complex machinery across the cell envelope barrier in bacteria. The pathways for drug entry/exit and proton movement are indicated in solid lines. Drugs are incorporated via three possible pathways mostly from the periplasm and also possibly from the cytoplasmic membrane (CM) and directly pumped out of the outer membrane (OM) to the extracellular medium. A functional rotating mechanism with the ordered multidrug access, binding and extrusion changes has been proposed to illustrate the drug export process of the RND pumps. An accessory protein (termed AcrZ) for AcrB is not shown, and there is also information suggesting that AcrB and TolC may not be in direct contact. See text and relevant references for detail. (Figure courtesy of Satoshi Murakami, Tokyo Institute of Technology)

Numerous structural and biochemical studies of the individual components of the RND efflux complex such as AcrAB-TolC and MexAB-OprM have provided the elucidation of the structures and transport mechanisms of RND pump systems including substrate recognition, energy coupling, and pump conformational alterations [54, 57–60]. Figure 10.2 shows the AcrAB-TolC efflux complex across the cytoplasmic and outer membranes with the drug substrate access and exit pathway in conjunction with the proton translocation [57–59]. Each protomer of the trimeric AcrB possesses a large periplasmic domain with a large cleft that is close to the ligand binding area (also called the distal binding pocket) and faces the surrounding periplasm. Each promoter shows a unique conformation differing from its neighbor with a total of three different conformations for three protomers, i.e., Access, Binding, and Extrusion [57]. A three-step functionally rotating mechanism has been proposed to illustrate the transport mechanism of the RND pumps, in which each promoter undergoes conformational cycling during the transport process. The drug substrates are incorporated in one state of the trimer and are extruded in another state. The same occurs to the protons. Additionally, substrates are hypothesized to be taken by RND pumps through three pathways in the Access state from the periplasm and the cytoplasmic membrane (but not directly from the cytosol): the opened vestibule, the cleft opening between subdomains, and the central cavity [54, 57, 59]. In the Extrusion state, the distal binding pocket, where the pump substrates are located, becomes distorted or shrunken, allowing the extrusion of the bound substrates via the exit gate to the TolC channel [57, 59] TolC is a trimer that forms a long continuous channel-tunnel containing an OM-spanning β-barrel and a periplasmic 12-stranded α-barrel [61]. The AcrA adaptor protein is featured by four domains (including the long α-hairpin, a lipoyl domain, a short β-barrel, and an additional membrane-proximal domain) and serves to bridge AcrB and TolC [62]. More recently, a small protein named AcrZ was also found to interact with AcrB as an accessory protein for AcrB (thus perhaps for the AcrAB-TolC complex), and its inactivation renders *E. coli* more antibiotic-susceptible [60]. The exact role of AcrZ within the AcrAB-TolC machinery remains to be fully understood. Although a newer model of the AcrABZ-TolC complex by means of electron microscopy suggests that TolC may not be directly in contact with AcrB due to a possible AcrA hexameric tunnel between AcrB and TolC [60]; however, AcrB and TolC may still be possibly in direct contact in vivo [12].

3 Role of Efflux Pumps in Antimicrobial Resistance

Efflux pump-mediated mechanisms are just one amongst the several, important biochemical mechanisms responsible for antimicrobial resistance. The predominate role of drug efflux pumps in clinically relevant resistance have been demonstrated in the native hosts of these pumps—for example, in many clinical isolates of the ESKAPE pathogens (i.e., *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) [10, 12]. Table 10.1 lists some representative examples of drug efflux transporters that provide resistance in bacteria, fungi, and

Table 10.1 Examples of functionally characterized drug efflux transporters in bacteria, fungi, and parasites

Superfamily or family	Exporter	Species	Substrates	Reference
ABC superfamily	EfrAB	*Enterococcus faecalis*	Acriflavine, ciprofloxacin, daunorubicin, doxorubicin, doxycycline, norfloxacin, tetraphenylphophonium chloride	[128]
	LmrA	*Lactococcus lactis*	Daunorubicin, doxorubicin, ethidium bromide, olchicine, rhodamine 6G, vinblastine, vincristine	[129]
	Sav1866	*Staphylococcus aureus*	Ethidium bromide, tetraphenylphophonium chloride	[85, 130]
	MacAB-TolC	*Escherichia coli, Salmonella, Neisseria*	Macrolides	[131]
	FhaABC	*Stenotrophomonas maltophilia*	Fusaric acid	[132]
	PatAB	*Streptococcus pneumoniae*	Fluoroquinolones	[133]
	VcaM	*Vibrio cholerae*	Ciprofloxacin, doxorubicin, daunomycin, norfloxacin, tetracycline	[134]
	Rv1218c	*Mycobacterium tuberculosis*	Biaryl-piperazines, bisanilino-pyridines, pridones, pyrroles	[88, 89]
	AfuMdr4p	*Aspergillus fumigatus*	Itraconazole	[11, 135]
	AfuFp	*A. fumigatus*	Itraconazole	[11, 136]
	CaCdr1p	*Candida albicans*	Azoles, benomyl, cerulenin, clotrimazole, cycloheximide, nigericin, rhodamine 123 and 6G, tamoxifin, trifluoperazine, verapamil	[6, 11]
	CaCdr2p	*C. albicans*	Azoles, cycloheximide, rhodamine 6G, cerulenin, diamide	[11]
	CneMdr1p	*Cryptococcus neoformans*	Azoles, cycloheximide, rhodamine 6G	[11]
	CneAfr1p	*C. neoformans*	Azoles, cycloheximide, rhodamine 6G	[11]
	PfMDR1	*Plasmodium falciparum*	Chloroquine, piperaquine, mefloquine	[7, 8, 137]
	PfMRP	*P. falciparum*	Chloroquine, piperaquine, mefloquine, artemisinin	[106, 138]
	P-glycoprotein	Various parasites, e.g., *Haemonchus*, *Leishmania, Schistosoma*	Anthelmintics, macrocyclic lactone endectocides or praziquantel	[104, 138]
MFS Superfamily	LmrP	*L. lactis*	Clindamycin, macrolides, pentamidine, tetracyclines	[129]
	MefE	*S. pneumoniae, Streptococcus pyogenes*	Macrolides	[69, 70]
	NorA	*S. aureus*	Fluoroquinolones	[139]
	QacA	*S. aureus*, plasmid-encoded	Acriflavine, chlorhexidine, crystal violet, diamidines, ethidium bromide, quaternary ammonium compounds	[140]
	CraA	*Acinetobacter baumannii*	Chloramphenicol	[141]
	EmrAB-TolC	*E. coli, Salmonella*	Ethidium bromide, thiolactomycin	[142, 143]
	EmrKY-TolC	*E. coli*	Deoxycholate	[144–146]
	KpnGH	*Klebsiella pneumoniae*	Ceftazidime, cefepime, streptomycin, tetracycline	[147]
	LfrA	*Mycobacterium smegmatis*	Acriflavine, ethidium bromide, fluoroquinolones	[87]
	MdfA	*E. coli, Salmonella*	Chloramphenicol, doxorubicin, norfloxacin, tetracycline	[142]
	QepA, QepA2	*E. coli, Enterobacter* (plasmid-encoded)	Fluoroquinolones	[148]
	TetA	Various bacteria, often plasmid-encoded	Tetracyclines	[3]
	Tap	*M. tuberculosis, Mycobacterium bovis*	*p*-Aminosalicylate, diamide, specitinomycin, tetracycline	[149, 150]

(continued)

Table 10.1 (continued)

Superfamily or family	Exporter	Species	Substrates	Reference
	AfuMdr3p, AfuMdr4p	*A. fumigatus*	Itraconazole	[11, 135]
	CaMdr1p	*C. albicans*	Benomyl, brefeldin A, cerulenin, cyctoheximide, fluconazole, ketoconazole, methotrexate, voriconazole	[11]
MATE family	AbeM	*A. baumannii*	Acriflavine, aminoglycosides, daunomycin, doxorubicin, fluoroquinolones	[151]
	BexA	*Bacteroides thetaiotaomicron*	Ciprofloxacin, ethidium bromide, norfloxacin	[152]
	MepA	*S. aureus*	Centrimide, ethidium bromide, fluoroquinolones, monovalent and divalent biocides, tigecycline	[46, 47]
	NorM	*Vibrio, Neisseria*	Aminoglycosides, ethidium bromide, fluoroquinolones	[43, 85]
PACE family	AceI	*A. baumannii*	Acriflavine, benzalkonium, chlorhexidine, dequalinum, proflavine	[16]
SMR family	EbrAB	*Bacillus subtilis*	Acriflavine, ethidium bromide, pyronine Y, safranin O	[30]
	EmrE	*E. coli*	Acriflavine, ethidium bromide, methyl viologen, quaternary ammonium compounds	[29, 32]
	KpnEF	*K. pneumoniae*	Benzalkonium chloride, bile salts, cefepime, chlorhexidine, colistin, erythromycin, sodium dodecyl sulfate, streptomycin, tetracycline, triclosan	[34]
	TehAB	*E. coli*	Dequalinium, ethidium bromide, paraquat, proflavine, tellurite	[153]
	Mmr (Rv3065)	*M. tuberculosis*	Cetyltrimethylammonium bromide, clofazimine, pyrroles, tetraphenylphosphonium	[9, 89, 90]
	PfCRT (in DMT suprefamily)	*P. falciparum*	Chloroquine	[37, 38]
RND superfamily	AcrAB-TolC	*E. coli*, *Salmonella,* enteric bacteria	Most common antibiotics (β-lactams, fluoroquinolones, macrolides, amphenicols, rifamycins, tetracyclines), toxic agents, bile salts, fatty acids, detergents, organic solvents	[50, 154–156]
	AdeABC, AdeFGH, AdeIJK	*A. baumannii*	Most common antibiotics (β-lactams, aminoglycosides, fluoroquinolones, macrolides, phenicols, tetracyclines), biocides, toxic agents, bile salts, detergents	[77, 157–159]
	CusCBA	*E. coli*	Ag(I) and Cu(I) ions	[55]
	MexAB-OprM, MexXY-OprM/OprA, MexCD-OprJ, MexEF-OprN	*Pseudomonas aeruginosa*	Most common antibiotics (β-lactams, aminoglycosides, fluoroquinolones, macrolides, phenicols, sulfonamides, trimethoprim, tetracyclines), toxic agents, biocides, bile salts, detergents, organic solvents	[76, 79, 81, 160]
	MtrCDE	*Neisseria gonorrhoeae*	β-Lactams, cationic antibacterial peptides, ethidium bromide, macrolides, Triton X-100	[83–85]
	OqxAB	*E. coli* (plasmid-encoded), *K. pneumoniae*	Chloramphenicol, fluoroquinolones	[126]
	SmeABC, SmeDEF	*S. maltophilia*	Aminoglycosides, β-lactams, macrolides, fluoroquinolones, tetracyclines	[9, 161, 162]

parasites. As shown in Table 10.1, while drug exporters belonging to all of the five superfamilies described above are widely relevant in bacterial drug resistance [12], the pumps mediating antifungal resistance mainly fall into ABC and MFS transporters [11] and those pumps involving in antiparasitic resistance are often the ABC exporters [63]. Drug exporters also vary in substrate specificity with certain pumps being highly drug- or class-specific while others more accommodable to an incredibly broad range of the substrates. However, it should be noted that the substrate profiles of many efflux pumps have often been established through the heterologous expression of the relevant efflux genes in model organisms such as *E. coli* [12] or *Saccharomyces cerevisiae* [11]. In these cases, the clinical significance of these pumps in the native host should be examined carefully. For instance, despite that *E. coli* genomes possess a large number of genes encoding drug exporters, only a limited number of pumps (e.g., the predominate RND-type AcrAB-TolC system) can contribute to clinically relevant resistance [64, 65].

It is also important to discuss the contribution of drug efflux pumps to resistance in the conjunction with other resistance mechanisms. In particular, the competition between drug influx and efflux ultimately determines the steady-state drug level available to cytosolic drug targets. Figure 10.1 shows possible drug uptake and extrusion pathways in bacterial cells. The drug pathways in fungi or parasites are merely to cross the cytoplasmic membrane where the transporters are located. The drug may be taken from the cytoplasmic membrane or the cytosol and be extruded out of the cell.

3.1 Bacteria

Considering the drug influx and efflux pathways as shown in Fig. 10.1, it is easier to recognize the role of drug efflux pumps in resistance, including reasoning behind why many antibiotics (e.g., erythromycin, novobiocin, rifampicin, ketolides, and oxazolidinones) active against Gram-positive bacteria lack significant anti-Gram-negative activity [12]. The physical and chemical characteristics of drugs and membrane structures can themselves provide much explanation in terms of the spectrum activity of drugs against Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, drug molecules (particularly more hydrophobic agents) are expected to readily cross the cytoplasmic membrane, and hence, the role of efflux pumps is generally less significant than that in Gram-negative bacteria [12]. However, the efflux mechanism still contributes not only to basal susceptibility (or resistance) but also acquired resistance in Gram-positive bacteria. The basal resistance level can facilitate the development of higher, clinically relevant resistance levels. *S. aureus* strains possess multiple efflux pumps including MepA, MedA, NorA, NorB, NorC, and QacA/B [9, 10]. In one study from the USA, increased expression of these pumps was demonstrated in about 50 % of 232 clinical bloodstream isolates and correlated with resistance to fluoroquinolones, biocides, and dyes [66]. In another study carried out in Portugal, about ¼ of 52 hospital-isolated ciprofloxacin-resistant *S. aureus* displayed an elevated efflux activity toward an indicator substrate, ethidium bromide [67]. Single or multiple exposures to biocides and dyes that are used widely in clinical settings have also been found to readily select the multidrug efflux pump overproducers [68]. In *Streptococcus* spp., low-level resistance to 14- and 15-membraned macrolides mediated by MFS-type Mef efflux pump [69, 70]. Constitutive overexpression of ABC-type PatAB transporter in *Streptococcus pneumoniae* also caused a fourfold increase in MIC values of ciprofloxacin, norfloxacin, and levofloxacin [71] and PatAB overproduction was confirmed in clinical isolates with fluoroquinolone resistance [72].

In Gram-negative bacteria, hydrophilic agents predominantly pass through the water-filled channels of porins, which impose several restrictions to the influx of solutes, including those to the size, hydrophobicity, charge, and number of the open channels [73, 74]. Thus, small hydrophilic drugs penetrate the outer membrane via porins, while the larger and/or hydrophobic drugs need to cross the outer membrane through the lipid bilayer region (Fig. 10.1). Moreover, in comparison with the classical porins (i.e., OmpF and OmpC) of *E. coli*, certain Gram-negative species such as *A. baumannii* and *P. aeruginosa* mainly possess low-permeability porins (i.e., "slow porins" such as OprF and OmpA) [18]. In these bacteria, the efflux mechanism becomes very effective in producing high-level resistance. In particular, the tripartite RND pumps can bypass the outer membrane barrier to directly pump their substrates out of the cell. (For single-component pumps, their substrates would be only pumped to the periplasm and may be further taken up by RND pumps for extrusion out of the cell.) Thus, single-component and multicomponent pumps can interplay to increase the efflux effectiveness [17, 75]. The substrate molecules may also diffuse through porin channels or the lipid bilayer region of the outer membrane. Moreover, the clinical significance of RND pumps is not only attributed to the effectiveness of RND machinery but also to the extremely broad substrate specificity. The latter, as shown for AcrB and MexB pumps, includes practically all types of clinically relevant antibiotics, biocides, detergents, dyes, free fatty acids, and organic solvents (Table 10.1) [12]. Inactivation of the MexAB-OprM system in the wild-type *P. aeruginosa* PAO1 strain caused a 8- to 128-fold reduction of the minimal inhibitory concentration (MIC) values of various antipseudomonal agents such as azlocillin, carbenicillin, cefoperazone, ceftriaxzone, and ciprofloxacin [76]. Similarly, in *A. baumannii*, disruption of AdeIJK pump in a wild-type strain produced a 4- to 16-fold

MIC reduction of β-lactams (aztreonam, ceftazidime, cefepime, and ticarcillin), chloramphenicol, ciprofloxacin, minocycline, and tigecycline [77, 78].

Moreover, numerous studies have confirmed the significance of efflux mechanisms in the resistance of Gram-negative isolates from patients [12]. In one French study, the overproduction of MexAB-OprM [76] and MexXY [79] pumps was highly prevalent in clinical isolates with a reduced susceptibility to ticarcillin (MIC ≥32 μg/ml) [80]. If considering the susceptibility breakpoints from the Clinical and Laboratory Standards Institute (CLSI), the impact of an eightfold MIC increase due to the efflux mechanism alone can change the strain categorization for several antipseudomonal drugs (aztreonam, meropenem, ticarcillin, ciprofloxacin, and levofloxacin) from susceptible ("S") to intermediate ("I") or resistant ("R") [12]. Similarly, in another study from Switzerland, a mechanically ventilated patient treated by two drug substrates of the MexCD-OprJ pump [81], cefepime (two durations, first 8 days, another 11 days) and ciprofloxacin (14 days between two cefepime treatments), was found to have MexCD-OprJ-overproducing *nfxB* mutants, which were categorized as "R" as opposed to "S" with respect to susceptibility to fluoroquinolones (based on CLSI breakpoints) [82]. In *Neisseria gonorrhoeae*, MtrCDE [83, 84], MacAB, and NorM expression also influence the extensive or multiple drug resistance of clinical isolates [85]. Inactivation of MtrCDE altered the susceptibility status for azithromycin, penicillin, and tetracycline from "R" to "S" or "I." The MacAB disruption rendered the resistant isolate from "R" to "S", providing an important example for the clinical significance of ABC transporters in bacterial resistance. The NorM deficiency changed an "R" status to "I" for tetracycline [85]. A new study on post-therapy multidrug-resistant isolates of *Salmonella enterica* serova Typhimurium revealed that a single amino acid substitution (Gly288Asp) of the AcrB drug-binding pocket leads to clinically relevant resistance to ciprofloxacin (16-fold MIC increase) and other agents but also confers increased susceptibility to minocycline (fourfold MIC decrease; when the mutant AcrB was expressed in a complementation study) [86].

Mycobacteria exhibit high-level intrinsic resistance to many commonly used antimicrobial agents. This is attributed to the interplay between the multiple mechanisms, including a highly impermeable cell wall and the presence of multiple drug exporters (Fig. 10.1) [10]. The cell wall contains long-chain fatty acids (mycolic acids) covalently linked to the peptidoglycan-associated polysaccharide arabinogalactan [19]. Mycobacterial channels of porins are also sparse [19]. Inactivation of several efflux pumps of various families renders mycobacteria (including *Mycobacterium tuberculosis*) more susceptible to multiple classes of antimicrobial agents [87–90]. Elevated efflux pump expression has been found as a general first step in the evolution of high-level mycobacterial drug resistance [91]. Overexpression of the Rv1218c ABC pump was also observed in clinical tuberculosis isolates with MDR phenotype [92]. Efflux also contributes to the development of resistance to isoniazid and pyrazinamide [93, 94].

3.2 Fungi

In addition to being a major pathogen of plants, fungi are also important opportunistic pathogens of humans which predominantly include: *C. albicans*, *Cryptococcus neoformans,* and *A. fumigatus* [11]. The occurrence of fungal infections has been increasing in clinical settings and their treatment has also been threatened by the development of resistance, in particular MDR, in fungi [95, 96]. Several mechanisms are responsible for fungal drug resistance and these include, for example, overexpression or alteration of drug targets, elevated cellular stress response, and upregulation of multidrug transporters [96–98]. The active efflux process decreases effectively the intracellular drug concentrations and allows the fungi to survive against fungicides [11, 95]. The fungal genomes show the presence of a large number of efflux transporters. These efflux pumps mainly fall into the superfamilies of the ABC and MFS transporters (Table 10.1) [11, 95]. Fungal ABC transporters contain three families involved in efflux of cytotoxic agents, i.e., the MDR, multidrug resistance-associated protein (MRP), and the pleiotropic drug resistance (PDR) families. Belonging to the PDR family, the most studied Cdr1p and Cdr2p pumps of *Candida* spp. confer resistance to multiple agents including azoles such as fluconazole, ketoconazole, itraconazole, and voriconazole (Table 10.1). Many studies have confirmed the significance of these pumps (particularly Cdr1p) as a major, clinically relevant mechanism of azole resistance in *C. albicans* [99]. Inactivation of Cdr1p in a clinical isolates reduced resistance to fluconazole, ketoconazole, and itraconazole by 4- to 8-fold, while the disruption of Cdr2p decreased resistance to fluconazole and ketoconazole by merely less than twofold [100]. Another transporter of the PDR family, AtrB of *A. nidulans*, mediates resistance of all major classes of fungicides including certain azoles [101]. The fungal MFS drug transporters also possess 12 or 14 TMSs [95] and the well-studied MFS pumps, Mdr1 and its homologues, provide resistance to azoles and multiple other agents (Table 10.1). CaMdr1 overexpression due to a gain of function in its regulator *MMR1* is the major mechanism for azole resistance in clinical isolates of *C. albicans* [102]. The broad substrate profiles of fungal efflux pumps also pose a key challenge for the development of antifungal agents that are effective against fungi but remain safe to the human hosts because of the biochemical similarity between the two.

3.3 Parasites

Drug resistance in parasites is also caused by multiple mechanisms which include the alteration of binding affinities of drugs to their targets and reduced intracellular drug concentrations. ABC transporters such as those with P-glycoprotein activity constitute a major mechanism of drug resistance in various parasites including those causing malaria, helminth diseases, leishmaniasis, schistosomiasis, and trichomoniasis [63, 103, 104]. For instance, the expressional or structural changes of ABC transporters are linked to resistance of *P. falciparum*, helminthes, schistosomes to agents that are used to treat the relevant parasitic diseases such as antimalarials (e.g., chloroquine) [63], anthelmintic macrocyclic lactone endectocides (e.g., ivermectin) [103], antischistosome praziquantel [104]. Inhibition or inactivation of ABC transporters can restore susceptibility to praziquantel in schistosomes [105].

However, in chloroquine-resistant *P. falciparum* strains, in addition to the contribution of Pgp (PfMDR1; located in the membrane of digestive vacuole of the parasite) to resistance, mutated PfCRT of the DMT superfamily (also resided in the same membrane as PfMDR1) is widely responsible for chloroquine resistance [37, 106]. A third transporter, PfMRP (located in the plasma membrane), functions as a general drug exporter. Together, the state of these three transporters (PfMDR1, PfCRT, and PfMRP) determines the parasite's susceptibility to antimalarials including chloroquine, amodiaquine, mefloquine, lumefantrine, and artemisinins [106]. The homolog of PfMDR1, PvMDR1, has been identified as a resistance marker for *Plasmodium vivax* which should have been drawn more attention because of the increasingly observed severity of the disease caused by this species [106].

4 Interplay Between Antimicrobial Efflux and Other Resistance Mechanisms

Microbes possess multiple molecular and biochemical mechanisms of drug resistance, and these mechanisms can cooperate with each other to enhance resistance levels and profiles [9]. Efflux exporters themselves can interplay with each other. For example, additive or multiplicative effects of simultaneous expression of at least two efflux pumps were observed in *E. coli* and *P. aeruginosa* [17]. The increases of 4-, 32-, 64-, 128-, and 256-fold MIC values of antipseudomonal levofloxacin were reported for *P. aeruginosa*, respectively, with wild-type constitutive MexAB-OprM expression, MexAB-OprM overexpression, MexAB-OprM/MexCD-OprJ, MexAB-OprM/MexEF-OprN and MexAB-OprM/MexCD-OprJ/MexEF-OprN co-overexpressions [17]. Fungal transporters Cdr1p, Cdr2p, and Mdr1 pumps of *Candida* spp. also appear to interact to enhance azole resistance [100, 102]. Multiple pumps also interplay in ensuring adequate resistance to antimalarial agents [106].

In Gram-negative bacteria, the interplay between the outer membrane permeability barrier and efflux pumps likely provides the best example of how two different mechanisms contribute to clinically relevant high-level drug resistance (Fig. 10.1). As mentioned earlier, this type of interplay largely explains the important role of Gram-negative efflux pumps in resistance [12]. *E. coli* strains deficient in both AcrAB pump and intact LPS are more susceptible than strains only lacking one of the two mechanisms to a number of hydrophobic antibiotics. The latter agents penetrate the LPS-containing bilayer region and are the substrates for AcrAB. For example, inactivation of *acrAB* genes, *waaP* gene (its product is to modify LPS core), and *acrAB/waaP* genes decreased azithromycin MIC values, respectively, by 16-, 8-, and >64-fold (X.-Z. Li, unpublished data). Similarly, an early well-studied hypersusceptible *P. aeruginosa* strain Z61 was found to have an altered LPS structure and to be deficient in MexAB-OprM production [52, 107, 108]. The reduction or loss of porin expression and the pump overexpression are well documented in clinical isolates of resistant *Enterobacteriaceae* such as *Enterobacter* and *Klebsiella* species [12].

Target alterations are another major mechanism of resistance. However, efflux pump overproduction and mutations in DNA gyrase and topoisomerase IV, the targets of fluoroquinolone agents, exist in high-level fluoroquinolone-resistant isolates of various bacterial species including *S. aureus*, *E. coli*, and *P. aeruginosa* [9]. The synergy between RND pump overexpression and ribosomal target protein modifications was required for high-level macrolide resistance in *Campylobacter* spp. during an in vivo resistance selection study [109]. Mutations or overexpression of the azole target, ergosterol biosynthetic enzyme lanosterol demethylase, encoded by *ERG11*, can contribute together with drug transporters (and mediators of stress response pathways) to high-level azole resistance in clinical fungal isolates [97].

Enzymatic inactivation is the predominate mechanism for β-lactam resistance and may often mask the role of other resistance mechanisms. Yet, activation of AcrAB and AmpC β-lactamase production was reported to augment susceptibility to several β-lactams [110]. An aminoglycoside-modifying enzyme, acetyltransferase AAC(6′), also interacts with an efflux mechanism to enhance aminoglycoside resistance [9].

5 Role of Antimicrobial Efflux Pumps Beyond Drug Resistance

Drug transporters can play a duel role in resistance and other functions such as pathogenicity, and this fact would facilitate microorganism's exploitation and transmission among hosts. Research in the nonresistance role of drug efflux pumps has drawn much attention in the last decade. Indeed, drug

transporters are also involved in biofilm formation, stress response, colonization, and/or pathogenicity of microbes [12, 97], which can have adverse implications in clinical outcomes of the infectious diseases. Many bacterial RND pumps play important role in stress response such as those induced by cell envelope stress, oxidative or nitrosative stress [12, 111]. The AcrAB-TolC system allows the survival of enteric bacterial cells against bile salt stress in the intestinal tract. Several pumps including MacAB of *Salmonella* and *Stenotrophomonas* and NorM of *E. coli* protect cells against oxidative stress such as caused by excessive exposure to hydrogen peroxide [12]. As part of more general regulatory circuit, the elevated activities from the MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY pump systems of *P. aeruginosa* are able to respond to diverse challenges from reactive oxygen species, membrane-damaging compounds, nitrosative agents, and/or ribosome-disruptive agents [12]. Similarly, the efflux pump expression status may also influence the fitness, colonization, and virulence of microbial pathogens. In many cases, the basal or constitutive expression of a drug pump is essential for bacterial colonization and virulence such as seen in many enteric bacteria. Overexpressed pump activity may also come with a fitness cost for microbes. One may have to determine the impact of the status of an efflux pump via a case-by-case assessment. An epidemic *P. aeruginosa* strain overproducing MexAB-OprM and MexXY was found to have enhanced virulence [112]. The MFS pump Tap (Rv1258c) in *M. tuberculosis* is a macrophage-induced efflux pump needed for pathogen's intracellular growth and virulence [10, 113].

Similarly, the increased pathogenicity of *C. albicans* for systematic infection is associated with the overexpression of CaMdr1p in fluconazole- or micafungin-resistant strains that show several biochemical and physiological changes in cell wall, hypha formation, adherence, and biofilm formation [114]. Upregulation of the fungal ABC transporter AFR1 in *C. neoformans* not only confers resistance to fluconazole but also enhances virulence, possibly due to a reduced vulnerability to the antimicrobial factors generated by phagocytic cells [115]. In malaria parasites, drug resistance and fitness may coincide [116]. PfMdr1-mediated chloroquine-resistant *P. falciparum* was found to be outcompeted in vitro by the sensitive wild-type strains. Clinical observations also suggest a fitness advantage of chloroquine-sensitive parasites over the resistant ones [116]. Yet, fitness cost of resistance can be offset by compensatory mutations such that high polymorphisms of PfCRT transporter gene are accompanied by other mutations to allow the chloroquine-resistant mutant strains to retain adequate fitness [116].

6 Regulation and Induction of Antimicrobial Efflux Pump Expression

The presence of multiple drug transporters in a given species and their broad substrate profile (including overlapping substrate specificity) require a well-coordinated regulation of their expression. Such regulation is known to be diverse and complex. In bacteria, the regulation is controlled at multiple levels by various local and/or global regulators [9, 10]. Local regulators refer to protein products encoded by the genes adjacent to the genes encoding the efflux transporters. Many regulatory proteins fall into the transcriptional regulators (either as repressors or as activators) such as those single-component proteins belonging to the TetR family [117], which can bind DNA and small-molecule ligands. Two-component regulatory systems are also involved in regulation of efflux pump expression [10]. The pump expression can thus be influenced by mutational changes in the regulatory genes and also induced by certain ligands. More recently, noncoding small RNA molecules have also been also shown to play an important role in influencing antibiotic resistance including in regulation of the expression of efflux pumps [12]. For example, the AcrAB expression is subjected to the regulation by the local repressor AcrR, global activators MarA, SoxS, and Rob, two-component systems EvgAS and PhoQP, and noncoding small RNA molecules [12]. (Interestingly, the expression of the newly identified small protein of AcrZ [60] is transcriptionally co-regulated with that of AcrAB-TolC by global activators MarA, SoxS, and Rob [118].) Similar complex regulation is also demonstrated with several Mex pumps of *P. aeruginosa* [12]. The expression of the *mexAB-oprM* operon is regulated by several regulators or modulators, MexR, NalD, ArmR, MexT, AmpR, and BrlR [12]. Expression of staphylococcal efflux pumps is also often controlled by regulators [10]. For example, the MFS-type NorA pump is subjected to the regulation by several regulators [10]. The MATE-type MepA pump is negatively controlled at the transcriptional level by MepR repressor [46, 47], whose expression can be induced by MepA substrates [119]. Nevertheless, the regulation of many MATE and SMR pump expressions still remains unknown. It is important to note that the expression of drug pumps can be induced by small molecules such as antimicrobials, biocides, and bile salts, which may be the substrate of efflux pumps and bind the relevant regulatory proteins. Several classes of ribosome-disrupting antibiotics (e.g., aminoglycosides, amphenicols, marcolides, and tetracycleins) are often the substrates of the Mex pumps and can induce expression of MexXY pump [10, 56].

The expression of fungal drug transporters is also controlled by multiple mechanisms. Cdr1p and Cdr2p expression is regulated by the transcription factor Tac1 [97]. Yet, for certain pumps such as PfCRT, it is the mutation of the transporter proteins that possess a gain of function to extrude the substrates [11].

7 Implications of Drug Efflux Pumps in Antimicrobial Development and Stewardship

Many clinically used and novel antimicrobials are substrates for drug efflux transporters, thus requiring strategies for development of novel antimicrobials that can bypass the action of the pumps and other inhibitory agents that can block the efflux machinery to serve as antibiotic adjuvants [12]. In this regard, antimicrobial research has been quite successful in developing semisynthetic and synthetic agents that are stable against inactivation such as many newer β-lactams and fluoroquinolones. However, these agents can readily select for drug efflux mutants such as those Mex pump overproducers [9]. Our recognition of antimicrobial influx and efflux mechanisms (e.g., Fig. 10.1) helps the search for new antimicrobial drugs. Mutants deficient in drug efflux pumps and cell membrane penetration barrier can be used for screening possible antimicrobial substances. Moreover, one needs to also consider the size, charge, and hydrophobicity of the potential drug molecules. This reasoning is because drug molecules would somehow be lipophilic in order to cross the cytoplasmic membrane (and outer membrane) before exerting their action [73, 74]. This often also becomes a problem since these molecules are more likely to be substrates of drug efflux. Hence, small hydrophilic molecules may less likely act as substrates for drug efflux pump, and yet, this type of molecules may not be able to effectively penetrate the cytoplasmic membrane [12]. Overall, these considerations highlight the challenge for antimicrobial development to optimize the drug access by increasing influx and decreasing efflux of drug molecules. Indeed, the new ribosome-targeting omadacyclines that display activity against tetracycline-specific efflux and ribosome protection mechanisms [120, 121] are still the substrate of RND pumps [122]. Several newly developed classes of antibiotics are also substrates of Gram-negative RND pumps and thus are mainly active against Gram-positive bacteria. But some newer fluoroquinolones may have become less subjected to drug efflux [12].

An alternative approach for combating drug efflux mechanisms is to identify efflux pump inhibitors. This approach has been highly appreciated since the discovery of drug efflux pumps with an aim to understand the natural substrates of the MDR transporters and to develop combinatorial antimicrobial therapies to rejuvenate the existing antimicrobials. Staphylococcal NorA and Gram-negative AcrAB and Mex pumps have been widely used as models for the identification and characterization of the efflux pump inhibitors [10]. Current in-depth understanding of the pump structures and transport mechanisms should fundamentally help us in such a search of the pump inhibitors [12, 123]. In fact, various inhibitors from natural or synthetic sources have been identified to sensitize in vitro or in animal models the resistant microbes or parasites to antimicrobial agents [9, 10]. However, despite scientific efforts of at least two decades, it still remains a great challenge to find clinically suitable efflux pump inhibitors for possible combinational therapy like those β-lactam-β-lactamase inhibitor combinations. Nevertheless, several inhibitors have been used routinely in laboratories to characterize the efflux contribution to resistance in clinical isolates (although standardized methods should be established as discussed in a recent review for bacteria [12]), and these include, for example, phenylalanine-arginine-β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine as inhibitors of Gram-negative RND efflux pumps, and reserpine and verapamil for inhibiting ABC or MFS transporters in bacteria (including mycobacteria), fungi, parasites, and cancer cells [10–12, 124]. With the available crystallographic structures of AcrB pump, molecular dynamics simulation studies of several RND pump inhibitors including PAβN, NMP, the pyridopyrimidine derivative D13-9001, and the pyranopyridine derivative MBX2319 have provided insights about the mechanisms of RND pump inhibition, e.g., most inhibitors likely distort the structure of the AcrB distal pocket and impair the proper binding of the pump substrates [12, 125]. Various fungal pump inhibitors such as milbemycins, quinazolinones, and unarmicins have also been reported [11]. Although the anthelmintic macrocyclic lactone endectocides such as ivermectin are subjected to efflux by P-glycoprotein and MRP transporters, they are slowly transported and interact with these pumps to serve as strong inhibitors [103]. This fact supports the combinational therapy approach to reverse the efflux of MDR transporters [103]. Interestingly, a number of agents other than avermectins can also inhibit MDR transporters and their use with avermectins may improve anthelmintic efficacy of avermectins because of possible pharmacological reversal of MDR transporter-mediated efflux [103]. Efflux pump inhibitors have also been found to decrease emergence of resistance as well as to decrease biofilm formation and pathogenicity as evident with bacteria [12]. These effects provide more incentive for pump inhibitor development. It is also important to indicate that the outer membrane permeability barrier of Gram-negative bacteria interacts with efflux pumps and hence its permeabilization by

cationic peptides and other permeabilizers will also sensitize bacteria especially to lipophilic antimicrobial drugs [73].

The important role of drug efflux pumps in resistance certainly has implications in antimicrobial stewardship for promoting prudent drug use. This is because that the exposures of microbes or parasites can readily select both in vitro and in vivo the mutant strains that overproduce drug efflux pumps with resistance phenotype and other pathogenic traits [9]. Multidrug-resistant bacteria (e.g., *Acinetobacter*, *Pseudomonas,* and *Stenotrophomonas*) can be selected not only by conventional antibiotics agents, but also by antiseptics or biocides. Multiple antibacterial resistance can also be induced transiently by salicylate, a compound existing in plants and used widely in patients. This may facilitate emergence of acquired resistance [9]. In addition to limiting antimicrobial use, the optimization of the pharmacokinetics and pharmacodynamics of antimicrobial therapy should also be considered; in particular, efflux mechanisms may be the first step in providing initial low-level resistance for subsequent development of high-level resistance that produces severe clinical consequences. Additionally, plasmid-borne efflux pump genes have been increasingly identified in bacteria, suggesting potential for rapid horizontal spread among multiple species and raising an additional concern [12, 126].

8 Concluding Remarks

Efflux mechanisms play an important role as a key mechanism of antimicrobial drug resistance [127]. In the past two decades, in-depth knowledge has been gained to understand the pieces of the drug efflux puzzle. The contribution from drug efflux transporters of various families to resistance in either microbes or parasites is complex. Nevertheless, targeting efflux mechanisms is already an experimentally proven strategy to intervene efflux-mediated mechanism by developing novel agents (either pump-by passing antimicrobials or efflux-inhibiting antimicrobial adjuvants). Yet, there is no or little clinical assessments regarding efflux pump inhibitors. Furthermore, although efforts should be made to understand more about the impact of efflux pump-overproducing pathogens on the clinical efficacy of antimicrobial therapy, the fact that efflux-overexpressing resistant isolates can be readily selected after exposures of microorganisms to various classes of antimicrobial drugs continue to speak for the importance of responsible antimicrobial use in all environments particularly including clinical settings.

Acknowledgements The views in this chapter do not necessarily reflect those of the author's affiliation, Health Canada.

References

1. Gottesman MM, Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. FEBS Lett. 2006;580(4):998–1009. doi:10.1016/j.febslet.2005.12.060.
2. McMurry L, Petrucci Jr RE, Levy SB. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc Natl Acad Sci U S A. 1980; 77(7):3974–7. doi:10.1073/pnas.77.7.3974.
3. Levy SB. Active efflux mechanisms for antimicrobial resistance. Antimicrob Agents Chemother. 1992;36(4):695–703. doi:10.1128/AAC.36.4.695.
4. De Waard MA, Van Nistelrooy JG. Induction of fenarimol-efflux activity in *Aspergillus nidulans* by fungicides inhibiting sterol biosynthesis. J Gen Microbiol. 1981;126(2):483–9. doi: 10.1099/00221287-126-2-483.
5. Shearer Jr G, Sypherd PS. Cycloheximide efflux in antibiotic-adapted cells of the fungus *Mucor racemosus*. Antimicrob Agents Chemother. 1988;32(3):341–5. doi:10.1128/AAC.32.3.341.
6. Fling ME, Kopf J, Tamarkin A, Gorman JA, Smith HA, Koltin Y. Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. Mol Gen Genet. 1991;227(2):318–29. doi: 10.1007/BF00259685.
7. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK, Schlesinger PH. Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science. 1987;238(4831):1283–5. doi:10.1126/science.3317830.
8. Krogstad DJ, Gluzman IY, Herwaldt BL, Schlesinger PH, Wellems TE. Energy dependence of chloroquine accumulation and chloroquine efflux in *Plasmodium falciparum*. Bioch Pharmacol. 1992;43(1):57–62. doi: 10.1016/0006-2952(92)90661-2.
9. Li X-Z, Nikaido H. Efflux-mediated drug resistance in bacteria. Drugs. 2004;64(2):159–204. doi:10.2165/00003495-200464020-00004.
10. Li X-Z, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs. 2009;69(12):1555–623. doi:10.2165/11317030-000000000-00000.
11. Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, Keniya MV, Tanabe K, Niimi M, Goffeau A, Monk BC. Efflux-mediated antifungal drug resistance. Clin Microbiol Rev. 2009;22(2):291–321. doi:10.1128/CMR.00051-08.
12. Li X-Z, Plésiat P, Nikaido H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev. 2015;28(2):337–418. doi: 10.1128/CMR.00117-14.
13. Saier Jr MH, Paulsen IT, Sliwinski MK, Pao SS, Skurray RA, Nikaido H. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. FASEB J. 1998;12(3):265–74.
14. Saier Jr MH, Reddy VS, Tamang DG, Vastermark A. The transporter classification database. Nucleic Acids Res. 2014;42:D251–8. doi:10.1093/nar/gkt1097.
15. Yen MR, Chen JS, Marquez JL, Sun EI, Saier MH. Multidrug resistance: phylogenetic characterization of superfamilies of secondary carriers that include drug exporters. Methods Mol Biol. 2010;637:47–64. doi:10.1007/978-1-60761-700-6_3.
16. Hassan KA, Liu Q, Henderson PJ, Paulsen IT. Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. mBio. 2015;6(1):e01982–14. doi:10.1128/mBio.01982-14.
17. Lee A, Mao W, Warren MS, Mistry A, Hoshino K, Okumura R, Ishida H, Lomovskaya O. Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. J Bacteriol. 2000;182(11):3142–50. doi:10.1128/JB.182.11.3142-3150.2000.

18. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593–656. doi:10.1128/MMBR.67.4.593-656.2003.
19. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem. 1995;64:29–63. doi:10.1146/annurev.bi.64.070195.000333.
20. van Veen HW, Callaghan R, Soceneantu L, Sardini A, Konings WN, Higgins CF. A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene. Nature. 1998;391(6664):291–5. doi:10.1038/34669.
21. Dawson RJP, Locher KP. Structure of a bacterial multidrug ABC transporter. Nature. 2006;443(7108):180–5. doi:10.1038/nature05155.
22. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science. 2009;323(5922):1718–22. doi:10.1126/science.1168750.
23. Jin MS, Oldham ML, Zhang Q, Chen J. Crystal structure of the multidrug transporter P-glycoprotein from *Caenorhabditis elegans*. Nature. 2012;490(7421):566–9. doi:10.1038/nature11448.
24. George AM, Jones PM. Perspectives on the structure-function of ABC transporters: the switch and constant contact models. Prog Biophys Mol Biol. 2012;109(3):95–107. doi:10.1016/j.pbiomolbio.2012.06.003.
25. Jones PM, George AM. Opening of the ADP-bound active site in the ABC transporter ATPase dimer: evidence for a constant contact, alternating sites model for the catalytic cycle. Proteins. 2009;75(2):387–96. doi:10.1002/prot.22250.
26. Jones PM, George AM. A reciprocating twin-channel model for ABC transporters. Q Rev Biophys. 2014;47(3):189–220. doi:10.1017/S0033583514000031.
27. Gbelska Y, Krijger JJ, Breunig KD. Evolution of gene families: the multidrug resistance transporter genes in five related yeast species. FEMS Yeast Res. 2006;6(3):345–55. doi:10.1111/j.1567-1364.2006.00058.x.
28. Jack DL, Yang NM, Saier Jr MH. The drug/metabolite transporter superfamily. Eur J Biochem. 2001;268(13):3620–39. doi:10.1046/j.1432-1327.2001.02265.x.
29. Schuldiner S. EmrE, a model for studying evolution and mechanism of ion-coupled transporters. Biochim Biophys Acta. 2009;1794(5):748–62. doi:10.1016/j.bbapap.2008.12.018.
30. Masaoka Y, Ueno Y, Morita Y, Kuroda T, Mizushima T, Tsuchiya T. A two-component multidrug efflux pump, EbrAB, in *Bacillus subtilis*. J Bacteriol. 2000;182(8):2307–10. doi:10.1128/JB.182.8.2307-2310.2000.
31. Grinius L, Dreguniene G, Goldberg EB, Liao CH, Projan SJ. A staphylococcal multidrug resistance gene product is a member of a new protein family. Plasmid. 1992;27(2):119–29. doi: 10.1016/0147-619X(92)90012-Y.
32. Beketskaia MS, Bay DC, Turner RJ. Outer membrane protein OmpW participates with small multidrug resistance protein member EmrE in quaternary cationic compound efflux. J Bacteriol. 2014;196(10):1908–14. doi:10.1128/JB.01483-14.
33. Nishino K, Yamaguchi A. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J Bacteriol. 2001;183(20):5803–12. doi:10.1128/JB.183.20.5803-5812.2001.
34. Srinivasan VB, Rajamohan G. KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. Antimicrob Agents Chemother. 2013;57(9):4449–62. doi:10.1128/AAC.02284-12.
35. Srinivasan VB, Rajamohan G, Gebreyes WA. Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2009;53(12):5312–6. doi:10.1128/AAC.00748-09.
36. Li X-Z, Poole K, Nikaido H. Contributions of MexAB-OprM and an EmrE homolog to intrinsic resistance of *Pseudomonas aeruginosa* to aminoglycosides and dyes. Antimicrob Agents Chemother. 2003;47(1):27–33. doi:10.1128/AAC.47.1.27-33.2003.
37. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Coulibaly D, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med. 2001;344(4):257–63. doi:10.1056/NEJM200101253440403.
38. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, Kirk K. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science. 2009;325(5948):1680–2. doi:10.1126/science.1175667.
39. Pao SS, Paulsen IT, Saier Jr MH. Major facilitator superfamily. Microbiol Mol Biol Rev. 1998;62(1):1–34.
40. Reddy VS, Shlykov MA, Castillo R, Sun EI, Saier Jr MH. The major facilitator superfamily (MFS) revisited. FEBS J. 2012;279(11):2022–35. doi:10.1111/j.1742-4658.2012.08588.x.
41. Sapunaric FM, Aldema-Ramos M, McMurry LM. Tetracycline resistance: efflux, mutation, and other mechanisms. In: White DG, Alekshun MN, McDermot PF, editors. Frontiers in Antimicrobial Resistance, a tribute to Stuart B. Levy. Washington, DC: ASM Press; 2005. p. 3–18.
42. Hvorup RN, Winnen B, Chang AB, Jiang Y, Zhou XF, Saier Jr MH. The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily. Eur J Biochem. 2003;270(5):799–813. doi:10.1046/j.1432-1033.2003.03418.x.
43. Morita Y, Kodama K, Shiota S, Mine T, Kataoka A, Mizushima T, Tsuchiya T. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob Agents Chemother. 1998;42(7):1778–2.
44. Kuroda T, Tsuchiya T. Multidrug efflux transporters in the MATE family. Biochim Biophys Acta. 2009;1794(5):763–8. doi:10.1016/j.bbapap.2008.11.012.
45. He X, Szewczyk P, Karyakin A, Evin M, Hong WX, Zhang Q, Chang G. Structure of a cation-bound multidrug and toxic compound extrusion transporter. Nature. 2010;467(7318):991–4. doi:10.1038/nature09408.
46. Kaatz GW, McAleese F, Seo SM. Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. Antimicrob Agents Chemother. 2005;49(5):1857–64. doi:10.1128/AAC.49.5.1857-1864.2005.
47. McAleese F, Petersen P, Ruzin A, Dunman PM, Murphy E, Projan SJ, Bradford PA. A novel MATE family efflux pump contributes to the reduced susceptibility of laboratory-derived *Staphylococcus aureus* mutants to tigecycline. Antimicrob Agents Chemother. 2005;49(5):1865–71. doi:10.1128/AAC.49.5.1865-1871.2005.
48. Minematsu T, Giacomini KM. Interactions of tyrosine kinase inhibitors with organic cation transporters and multidrug and toxic compound extrusion proteins. Mol Cancer Ther. 2011;10(3):531–9. doi:10.1158/1535-7163.MCT-10-0731.
49. Tseng TT, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier Jr MH. The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J Mol Microbiol Biotechnol. 1999;1(1):107–25.
50. Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J Bacteriol. 1993;175(19):6299–313. doi: 10.1128/jb.175.19.6299-6313.1993.
51. Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J Bacteriol. 1993;175(22):7363–72. doi: 10.1128/jb.175.22.7363-7372.1993.
52. Li X-Z, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob

Agents Chemother. 1994;38(8):1732–41. doi:10.1128/AAC.38.8.1732.
53. Li X-Z, Ma D, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob Agents Chemother. 1994;38(8):1742–52. doi:10.1128/AAC.38.8.1742.
54. Nikaido H, Takatsuka Y. Mechanisms of RND multidrug efflux pumps. Biochim Biophys Acta. 2009;1794(5):769–81. doi:10.1016/j.bbapap.2008.10.004.
55. Long F, Su CC, Lei HT, Bolla JR, Do SV, Yu EW. Structure and mechanism of the tripartite CusCBA heavy-metal efflux complex. Philos Trans R Soc Lond B Biol Sci. 2012;367(1592):1047–58. doi:10.1098/rstb.2011.0203.
56. Poole K. Stress responses as determinants of antimicrobial resistance in *Pseudomonas aeruginosa*: multidrug efflux and more. Can J Microbiol. 2014;60(12):783–91. doi:10.1139/cjm-2014-0666.
57. Murakami S, Nakashima R, Yamashita E, Matsumoto T, Yamaguchi A. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature. 2006;443(7108):173–9. doi:10.1038/nature05076.
58. Murakami S. Multidrug efflux transporter, AcrB-the pumping mechanism. Curr Opin Struct Biol. 2008;18(4):459–65. doi:10.1016/j.sbi.2008.06.007.
59. Nakashima R, Sakurai K, Yamasaki S, Nishino K, Yamaguchi A. Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. Nature. 2011;480(7378):565–9. doi:10.1038/nature10641.
60. Du D, Wang Z, James NR, Voss JE, Klimont E, Ohene-Agyei T, Venter H, Chiu W, Luisi BF. Structure of the AcrAB-TolC multidrug efflux pump. Nature. 2014;509(7501):512–5. doi:10.1038/nature13205.
61. Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature. 2000;405(6789):914–9. doi:10.1038/35016007.
62. Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V. The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc Natl Acad Sci U S A. 2009;106(17):7173–8. doi:10.1073/pnas.0900693106.
63. Koenderink JB, Kavishe RA, Rijpma SR, Russel FG. The ABCs of multidrug resistance in malaria. Trends Parasitol. 2010;26(9):440–6. doi:10.1016/j.pt.2010.05.002.
64. Sulavik MC, Houseweart C, Cramer C, Jiwani N, Murgolo N, Greene J, DiDomenico B, Shaw KJ, Miller GH, Hare R, Shimer G. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother. 2001;45(4):1126–36. doi:10.1128/AAC.45.4.1126-1136.2001.
65. Nichols RJ, Sen S, Choo YJ, Beltrao P, Zietek M, Chaba R, Lee S, Kazmierczak KM, Lee KJ, Wong A, Shales M, Lovett S, Winkler ME, Krogan NJ, Typas A, Gross CA. Phenotypic landscape of a bacterial cell. Cell. 2011;144(1):143–56. doi:10.1016/j.cell.2010.11.052.
66. DeMarco CE, Cushing LA, Frempong-Manso E, Seo SM, Jaravaza TA, Kaatz GW. Efflux-related resistance to norfloxacin, dyes, and biocides in bloodstream isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2007;51(9):3235–9. doi:10.1128/AAC.00430-07.
67. Costa SS, Falcao C, Viveiros M, Machado D, Martins M, Melo-Cristino J, Amaral L, Couto I. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. BMC Microbiol. 2011;11:241. doi:10.1186/1471-2180-11-241.
68. Huet AA, Raygada JL, Mendiratta K, Seo SM, Kaatz GW. Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple in vitro exposures to biocides and dyes. Microbiology. 2008;154(10):3144–53. doi:10.1099/mic.0.2008/021188-0.
69. Sutcliffe J, Tait-Kamradt A, Wondrack L. *Streptococcus pneumoniae* and *Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimicrob Agents Chemother. 1996;40(8):1817–24.
70. Tait-Kamradt A, Clancy J, Cronan M, Dib-Hajj F, Wondrack L, Yuan W, Sutcliffe J. *mefE* is necessary for the erythromycin-resistant M phenotype in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 1997;41(10):2251–5.
71. Garvey MI, Piddock LJ. The efflux pump inhibitor reserpine selects multidrug-resistant *Streptococcus pneumoniae* strains that overexpress the ABC transporters PatA and PatB. Antimicrob Agents Chemother. 2008;52(5):1677–85. doi:10.1128/AAC.01644-07.
72. Garvey MI, Baylay AJ, Wong RL, Piddock LJ. Overexpression of *patA* and *patB*, which encode ABC transporters, is associated with fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2011;55(1):190–6. doi:10.1128/AAC.00672-10.
73. Nikaido H. The role of outer membrane and efflux pumps in the resistance of Gram-negative bacteria. Can we improve drug access? Drug Resist Updat. 1998;1(2):93–8. doi:10.1016/S1368-7646(98)80023-X.
74. Brown DG, May-Dracka TL, Gagnon MM, Tommasi R. Trends and exceptions of physical properties on antibacterial activity for Gram-positive and Gram-negative pathogens. J Med Chem. 2014;57(23):10144–61. doi:10.1021/jm501552x.
75. Palmer M. Efflux of cytoplasmically acting antibiotics from Gram-negative bacteria: periplasmic substrate capture by multicomponent efflux pumps inferred from their cooperative action with single-component transporters. J Bacteriol. 2003;185(17):5287–9. doi:10.1128/JB.185.17.5287-5289.2003.
76. Li X-Z, Nikaido H, Poole K. Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1995;39(9):1948–53. doi:10.1128/AAC.39.9.1948.
77. Damier-Piolle L, Magnet S, Bremont S, Lambert T, Courvalin P. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2008;52(2):557–62. doi:10.1128/AAC.00732-07.
78. Coyne S, Courvalin P, Périchon B. Efflux-mediated antibiotic resistance in *Acinetobacter* spp. Antimicrob Agents Chemother. 2011;55(3):947–53. doi:10.1128/AAC.01388-10.
79. Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob Agents Chemother. 1999;43(11):2624–8.
80. Hocquet D, Roussel-Delvallez M, Cavallo JD, Plésiat P. MexAB-OprM- and MexXY-overproducing mutants are very prevalent among clinical strains of *Pseudomonas aeruginosa* with reduced susceptibility to ticarcillin. Antimicrob Agents Chemother. 2007;51(4):1582–3. doi: 10.1128/AAC.01334-06.
81. Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, Neshat S, Yamagishi J, Li X-Z, Nishino T. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol Microbiol. 1996;21(4):713–24. doi:10.1046/j.1365-2958.1996.281397.x.
82. Reinhardt A, Köhler T, Wood P, Rohner P, Dumas JL, Ricou B, van Delden C. Development and persistence of antimicrobial resistance in *Pseudomonas aeruginosa*: a longitudinal observation in mechanically ventilated patients. Antimicrob Agents Chemother. 2007;51(4):1341–50. doi:10.1128/AAC.01278-06.
83. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system.

Microbiology. 1995;141(3):611–22. doi:10.1099/13500872-141-3-611.
84. Shafer WM, Qu X, Waring AJ, Lehrer RI. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc Natl Acad Sci U S A. 1998;95(4):1829–33.
85. Golparian D, Shafer WM, Ohnishi M, Unemo M. Importance of multidrug efflux pumps in the antimicrobial resistance property of clinical multidrug-resistant isolates of *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 2014;58(6):3556–9. doi:10.1128/AAC.00038-14.
86. Blair JM, Bavro VN, Ricci V, Modi N, Cacciotto P, Kleinekathfer U, Ruggerone P, Vargiu AV, Baylay AJ, Smith HE, Brandon Y, Galloway D, Piddock LJ. AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. Proc Natl Acad Sci U S A. 2015;112(11):3511–6. doi:10.1073/pnas.1419939112.
87. Li X-Z, Zhang L, Nikaido H. Efflux pump-mediated intrinsic drug resistance in *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 2004;48(7):2415–23. doi:10.1128/AAC.48.7.2415-2423.2004.
88. Balganesh M, Kuruppath S, Marcel N, Sharma S, Nair A, Sharma U. Rv1218c, an ABC transporter of *Mycobacterium tuberculosis* with implications in drug discovery. Antimicrob Agents Chemother. 2010;54(12):5167–72. doi:10.1128/AAC.00610-10.
89. Balganesh M, Dinesh N, Sharma S, Kuruppath S, Nair AV, Sharma U. Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis activity of potential drug candidates. Antimicrob Agents Chemother. 2012;56(5):2643–51. doi:10.1128/AAC.06003-11.
90. Rodrigues L, Villellas C, Bailo R, Viveiros M, Ainsa JA. Role of the Mmr efflux pump in drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2013;57(2):751–7. doi:10.1128/AAC.01482-12.
91. Schmalstieg AM, Srivastava S, Belkaya S, Deshpande D, Meek C, Leff R, van Oers NS, Gumbo T. The antibiotic resistance arrow of time: efflux pump induction is a general first step in the evolution of mycobacterial drug resistance. Antimicrob Agents Chemother. 2012;56(9):4806–15. doi:10.1128/AAC.05546-11.
92. Wang K, Pei H, Huang B, Zhu X, Zhang J, Zhou B, Zhu L, Zhang Y, Zhou FF. The expression of ABC efflux pump, Rv1217c-Rv1218c, and its association with multidrug resistance of *Mycobacterium tuberculosis* in China. Curr Microbiol. 2013;66(3):222–6. doi:10.1007/s00284-012-0215-3.
93. Machado D, Couto I, Perdigao J, Rodrigues L, Portugal I, Baptista P, Veigas B, Amaral L, Viveiros M. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. PLoS ONE. 2012;7(4):e34538. doi:10.1371/journal.pone.0034538.
94. Zimic M, Fuentes P, Gilman RH, Gutierrez AH, Kirwan D, Sheen P. Pyrazinoic acid efflux rate in *Mycobacterium tuberculosis* is a better proxy of pyrazinamide resistance. Tuberculosis. 2012;92(1):84–91. doi:10.1016/j.tube.2011.09.002.
95. Coleman JJ, Mylonakis E. Efflux in fungi: la piece de resistance. PLoS Pathog. 2009;5(6):e1000486. doi:10.1371/journal.ppat.1000486.
96. Pfaller MA. Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med. 2012;125 Suppl 1:S3–13. doi:10.1016/j.amjmed.2011.11.001.
97. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev. 2011;75(2):213–67. doi:10.1128/MMBR.00045-10.
98. Blum G, Hortnagl C, Jukic E, Erbeznik T, Pumpel T, Dietrich H, Nagl M, Speth C, Rambach G, Lass-Florl C. New insight into amphotericin B resistance in *Aspergillus terreus*. Antimicrob Agents Chemother. 2013;57(4):1583–8. doi:10.1128/AAC.01283-12.
99. Morschhauser J. Regulation of multidrug resistance in pathogenic fungi. Fungal Genet Biol. 2010;47(2):94–106. doi:10.1016/j.fgb.2009.08.002.
100. Tsao S, Rahkhoodaee F, Raymond M. Relative contributions of the *Candida albicans* ABC transporters Cdr1p and Cdr2p to clinical azole resistance. Antimicrob Agents Chemother. 2009;53(4):1344–52. doi:10.1128/AAC.00926-08.
101. Andrade AC, Del Sorbo G, Van Nistelrooy JG, Waard MA. The ABC transporter AtrB from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds. Microbiology. 2000;146(8):1987–97. doi: 10.1099/00221287-146-8-1987.
102. Eddouzi J, Parker JE, Vale-Silva LA, Coste A, Ischer F, Kelly S, Manai M, Sanglard D. Molecular mechanisms of drug resistance in clinical *Candida* species isolated from Tunisian hospitals. Antimicrob Agents Chemother. 2013;57(7):3182–93. doi:10.1128/AAC.00555-13.
103. Lespine A, Menez C, Bourguinat C, Prichard RK. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: prospects for reversing transport-dependent anthelmintic resistance. Int J Parasitol Drugs Drug Resist. 2012;2:58–75. doi:10.1016/j.ijpddr.2011.10.001.
104. Greenberg RM. ABC multidrug transporters in schistosomes and other parasitic flatworms. Parasitol Int. 2013;62(6):647–53. doi:10.1016/j.parint.2013.02.006.
105. Kasinathan RS, Sharma LK, Cunningham C, Webb TR, Greenberg RM. Inhibition or knockdown of ABC transporters enhances susceptibility of adult and juvenile schistosomes to praziquantel. PLoS Negl Trop Dis. 2014;8(10):e3265. doi:10.1371/journal.pntd.0003265.
106. Petersen I, Eastman R, Lanzer M. Drug-resistant malaria: molecular mechanisms and implications for public health. FEBS Lett. 2011;585(11):1551–62. doi:10.1016/j.febslet.2011.04.042.
107. Li X-Z, Zhang L, Poole K. Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. J Antimicrob Chemother. 2000;45(4):433–6. doi:10.1093/jac/45.4.433.
108. Kropinski AM, Kuzio J, Angus BL, Hancock RE. Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersusceptible mutant of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1982;21(2):310–9. doi: 10.1128/AAC.21.2.310.
109. Cagliero C, Mouline C, Cloeckaert A, Payot S. Synergy between efflux pump CmeABC and modifications in ribosomal proteins L4 and L22 in conferring macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob Agents Chemother. 2006;50(11):3893–6. doi:10.1128/AAC.00616-06.
110. Mazzariol A, Cornaglia G, Nikaido H. Contributions of the AmpC β-lactamase and the AcrAB multidrug efflux system in intrinsic resistance of *Escherichia coli* K-12 to β-lactams. Antimicrob Agents Chemother. 2000;44(5):1387–90. doi:10.1128/AAC.44.5.1387-1390.2000.
111. Poole K. Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol. 2012;20(5):227–34. doi:10.1016/j.tim.2012.02.004.
112. Salunkhe P, Smart CH, Morgan JA, Panagea S, Walshaw MJ, Hart CA, Geffers R, Tummler B, Winstanley C. A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. J Bacteriol. 2005;187(14):4908–20. doi:10.1128/JB.187.14.4908-4920.2005.
113. Szumowski JD, Adams KN, Edelstein PH, Ramakrishnan L. Antimicrobial efflux pumps and *Mycobacterium tuberculosis*

drug tolerance: evolutionary considerations. Curr Top Microbiol Immunol. 2013;374:81–108. doi:10.1007/82_2012_300.
114. Angiolella L, Stringaro AR, De Bernardis F, Posteraro B, Bonito M, Toccacieli L, Torosantucci A, Colone M, Sanguinetti M, Cassone A, Palamara AT. Increase of virulence and its phenotypic traits in drug-resistant strains of *Candida albicans*. Antimicrob Agents Chemother. 2008;52(3):927–36. doi:10.1128/AAC.01223-07.
115. Sanguinetti M, Posteraro B, La Sorda M, Torelli R, Fiori B, Santangelo R, Delogu G, Fadda G. Role of AFR1, an ABC transporter-encoding gene, in the in vivo response to fluconazole and virulence of *Cryptococcus neoformans*. Infect Immun. 2006;74(2):1352–9. doi:10.1128/IAI.74.2.1352-1359.2006.
116. Rosenthal PJ. The interplay between drug resistance and fitness in malaria parasites. Mol Microbiol. 2013;89(6):1025–38. doi:10.1111/mmi.12349.
117. Cuthbertson L, Nodwell JR. The TetR family of regulators. Microbiol Mol Biol Rev. 2013;77(3):440–75. doi:10.1128/MMBR.00018-13.
118. Hobbs EC, Yin X, Paul BJ, Astarita JL, Storz G. Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects antibiotic resistance. Proc Natl Acad Sci U S A. 2012;109(41):16696–701. doi:10.1073/pnas.1210093109.
119. Schindler BD, Seo SM, Birukou I, Brennan RG, Kaatz GW. Mutations within the *mepA* operator affect binding of the MepR regulatory protein and its induction by MepA substrates in *Staphylococcus aureus*. J Bacteriol. 2015;197(6):1104–14. doi:10.1128/JB.02558-14.
120. Macone AB, Caruso BK, Leahy RG, Donatelli J, Weir S, Draper MP, Tanaka SK, Levy SB. In vitro and in vivo antibacterial activities of omadacycline, a novel aminomethylcycline. Antimicrob Agents Chemother. 2014;58(2):1127–35. doi:10.1128/AAC.01242-13.
121. Draper MP, Weir S, Macone A, Donatelli J, Trieber CA, Tanaka SK, Levy SB. Mechanism of action of the novel aminomethylcycline antibiotic omadacycline. Antimicrob Agents Chemother. 2014;58(3):1279–83. doi:10.1128/AAC.01066-13.
122. Pucci MJ, Bush K. Investigational antimicrobial agents of 2013. Clin Microbiol Rev. 2013;26(4):792–821. doi:10.1128/CMR.00033-13.
123. Nakashima R, Sakurai K, Yamasaki S, Hayashi K, Nagata C, Hoshino K, Onodera Y, Nishino K, Yamaguchi A. Structural basis for the inhibition of bacterial multidrug exporters. Nature. 2013;500(7460):102–6. doi:10.1038/nature12300.
124. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H, Lee VJ. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob Agents Chemother. 2001;45(1):105–16. doi:10.1128/AAC.45.1.105-116.2001.
125. Vargiu AV, Ruggerone P, Opperman TJ, Nguyen ST, Nikaido H. Inhibition of *E. coli* AcrB multidrug efflux pump by MBX2319: molecular mechanism and comparison with other inhibitors. Antimicrob Agents Chemother. 2014;58(10):6224–34. doi:10.1128/AAC.03283-14.
126. Hansen LH, Johannesen E, Burmølle M, Sørensen AH, Sørensen SJ. Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in *Escherichia coli*. Antimicrob Agents Chemother. 2004;48(9):3332–7. doi:10.1128/AAC.48.9.3332-3337.2004.
127. Li X-Z, Elkins CA, Zgurskaya HI, editors. Efflux-mediated antimicrobial resistance in bacteria: mechanisms, regulation and clinical implications. Adis, Springer Nature; 2016. doi:10.1007/978-3-319-39658-3.
128. Lee EW, Huda MN, Kuroda T, Mizushima T, Tsuchiya T. EfrAB, an ABC multidrug efflux pump in *Enterococcus faecalis*. Antimicrob Agents Chemother. 2003;47(12):3733–8. doi:10.1128/AAC.47.12.3733-3738.2003.
129. Poelarends GJ, Mazurkiewicz P, Konings WN. Multidrug transporters and antibiotic resistance in *Lactococcus lactis*. Biochim Biophys Acta. 2002;1555(1–3):1–7. doi:10.1016/S0005-2728(02)00246-3.
130. Velamakanni S, Yao Y, Gutmann DA, van Veen HW. Multidrug transport by the ABC transporter Sav 1866 from *Staphylococcus aureus*. Biochemistry. 2008;47(35):9300–8. doi:10.1021/bi8006737.
131. Kobayashi N, Nishino K, Yamaguchi A. Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. J Bacteriol. 2001;183(19):5639–44. doi: 10.1128/JB.183.19.5639-5644.2001.
132. Hu RM, Liao ST, Huang CC, Huang YW, Yang TC. An inducible fusaric acid tripartite efflux pump contributes to the fusaric acid resistance in *Stenotrophomonas maltophilia*. PLoS ONE. 2012;7(12):e51053. doi:10.1371/journal.pone.0051053.
133. Marrer E, Schad K, Satoh AT, Page MG, Johnson MM, Piddock LJ. Involvement of the putative ATP-dependent efflux proteins PatA and PatB in fluoroquinolone resistance of a multidrug-resistant mutant of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2006;50(2):685–93. doi:10.1128/AAC.50.2.685-693.2006.
134. Huda N, Lee EW, Chen J, Morita Y, Kuroda T, Mizushima T, Tsuchiya T. Molecular cloning and characterization of an ABC multidrug efflux pump, VcaM, in Non-O1 *Vibrio cholerae*. Antimicrob Agents Chemother. 2003;47(8):2413–7. doi:10.1128/AAC.47.8.2413-2417.2003.
135. Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother. 2003;47(5):1719–26. doi:10.1128/AAC.47.5.1719-1726.2003.
136. Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol. 2002;36(3):199–206. doi:10.1016/S1087-1845(02)00016-6.
137. Henry M, Alibert S, Orlandi-Pradines E, Bogreau H, Fusai T, Rogier C, Barbe J, Pradines B. Chloroquine resistance reversal agents as promising antimalarial drugs. Curr Drug Targets. 2006;7(8):935–48. doi:10.2174/138945006778019372.
138. Veiga MI, Osorio NS, Ferreira PE, Franzen O, Dahlstrom S, Lum JK, Nosten F, Gil JP. Complex polymorphisms in the *Plasmodium falciparum* multidrug resistance protein 2 gene and its contribution to antimalarial response. Antimicrob Agents Chemother. 2014;58(12):7390–7. doi:10.1128/AAC.03337-14.
139. Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J Bacteriol. 1990;172(12):6942–9. doi: 10.1128/jb.172.12.6942-6949.1990.
140. Littlejohn TG, Paulsen IT, Gillespie MT, Tennent JM, Midgley M, Jones IG, Purewal AS, Skurray RA. Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. FEMS Microbiol Lett. 1992;95(2–3):259–65. doi:10.1016/0378-1097(92)90439-U.
141. Roca I, Marti S, Espinal P, Martínez P, Gibert I, Vila J. CraA, an MFS efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2009;53(9):4013–4. doi:10.1128/AAC.00584-09.
142. Nishino K, Latifi T, Groisman EA. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. Mol Microbiol. 2006;59(1):126–41. doi:10.1111/j.1365-2958.2005.04940.x.

143. Horiyama T, Yamaguchi A, Nishino K. TolC dependency of multidrug efflux systems in *Salmonella* enterica serovar Typhimurium. J Antimicrob Chemother. 2010;65(7):1372–6. doi:10.1093/jac/dkq160.
144. Nishino K, Yamaguchi A. Overexpression of the response regulator *evgA* of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. J Bacteriol. 2001;183(4):1455–8. doi:10.1128/JB.183.4.1455-1458.2001.
145. Eguchi Y, Oshima T, Mori H, Aono R, Yamamoto K, Ishihama A, Utsumi R. Transcriptional regulation of drug efflux genes by EvgAS, a two-component system in *Escherichia coli*. Microbiology. 2003;149(10):2819–28. doi:10.1099/mic.0.26460-0.
146. Nishino K, Yamaguchi A. Role of histone-like protein H-NS in multidrug resistance of *Escherichia coli*. J Bacteriol. 2004;186(5):1423–9. doi:10.1128/JB.186.5.1423-1429.2004.
147. Srinivasan VB, Singh BB, Priyadarshi N, Chauhan NK, Rajamohan G. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. PLoS ONE. 2014;9(5):e96288. doi:10.1371/journal.pone.0096288.
148. Cattoir V, Poirel L, Nordmann P. Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. Antimicrob Agents Chemother. 2008;52(10):3801–4. doi:10.1128/AAC.00638-08.
149. Ainsa JA, Blokpoel MC, Otal I, Young DB, De Smet KA, Martin C. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. J Bacteriol. 1998;180(22):5836–43.
150. Ramón-García S, Mick V, Dainese E, Martin C, Thompson CJ, De Rossi E, Manganelli R, Ainsa JA. Functional and genetic characterization of the Tap efflux pump in *Mycobacterium bovis* BCG. Antimicrob Agents Chemother. 2012;56(4):2074–83. doi:10.1128/AAC.05946-11.
151. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H^+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. Antimicrob Agents Chemother. 2005;49(10):4362–4. doi:10.1128/AAC.49.10.4362-4364.2005.
152. Miyamae S, Ueda O, Yoshimura F, Hwang J, Tanaka Y, Nikaido H. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides thetaiotaomicron*. Antimicrob Agents Chemother. 2001;45(12):3341–6. doi:10.1128/AAC.45.12.3341-3346.2001.
153. Turner RJ, Taylor DE, Weiner JH. Expression of *Escherichia coli* TehA gives resistance to antiseptics and disinfectants similar to that conferred by multidrug resistance efflux pumps. Antimicrob Agents Chemother. 1997;41(2):440–4.
154. Swick MC, Morgan-Linnell SK, Carlson KM, Zechiedrich L. Expression of multidrug efflux pump genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob Agents Chemother. 2011;55(2):921–4. doi:10.1128/AAC.00996-10.
155. Gomes AR, Westh H, de Lencastre H. Origins and evolution of methicillin-resistant *Staphylococcus aureus* clonal lineages. Antimicrob Agents Chemother. 2006;50(10):3237–44. doi:10.1128/AAC.00521-06.
156. Lennen RM, Politz MG, Kruziki MA, Pfleger BF. Identification of transport proteins involved in free fatty acid efflux in *Escherichia coli*. J Bacteriol. 2013;195(1):135–44. doi:10.1128/JB.01477-12.
157. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrob Agents Chemother. 2001;45(12):3375–80. doi:10.1128/AAC.45.12.3375-3380.2001.
158. Coyne S, Rosenfeld N, Lambert T, Courvalin P, Perichon B. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2010;54(10):4389–93. doi:10.1128/AAC.00155-10.
159. Rajamohan G, Srinivasan VB, Gebreyes WA. Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. J Antimicrob Chemother. 2010;65(2):228–32. doi:10.1093/jac/dkp427.
160. Köhler T, Michéa-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechère JC. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol. 1997;23(2):345–54. doi:10.1046/j.1365-2958.1997.2281594.x.
161. Li X-Z, Zhang L, Poole K. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother. 2002;46(2):333–43. doi: 10.1128/AAC.46.2.333-343.2002.
162. Zhang L, Li XZ, Poole K. SmeDEF multidrug efflux pump contributes to intrinsic multidrug resistance in *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother. 2001;45(12):3497–503. doi: 10.1128/AAC.45.12.3497-3503.2001.

The Functional Resistance of Biofilms

11

Elias K. Manavathu and Jose A. Vazquez

1 Introduction

Antimicrobial drug tolerance/resistance exhibited by cells embedded in biofilm appears to be multifactorial, involving mechanisms similar to conventional planktonic antimicrobial drug resistance due to spontaneous mutations, secondary acquisition of drug resistance determinants from external sources through mobile genetic elements such as plasmids or transposons, and increased efflux pump activity governed by biofilm specific transcriptional regulators. In addition, mechanisms specific to the biofilm lifestyle of the organism can also confer unprecedented antimicrobial drug tolerance (otherwise known as resistance to the killing action of the antibiotic) even at high concentrations. Microbial biofilms have emerged as a significant clinical problem in the treatment and management of many infectious diseases over the last two decades. There are several reasons for the steady increase in the frequency of microbial biofilm being recognized in clinical settings, especially the increased use of life-saving or quality of life improving artificial devices. The existence of microbial biofilms impacts the treatment and care of patients suffering from infectious diseases in numerous ways. This chapter describes the known mechanisms of high level drug tolerance/resistance exhibited by biofilm and its implications in areas affecting the treatment of infectious diseases.

E.K. Manavathu, Ph.D.
Division of Infectious Diseases, Department of Medicine, Medical College of Georgia, Augusta University, Augusta, GA, USA

J.A. Vazquez, M.D. (✉)
Division of Infectious Diseases, Medical College of Georgia, Augusta University, Augusta, GA, USA
e-mail: jvazquez@augusta.edu

1.1 Definition and General Description

Biofilms are highly organized communities of microorganisms made up of one or more species [1–6] attached to a biotic or abiotic solid surface and encased in a self-synthesized extracellular matrix (ECM). The ECM varies in chemical composition depending on the organism, but is primarily made up of complex exopolysaccharides [7–11], proteins [12, 13], and nucleic acids [14–17]. Microbial biofilm formation is a multistep process [18–22] initiated by the presence of a critical number of cells (often sensed by the extraordinary chemical communication ability among individual cells or quorum sensing) in a localized niche [23–28]. The biofilm life cycle has distinct developmental stages including attachment of planktonic cells to a solid surface, growth of the cells into a mature biofilm community, and the eventual dispersal of the cells from the microbial community into the surrounding environment [19]. The initial adherence to a solid surface is a transient step, and is followed by the firm attachment, which is promoted by bacterial adhesion molecules. The attached microbial cells multiply in a favorable niche and form a complex three-dimensional microbial community with an elaborate chemical communication network. Microbial cells that make up the biofilm adhere to each other and to the surface with the ECM which often incorporates host derived cellular components. In bacterial biofilm communities, it is estimated that they are composed of 75–95 % ECM and only 5–25 % bacteria. In a fully matured biofilm, the microbial community is encased in the ECM that provides the cells protection from harsh environments, including the presence of antimicrobial drugs [2, 29–31] and evasion from the immune system of the host [32–35]. Under the appropriate conditions, the biofilm can persist for a long period of time. Individual cells or groups of cells will be eventually detached from the biofilm community and be dispersed to the neighboring environments subsequently forming other biofilm communities. It is the process of dispersal from an existing biofilm that makes

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_11

them clinically threatening by providing a reservoir of microbes capable of initiating recurrent and active infection [29, 36–38].

1.2 Monomicrobial and Mixed Microbial Biofilms

Microbial biofilm can be formed by a single species (monomicrobial biofilm) or multiple species (polymicrobial biofilms). Recent advancements in metagenomics and microbiome studies suggest that polymicrobial biofilms are much more likely to form in nature, whether it is the human body, the root system of an alpha-alpha plant, a shiny rock in a cold lake or a hot spring [18, 39–43]. Polymicrobial biofilm producing organisms belong to highly different taxonomic groups, including those belonging to different kingdoms forming an interkingdom (e.g., fungal-bacterial assemblage) interaction producing biofilm [44, 45]. The characteristics of monomicrobial biofilm produced ECM may be different from that of the polymicrobial biofilm and such variations will have profound impact on the susceptibility of the organism(s) within the biofilm to antimicrobial drugs [46, 47]. Moreover, a species-specific targeted antimicrobial therapy of a polymicrobial biofilm complex may eliminate one species and provide an opportunity for the more virulent organism to flourish and thrive. Figures 11.1 and 11.2 show typical examples of polymicrobial biofilms formed by *Aspergillus fumigatus* with *Pseudomonas aeruginosa* and *Scedosporium prolificans* with *P. aeruginosa*. These organisms are common inhabitants of the airways of cystic fibrosis patients.

2 Prevalence of Microbial Biofilms

In theory, all microbial species from Gram-negative to Gram-positive bacteria, pathogenic yeasts to filamentous fungi, and fresh water algae to marine phytoplankton are capable of adhering to a solid surface and are capable of initiating, producing, and sustaining biofilms in appropriate ecological niches. The majority of the biofilm studies have focused on those organisms causing diseases in man such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Candida albicans*, and *Aspergillus fumigatus* because of their prevalence and clinical importance. The National Institute of Health now estimates that as many as 80 % of the clinical infections seen in humans have a biofilm origin. Subsequently, this translates into a significant economic impact due to microbial biofilms in terms of increased morbidity and mortality, increased length of stay, and the additional economic cost associated with the care and treatment of recalcitrant microbial infections for prolonged periods of time.

Although many microorganisms of clinical importance are capable of producing biofilms, there are significant differences in the ability of even different members of a single genus in the production of biofilm. For example, most *Candida* species are capable of producing biofilms, but there is significant variability in their ability to form mature biofilm. For instance, *Candida glabrata* and *Candida krusei* are not prolific producers of dense, thick biofilm, like the biofilm produced by *C. albicans* [48–52]. Nevertheless, all *Candida* biofilms are known to exhibit the hallmark biofilm characteristics such as reduced susceptibility to antifungal drugs, decreased growth rate of the biofilm bound cells, the ability to survive under nutrient limiting conditions, and the presence of an extracellular matrix.

3 Antibiotic and Microbicide Tolerance/Resistance of Microbial Biofilms

Microbial cells in biofilm are generally highly tolerant/resistant to antimicrobial drugs and thus difficult to eradicate with standard antimicrobial therapy. In fact, biofilm cells show up to a 1000-fold more tolerance/resistance to antimicrobial drugs than their planktonic cell counterparts. Initially, it was believed that the ECM was the main culprit rendering resistance to the antimicrobial drug by acting as a physical barrier and limiting the accessibility of the drug to the target cells. However, recent evidence indicates that limiting the accessibility of the drug to the target cells by the ECM acting as a physical barrier is only a small part of the multitude of drug resistance mechanisms described within microbial biofilms.

Multidrug tolerance (MDT) is the ability of a microorganism to resist killing by antibiotics or microbicides and it is mechanistically and genetically distinct from multidrug resistance (MDR). Multidrug tolerance is a noninheritable phenotypic trait that is not passed on from parent to offspring and appears to be genetically unstable. Thus, it is not induced by a mutation. In contrast, MDR is an inheritable trait that is usually genetically stable and developed by spontaneous mutations or by the secondary acquisition of the trait from an external source such as mobile genetic elements (plasmids and transposons). Most microorganisms such as bacteria, fungi, and at times even parasites display MDT. Figure 11.3 shows a brief diagrammatic illustration of the known mechanisms of MDT/MDR in microbial biofilms.

3.1 Role of Low Level Cell Divisional and Metabolic Rates

A typical microbial biofilm is composed of a physiologically heterogeneous cell population ranging from slow growing cells in stationary phase, to non-growing, near dormant but

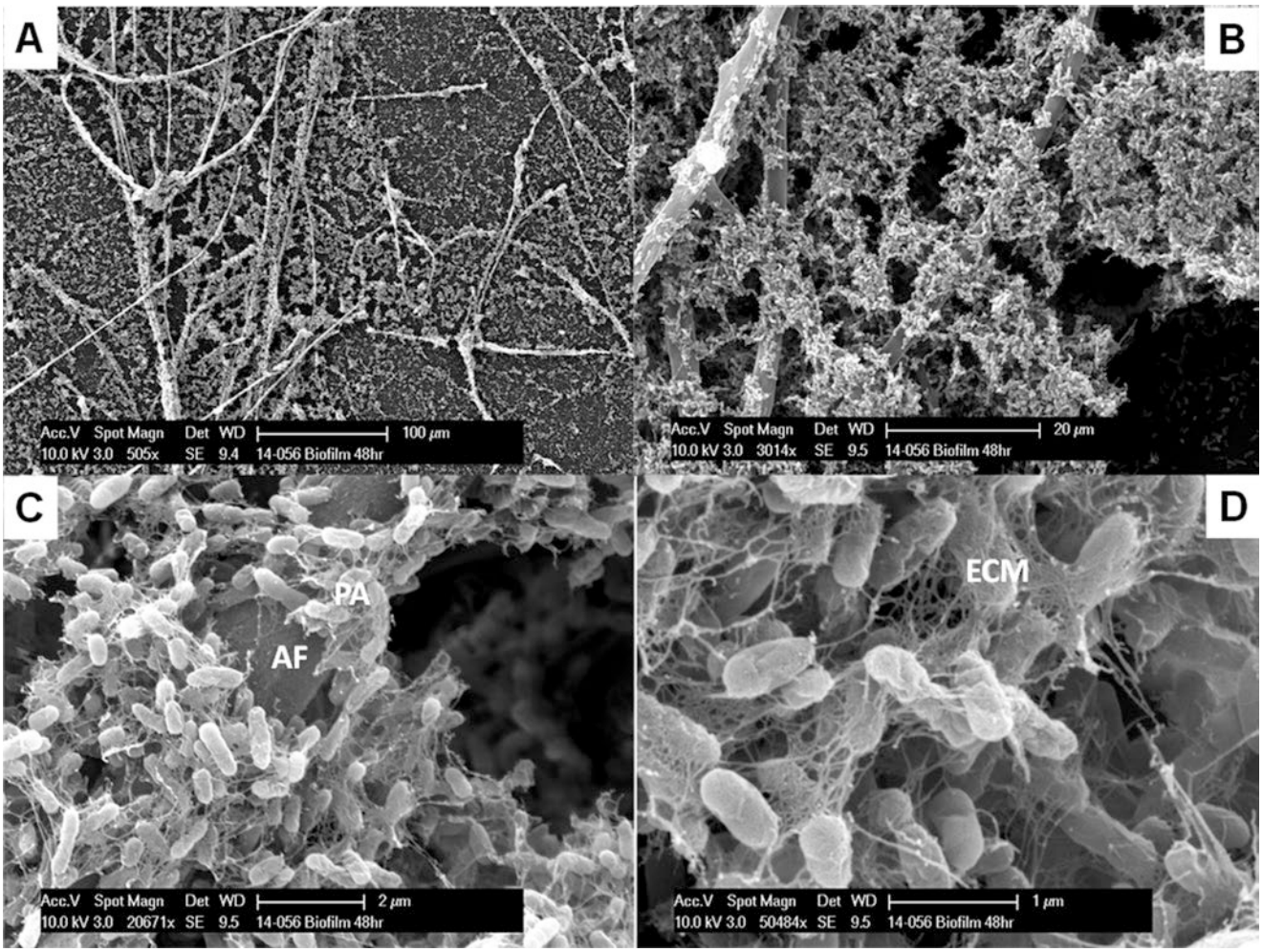


Fig. 11.1 Low (Panels A & B) and high (Panels C & D) magnification scanning electron micrograph of a polymicrobial biofilm formed by *Pseudomonas aeruginosa* and *Aspergillus fumigatus*. The biofilm was developed on Tissue Culture Thermanox 13 mm coverslips in SD broth in 12-well cell culture dishes at 35 °C for 48 h using *A. fumigatus* hyphae pregrown for 18 h. The biofilm was observed and imaged in a FEI XL30 scanning electron microscope (FEI, Hillsboro, OR) at 10 kV. Legends: AF, *Asergillus fumigatus* hypha; PA, *Pseudomonas aeruginosa* cells forming biofilm; ECM, extracellular matrix

live cells that persist [53]. A model theory has been proposed by Lewis et al. [54] to explain the unprecedented recalcitrance of biofilm to antimicrobial drugs and primarily focuses on two hallmark features of microbial biofilms: (1) the heterogeneity of the growth phases in the biofilm community and (2) the presence of the extracellular matrix that limits the effectiveness of antimicrobial drugs by preventing or limiting their accessibility to the target cells. It has been shown that a population of slow-growing, stationary-phase cells within the biofilm can tolerate the killing action of the fluoroquinolone antibiotic ofloxacin, in which a small population of cells within the biofilm was not killed by this agent [55].

Among the 100 s of antimicrobial drugs currently available on the market for treating microbial infections, the majority of them act on intracellular targets involved in various metabolic pathways, such as nucleic acid, protein, lipid, carbohydrate, cell membrane, and cell wall syntheses, just to name a few. The intracellular location of drug targets poses two problems for antibiotic activity: (1) the antimicrobial drug has to have easy access to the target molecule(s) to elicit its inhibitory action and (2) the need for physiologically active cells carrying out the targeted metabolic reaction(s), for the antimicrobial drug to be effective. This is particularly true in the case of cytocidal drugs, unless the antibiotic is able to cause lethal physical damage or injury to an existing cellular component in the absence of an active metabolic state. On the other hand, cytostatic drugs are not required to have physiologically active cells to arrest growth and prevent multiplication, but eradication of the infecting pathogen will not take place. In either case, low cellular divisional and metabolic rates within the biofilm cells make them less susceptible to antimicrobial drugs when compared to the

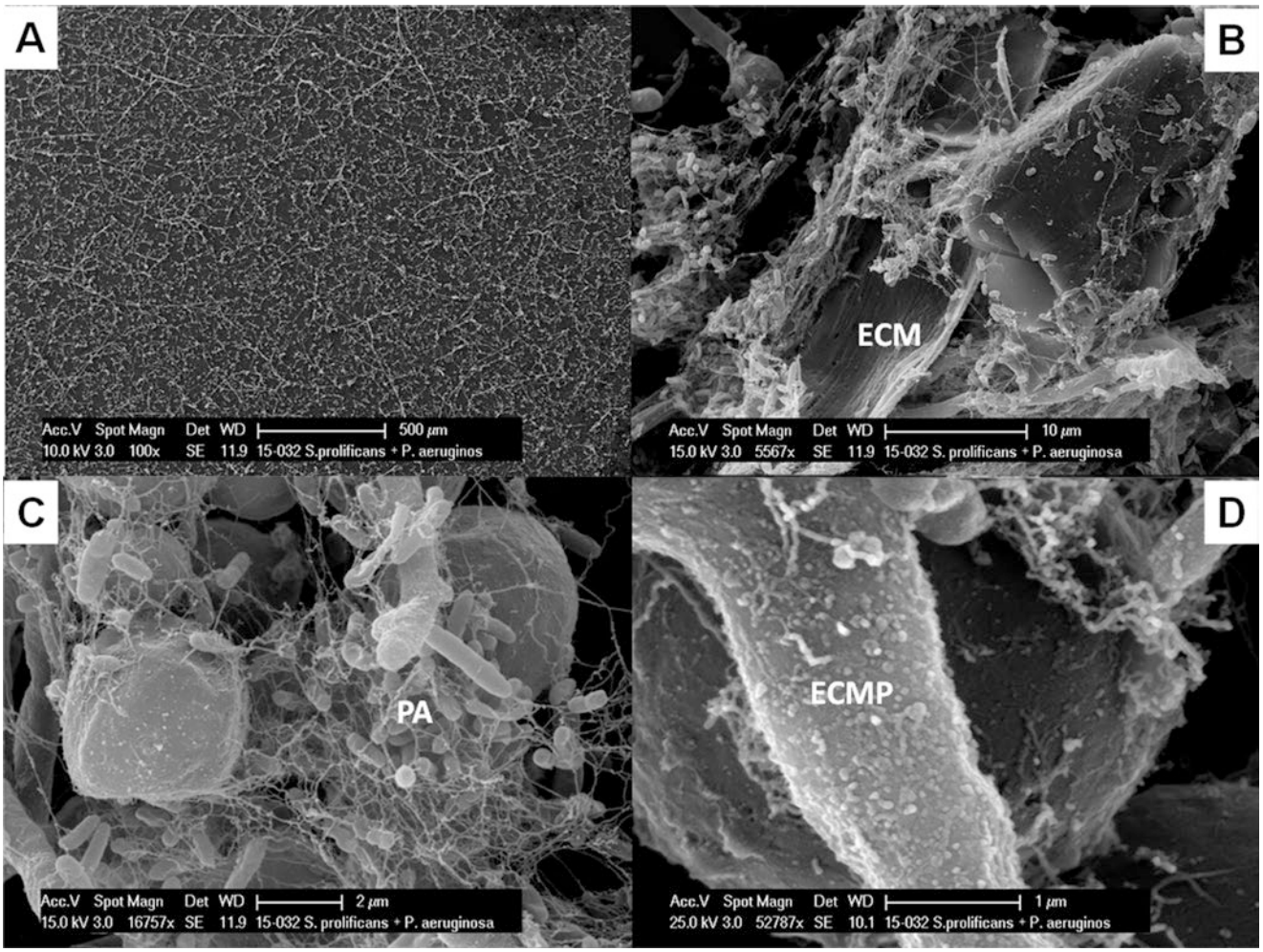


Fig. 11.2 Low (Panels A & B) and high (Panels C & D) magnification scanning electron micrograph of polymicrobial biofilm formed by *Pseudomonas aeruginosa* and *Scedosporium prolificans*. The biofilm was developed on Tissue Culture Thermanox 13 mm coverslips in SD broth in 12-well cell culture dishes at 35 °C for 48 h using *S. prolificans* hyphae pregrown for 18 h. Biofilm was observed and imaged in a FEI XL30 scanning electron microscope (FEI, Hillsboro, OR) at 10 kV. Legends: SP, *Scedosporium prolificans* hypha; PA, *Pseudomonas aeruginosa* cells forming biofilm; ECM, extracellular matrix; ECMP, extracellular matrix precursor produced by the fungal hyphae

actively growing cells. This reduced susceptibility is demonstrated by the cells within the biofilm by expressing tolerance to low levels of antimicrobial drugs by virtue of their metabolic inactivity.

3.2 Persister Cells

Persister cells are a very small subpopulation of isogenic cells (usually less than 1 %) present in stationary phase within the biofilm. Under biofilm conditions, the cultures show high level tolerance to multiple antimicrobials by virtue of their transient phenotypic expression. Persister cells neither grow nor die in the presence of high concentration of antimicrobial drugs [56–58]. A wide variety of microorganisms including bacteria and pathogenic yeasts are capable of producing persister cells in stationary phase planktonic and biofilm cultures [36, 47, 52, 55, 56, 58–63]. Since the genetic factor(s) that makes the persister cells tolerant to high concentrations of antibiotics is an unstable, nonheritable characteristic, it is different from the genetically stable inheritable antibiotic resistance trait that is passed on from the parent to the progeny from generation to generation. Persister cells that are recovered from stationary or biofilm cultures are susceptible to the same antibiotic when evaluated in planktonic cultures. Eventually, a new subpopulation of persister cells will develop during a new cycle of growth. Both biofilm and stationary phase cultures produce persister cells, but biofilm cultures often produce greater numbers of persister cells than stationary planktonic cultures. It has already been shown that persister cells are largely responsible for the drug recalcitrance and the development of chronic and recurrent microbial infections [57, 60, 64–69].

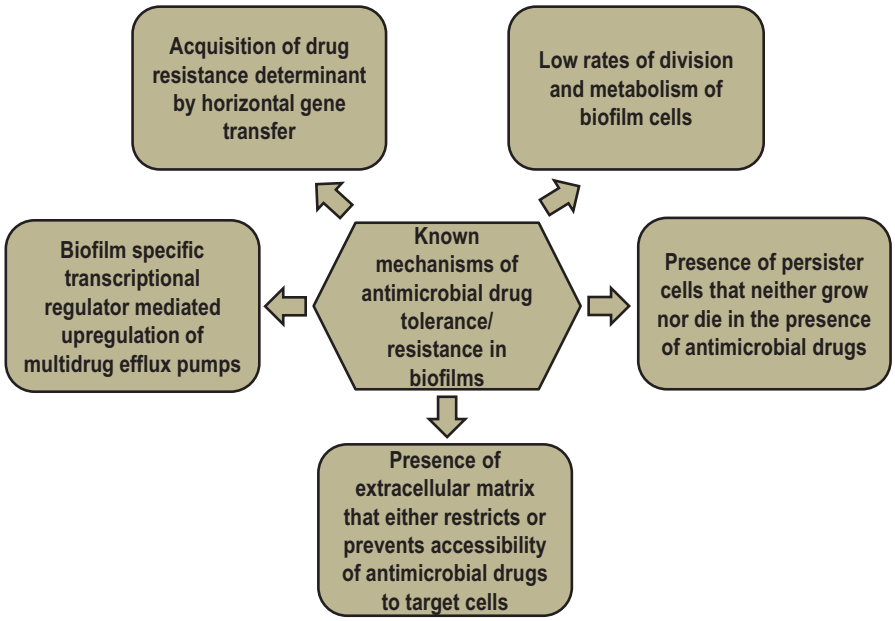


Fig. 11.3 Diagrammatic illustration of the known mechanisms of antimicrobial drug tolerance/resistance in biofilms. See the text for details

There is a clear difference between microbial resistance and microbial tolerance to antimicrobial drugs. In the former case, a resistant organism is able to grow and multiply in the presence of high concentrations of the antimicrobial that is inhibitory to drug-susceptible cells. However, in the case of drug tolerance, the tolerant cells are usually unable to grow and reproduce in the presence of the antimicrobial agent. These cells become refractory to the killing effect of a microbicidal drug and remain viable in a dormant state until the adverse pressure is removed. Afterwards, these cells become active again and resume a conventional growth cycle. The cells within the biofilm are highly resistant to killing by bactericidal antibiotics when compared to the logarithmic and stationary phase cells [55]. This unparalleled drug tolerance exhibited by the cells within the biofilm is due to the presence of persister cells.

3.2.1 Initiation of Persister Cells

In spite of the recent renewed interest in the developmental biology of persister cells, very little is known about the cell signal or the physiological cue that triggers the development of persistence in biofilms containing isogenic cells. Recently, Amato and Brynildsen [70] studied the effect of nutrient transition (or nutrient switching) on the development of persisters in *E. coli* biofilms. Nutrient transition (e.g., switching from one carbon source to another) induces a metabolic stress in both planktonic and biofilm cells. By switching the biofilm cells from one type of carbon source to another, these investigators showed that persister cell development in *E. coli* biofilm was dependent on nutrient transition which was mediated by guanosine tetraphosphate (ppGpp) (an alarmone which is involved in the stringent responses in bacteria causing the inhibition of RNA synthesis when there is a shortage of amino acids present in the cell) and a group of nucleoid-associated proteins. Alarmones are intracellular signal molecules that are produced during harsh environmental conditions [71]. The alarmones regulate gene expression at the transcription level. These signal molecules are produced in high concentrations when harsh environmental factors occur to organisms, such as lack of amino acids or carbon sources. Thus, one of the signature physiological cues that initiate the formation of persister cells in an isogenic biofilm culture is nutrient induced starvation stress. Interestingly, although both planktonic and biofilm cells are dependent on nutrient transition for the development of *E. coli* persister cells, the set of proteins and enzymes are significantly different and appeared to be independently regulated [70].

3.2.2 Microbicidal Drug Tolerance in Persister Cells

Although the presence of persister cells in stationary phase and biofilm cultures was discovered by Joseph Bigger in 1944 [72, 73], the mechanism(s) of their high level multidrug tolerance and the physiological nature of these cells have remained unexplained until recently. Persister cells are derived from a population of isogenic cells and their genetic makeup remains similar even after the development of persistence. Thus, differential gene expression appears to be the key to the phenotypic expression of persistence that allow the persisters to resist the killing effect of microbicidal drugs. One of the difficulties that have hampered progress in persister cell research is the inherent difficulty in isolating these special cells from an in vitro biofilm culture containing a population of isogenic cells. Using a special persister cell isolation method, Lewis et al. [56] and Keren et al. [58] performed a series of gene

profiling studies on *E. coli* persister cells. The expression profile studies indicate an elevated expression of a toxin-antitoxin gene module, as well as several other genes that possess the ability to block important cellular functions, including translation. This self-imposed shut down of the essential cellular functions prevents the microbicidal drug from corrupting the cellular functions and protects the cell from lethal consequences. The corollary is also true. Expression of the chromosomally encoded toxin genes such as RelE [74] and HipA [75] provides a sharp increase in the number of persister cells. Thus, these investigators suggested that a random fluctuation in levels of multidrug tolerance proteins may lead to the formation and preservation of rare persister cells.

In addition to the toxin-antitoxin gene module expression, other genes are also involved in the initiation and development of persister cells. De Groote et al. [76] applied a different strategy to identify potential candidate genes involved in the initiation and the development of persister cells. Using a high-throughput screening technique these investigators screened a mutant library of *P. aeruginosa* containing over 5000 clones. They identified nine mutants that demonstrated persistence in the presence of the antimicrobial; four insertion mutant in dinG, spuC, PA14_17880, and PA14_66140 showing low persister phenotype and five mutants (algR, pilH, ycgM, pheA, and PA14_13680) displaying high level persistence.

3.2.3 Eradication of Persister Cells

Elimination of persister cells is the key to the eradication of the biofilm and biofilm-related infection. Several approaches have been adopted by various investigators to achieve this goal [77–79]; however, none of the approaches appear to be highly effective and hold guarded promise for further research work in this area. One of the approaches is to reactivate the persister cells from their dormant metabolic state and treat the reactivated cells with an effective antibiotic. The fatty acid signaling molecule, cis-2-decenoic acid (cis-DA), is able to change the metabolic status of *P. aeruginosa* and *E. coli* persister cells from a dormant state to a metabolically active state without an increase in cell number by multiplication [80]. The cis-DA mediated reactivation of persister cells coincided with an increased respiratory rate and protein synthesis. Moreover, a combination treatment of *P. aeruginosa* and *E. coli* cells with cis-DA and an otherwise ineffective antibiotic was able to significantly decrease cell viability.

A second approach to increase the susceptibility of persister cells to antimicrobials is to expose the dormant cells to an osmotic agent such as mannitol. Treatment of *P. aeruginosa* cells with tobramycin in combination with mannitol (10–40 mM) increased the susceptibility of persister cells to tobramycin up to a 1000-fold [81]. Similarly, the addition of mannitol to pregrown biofilms was able to revert the persister phenotype and improve the efficacy of tobramycin. Therefore, the primary effect of mannitol in reverting biofilm associated persister cells appears to be primarily an active, physiological response, associated with a minor contribution of osmotic stress. A third rather ingenious approach for the elimination of persister cells in biofilm was reported by Conlon et al. [64]. The acyldepsipeptide antibiotic (ADEP4) has been shown to activate ClpP, a nonspecific protease usually resulting in the death of growing cells [64, 82, 83]. Treatment of *S. aureus* biofilm with ClpP kills persister cells by degrading over 400 proteins forcing the cells to self-digest. Moreover, combining ADEP4 with rifampicin produced complete eradication of *S. aureus* biofilm in vitro and in a mouse model of chronic infection [64].

3.2.4 Fungal Persister Cells

In addition to bacterial species such as *P. aeruginosa* [55, 84–86], *E. coli* [61, 70, 77], *S. aureus* [62, 87–89], and *B. cepacia* [90, 91] that readily produce persister cells, eukaryotic microbes that are capable of producing prolific biofilms (e.g., *Candida* species) are also known to produce persister cells [59, 63]. LaFleur et al. [59] have studied the presence of persister cells in *C. albicans* biofilm. Their investigation revealed that in young *Candida* biofilm expression of efflux pumps was mainly responsible for resistance to triazoles [59]. On the other hand, antifungal drug resistance in mature biofilm exhibited a biphasic killing pattern in response to exposure to microbicidal drugs such as amphotericin B and chlorhexidine (an antiseptic agent) indicating that a subpopulation of highly drug tolerant cells existed in mature biofilm. The extent of killing produced by these drugs individually and in combination was almost the same suggesting that the subpopulation was invulnerable to these drugs. Furthermore, reinoculation of cells that survived the killing of the biofilm by amphotericin B were able to produce a new biofilm with a new population of persister cells suggesting that the persisters are not genetic mutants, but are genuine phenotypic variants of the wild type. As in the case of bacteria, *C. albicans* also produces multidrug tolerant persister cells in both stationary phase planktonic and biofilm cultures suggesting that both yeasts and bacteria have evolved similar strategies for the survival and preservation of a small subpopulation of highly drug tolerant cells.

3.3 The Role of Extracellular Matrix

The extracellular matrix (ECM) is an integral part of the microbial biofilm and cells. In essence, the complex is enveloped by the ECM and is protected to an extent from the harsh external environment outside the matrix, including the presence of antimicrobial drugs. The ECM is primarily made up of complex exopolysaccharides that form the skeletal network of

the ECM to which other macromolecules such as proteins and DNA are incorporated, thus adding to the thickness and the structural integrity and stability of the ECM [17, 92–101]. There is no indication that the proteins and the nucleic acid molecules are specifically synthesized by the biofilm cells to be incorporated in ECM, but they appear to be scavenged by the biofilm building cells from the surrounding microenvironment left behind after cell lysis. The presence of DNA and proteins in the biofilm provides strength, integrity, and rigidity to the biofilm. It has been shown that treating microbial biofilms within the ECM with DNAase reduces biofilm mass, increases the permeability of certain antibiotics, and increases the susceptibility of the microbial biofilms to antibiotics [17, 96, 100].

The chemical composition and the architecture of the ECM are highly variable and mainly dependent on the type of biofilms formed by the microorganisms. The ECM produced by monomicrobial biofilm is believed to be simple in its chemical composition and structure consisting of repeated subunits of one or two types of carbohydrates [102–106]. On the other hand, the chemistry and the architecture of ECM formed by mixed microbial cultures producing polymicrobial biofilms are extremely complex. For instance, in a dual microbial biofilm both partners of the biofilm contribute to the physical-chemical properties of the ECM. In many cases, although both components of the biofilm actively contribute to the construction of the biofilm ECM, their contributions are disproportionally divided between the participating members of the mixed microbial community. Sometimes one component may contribute more to the building of the biofilm matrix than the other components. This appears to be the case for polymicrobial biofilms formed by the mixed cultures of *Aspergillus* and *Scedosporium* species with the pathogenic bacterium, *Pseudomonas aeruginosa*. Polymicrobial biofilms of *A. fumigatus* or *Scedosporium apiospermum* or *Scedosporium prolificans* with *P. aeruginosa* are prolific producers of biofilm ECM. High resolution scanning electron micrographs reveal that the basic frame work of the ECM in these cases is produced by the fungal hyphae, initially in the form of polysaccharides strands originated from vesicular bodies on the hyphae and subsequently weaved into an elaborate network resembling a "Fisherman's net" entrapping and encasing the bacterial cells within (Fig. 11.2d). Subsequently, the ECM encased cells benefit from the added protection by decreasing the inhibitory effects of the antimicrobial agent.

The exact mechanism(s) by which the ECM renders protection and tolerance for biofilm cells from the effects of antimicrobial drugs is largely unknown and has yet to be investigated to any degree. However, it is generally believed that the ECM acts as a molecular sieve providing physical protection from the drugs by allowing the selective permeation of certain antibiotic(s), but not others. For example, the *P. aeruginosa* cells in polymicrobial biofilms formed by *A. fumigatus and P. aeruginosa* is highly resistant to cefepime, but is not resistant to tobramycin or ciprofloxacin [45]. Inherent or acquired resistance mechanism(s) have not been identified and associated with Cefepime-resistance. This indicates that the ECM may be responsible for the selective protection of the encased *P. aeruginosa* cells in an *A. fumigatus-P. aeruginosa* polymicrobial biofilm. On the other hand, monomicrobial biofilms formed by *P. aeruginosa* failed to provide the same level of differential susceptibility to cefepime. The permeability characteristics of ECM may change with the alteration of its chemical composition and with altered chemical composition, the physical characteristics will be changed affecting its permeability properties. For instance, if the ECM contains a higher concentration of polar molecules [107] or hydrophobic molecules [108, 109], the permeability of nonpolar and hydrophilic antibiotics will be markedly affected by reducing their accessibility to cellular target of action. Thus, the low level of intracellular drug results in reduced activity and decreased susceptibility.

3.4 Acquired Resistance by Horizontal Gene Transfer

Horizontal Gene Transfer (HGT) is a frequent mode of intraspecies and interspecies transmission of antimicrobial drug resistance determinants among bacterial cells within biofilms [110–115]. HGT is the process by which bacteria can pass genetic material (mobile genetic elements and genomic DNA) from one cell to another horizontally (rather than from the parent to the progeny) by transformation, conjugation, or transduction. In transformation, bacterial DNA from lysed cells is transported across the cellular membrane by actively growing bacterial cells in a physiological state called "competence." The material is incorporated into their own genome by genetic recombination. Any genetic trait that is advantageous (e.g., antimicrobial drug resistance determinants, ability to utilize alternate nutrient sources, ability to metabolize toxic chemicals) to the survival and fitness of the cell will be retained by natural selection. The newly transformed cell will eventually outgrow the rest of the population under selection pressure and will eventually become the dominant strain because of the natural selection process. One of the earliest reported examples of transformation is the classic experiment by Frederick Griffith [116] showing that nonvirulent *Streptococcus pneumonia* became virulent with the addition of the cell extract from a virulent strain transferred to a growing *S. pneumonia* culture. This classic experiment not only paved the way to the discovery of transformation, but also provided evidence in support of DNA as the genetic material. Similarly, Aspiras et al. [117] have demonstrated

that biofilm grown *Streptococcus mutans* was transformed to erythromycin resistant phenotype by the addition of naked DNA. In fact, the rates of transformation were 10–600 times greater than those observed in planktonic cell culture.

The second HGT mechanism by which the biofilm embedded bacterial cells acquire resistance to antimicrobial drugs depends on their ability to transfer genetic traits from one cell to the other by conjugation. In contrast to transformation, conjugation requires direct physical contact between the donor and the recipient by a conjugation tube called an "F pilus." The F pilus is composed of proteins encoded by the F plasmid in the donor cell. It contracts drawing the cells closer together and the DNA passes through the conjugation tube to the recipient cell [118]. One of the prerequisites that facilitate conjugational transfer of genetic material is a stable undisturbed environment and close proximity between neighboring cells. The biofilm growth condition is ideal for providing a stable uninterrupted environment with close proximity to neighboring cells (Figs. 11.1 and 11.2). Dunny et al. [119] demonstrated the effective conjugational transfer of a tetracycline bearing plasmid in *Enterococcus faecalis* biofilm, with a frequency almost 100 times greater than that obtained in planktonic cultures. The close association between microbes of many different species found in naturally occurring (non-laboratory) biofilms would seem to promote the possibility of cross species conjugation. In fact, interspecies conjugation has been observed in the laboratory and in a dual species biofilm consisting of a tetracycline-resistant *Bacillus subtilis* strain and a tetracycline sensitive *Staphylococcus* species where the drug resistant *Bacillus* passed on the resistance trait to *Staphylococcus* [120].

The third mechanism of HGT is transduction. During the replication of a bacteriophage in the lytic cycle, a bacterial virus is accidently packaged with a piece of bacterial DNA together with the phage genome. When the bacterial DNA carrying phage infects another bacterial cell, at least a portion of the viral genomic DNA introduced into the cell is bacterial and not viral. The bacterial DNA thus introduced is subsequently incorporated into the bacterial genome either by recombination or by the integration of the bacterial virus into the host genome by lysogeny. If the heterologous DNA thus introduced to the new host bacterial recipient carries antibiotic resistance determinants, then the recipient cell would show resistance/tolerance to antimicrobial drug(s). Reports of transduction within biofilms are not as common as those of transformation or conjugation. However, numerous reports exist of bacterial virus genes being expressed in biofilms. There are also examples of bacterial genes carried by bacteriophages being expressed in the cells of biofilms.

HGT is extremely rare in planktonic cultures. The persistent biofilm growth provides not only a favorable environment for increased spontaneous mutation, but also an increased frequency of HGT. For instance, in *S. aureus* biofilms the frequency (1.9×10^{-4}) of HGT is increased by almost 16,000-fold compared to the frequency (1×10^{-9}) in planktonic cultures [121]. There are several examples of horizontal gene transfer coupled with acquisition of drug resistance under biofilm conditions in a wide variety of biofilm producing organisms [115, 122–124].

3.5 Biofilm Specific Upregulation of MDR Efflux Pumps

The enhanced frequency of spontaneous mutation and HGT within the biofilm community results in the increased antibiotic resistance/tolerance. In addition, the biofilm growth appears to employ some of the classic gene regulation mechanisms described in high level resistance/tolerance to antimicrobial drugs. BrlR is a biofilm specific MerR-like transcriptional regulator of multidrug transporters that plays a key role in the high-level drug resistance/tolerance in *Pseudomonas aeruginosa* biofilms. The dimeric MerR regulator binds to the operator region of the promoter and then recruits σ70 factor and RNA polymerase forming a ternary complex. Transcription is now repressed because the binding of MerR regulator dimer has bent the promoter DNA slightly such that RNA polymerase is unable to make proper contact with the promoter region. However, upon binding the cognate metal ions, the metal-bound MerR homodimer molecule causes a realignment of the promoter region to accommodate proper RNA polymerase binding to the −35 and −10 sequences leading to open complex formation and transcription [125].

BrlR binds to its own promoter, most likely in a manner similar to that of MerR involving palindromic sequence. Unlike the known MerR family of multidrug transport activators, BrlR is not activated by multidrug transporter substrates. Instead, BrlR–DNA binding is enhanced by the secondary messenger c-di-GMP. In addition, c-di-GMP promotes increased expression of *brl*R gene by enhancing its promoter activity. Binding studies have shown that one c-di-GMP molecule binds two BrlR [126]. Thus, *brl*R binds to its own promoter in the presence of the secondary messenger cyclic-di-GMP and autoinduces its own expression. The increased levels of BrlR in conjunction with another transcriptional regulator called *Sag*S [127] activate the multidrug efflux pump operons *mexAB-oprM* and *mexEF-oprN*, thus enhancing the synthesis of the multidrug efflux proteins MexA and MexE. The increased MexA and MexE levels result in active expulsion of intracellular antimicrobial drug(s) conferring high level resistance/tolerance to biofilm bound cells [126–130].

4 Implications of Microbial Biofilms in Healthcare (Infectious Diseases), Industry, and Environment

The formation of drug tolerant/resistant biofilms by microbial pathogens carries important clinical implications in at least four major areas related to the treatment and management of infectious diseases: (1) increased tolerance/resistance of the biofilm to antimicrobial drug therapy, (2) the ability of cells within the biofilm to withstand or even evade the host immune defenses, (3) biofilm formation on medical devices that can negatively impact the host by causing the failure of the device and/or by serving as a reservoir for disseminated and recurrent infections, and finally (4) persistence of certain chronic medical conditions due to the modulation of the host immune system.

4.1 Medical Device Related Infections

A significant percentage of nosocomial infections are directly related to the implantation of modern medical devices and prosthetics commonly used to either improve the quality of life or deliver improved medical care [131, 132]. However, the use of these devices on a long term or permanent basis comes with an increased risk for infection [132–139]. These medical devices are generally composed of plastic, steel, or other types of durable material to which microorganisms can attach to rapidly and with high efficiency [140–142]. In view of the long term use of these devices, the organisms commonly responsible for medical devices related infections are capable of producing sustainable and recalcitrant biofilms. The biofilm community of cells is not only highly resistant to commonly used antimicrobial drugs, but also provides a reservoir for recurrent and at times life-threatening bloodstream infections [36]. Moreover, at times the presence of a microbial biofilm causes the malfunction and failure of the device. The most commonly involved medical devices associated with the development of microbial biofilms are intravascular catheters (IVCs), prosthetic devices used in orthopedic surgery such as hip and knee replacements, metal plates and orthodontic devices. Studies have also shown that biofilms are able to rapidly recover from mechanical disruption of these devices and reform biofilm within 24 h. Thus, removal of the device involved followed by aggressive antimicrobial therapy is the only viable option to clear device-related biofilm-dependent infection.

4.2 Biofilm and Chronic Infection

Chronic infections are the breeding ground for microbial biofilm development. In chronic infections (as opposed to active infections) the organism(s) persist for prolonged periods of time (often multiple organisms in the same body site) providing ample opportunities for mutualistic or synergistic interactions resulting in the formation of polymicrobial biofilms. For instance, the chronically infected lungs of patients with cystic fibrosis are frequently culture positive for *P. aeruginosa* and *A. fumigatus*. Interestingly enough, these organisms are traditionally antagonistic in nature and in this case are able adapt to each other, producing a sustainable interaction which often leads to the formation of biofilm involving both species. Obviously, such duel-species microbial growth produces duel-species biofilms whose composition and characteristics are significantly different, including the production of a mixed microbial ECM. The inflammatory response of the host immune system to the monomicrobial and polymicrobial biofilms could have vastly different clinical implications.

Diabetic foot infections with associated chronic wounds are another important medical condition where microbial biofilms play a major role in the inability of chronic wound to heal. Chronic wound microbiology in this setting is extremely complex and a wide variety of microorganisms are implicated in delayed wound healing [65, 68, 69, 143–148]. *Staphylococcus aureus* (often in combination with *C. albicans*) is the most common bacteria in chronic wounds, followed by *Enterococcus faecalis*, *P. aeruginosa*, coagulase negative staphylococci, and *Proteus* species. However, in all likelihood, they represent only a fraction of the microbial community associated with chronic wounds in diabetics. Harrison-Balestra et al. [149] showed that wound-isolated *P. aeruginosa* displays characteristics of a mature biofilm within 10 h of in vitro growth suggesting that bacteria in wounds are capable of rapidly generating biofilms. In contrast, only 6 % of acute wounds contain active microbial biofilms whereas 60 % of chronic wounds exhibited biofilm formation. The polymicrobial nature of chronic wound infections and the possible role of anaerobic bacteria have also been described by several investigators [150, 151]. Several studies have found a correlation between the presence of multiple bacterial species and nonhealing wounds when compared to one bacterial species [150, 151].

4.3 Role of Biofilm in Modulation of Immune Response

The inflammatory response mounted by the immune system of the affected host against invading pathogens is intended to protect the host and damage the infectious agent. However, there are several clinical conditions involving chronic infections where the mounting proinflammatory immune response is actually detrimental to the host tissue due to the so-called *friendly fire* causing irreversible and sustained tissue damage. In many of these cases, the presence of microbial biofilms is the underlying cause for the misdirected “friendly

fire." Furthermore, the problem is aggravated by the presence of multiple organisms in the biofilm community [35, 152–156]. Chronically infected cystic fibrosis (CF) lung is a prime example of this. In effect, although these patients are chronically colonized with numerous organisms, the majority of the lung tissue destruction and damage is due to the proinflammatory response of the host immune system to the organisms and not due to the infectious agent(s) entirely. Certain chemical component(s) of the ECM produced by the microbial biofilm, including the exogenous DNA in the matrix, is directly responsible for the proinflammatory response leading to the lung tissue damage [157]. A second prime example is the chronic wounds in diabetics where they often contain polymicrobial biofilms as the root cause of the nonhealing, chronic wound. The proinflammatory factors produced by the immune system in response to the wound microbial biofilm are thought to be responsible for the fact that the wound healing process stagnates and becomes fixed in the "inflammatory wound healing stage" and thus the body is unable to successfully progress towards the proliferative stage of wound healing [152–154]. The inflammatory response is generally unsuccessful in eradicating the existing biofilm. On the contrary, it frequently increases biofilm survival and prolongs the chronicity of the wound. A third example of the unintended consequences of microbial biofilm mediated modulation of the immune system is periodontitis. Periodontitis is a chronic inflammatory condition of the periodontium. Periodontitis is frequently caused by the presence of numerous microbial biofilms formed on the teeth called dental plaque. Substances released from the dental plaque such as lipopolysaccharides, antigens, and other virulence factors gain access to the gingival tissues and initiate a chronic inflammatory immune response, leading to the activation of the host immune response. As a result of the cellular activation and release of inflammatory mediators such as cytokines, chemokines, arachidonic acid metabolites, and proteolytic enzymes collectively, all contribute to tissue inflammation and destruction [6, 158, 159].

5 Conclusions

Microbial biofilms and their impact on clinical infectious diseases have emerged as an important aspect in the management of biofilm-associated infections. The importance of biofilms and their role in generating chronic sustained immune activation and eventual chronic bacterial disease is of utmost importance in these infections. Furthermore, biofilm-associated infections are the "Achilles heel" of antimicrobial therapy because of the high-level drug resistance seen in biofilms and the lack of immunologic response seen in these patients.

Diagnosis, characterization, and implications of biofilms will have to be considered in all infections where biofilms have been described. In general, biofilms affect human health in relation to infectious diseases in four major areas: (1) development of antimicrobial resistance, (2) increases in medical device-associated infections, (3) increased persistence of chronic infections, and (4) alter the immune response to infections by immune modulation. Subsequently, suboptimal management of any biofilm-associated infection may result in increased morbidity and mortality, with increased healthcare cost.

6 Future Directions

The increasing dilemma of the management of biofilm-associated infections will force us to investigate and develop novel antibiofilm drugs directed at one or more of the major stages in the development of biofilm such as adhesion, maturation, dispersion, or quorum sensing. By directly interfering with one or more of these stages of biofilm, the persistence of a chronic infection and/or the spread of infection would be markedly reduced. Evaluation of antibiofilm drugs that prevent either adhesion or dispersal may be able to directly attack the source of infection without having to utilize an antimicrobial agent, and thus avoid antimicrobial drug resistance developing in chronic biofilm-associated infections. In addition, since a major "inciter" of biofilms are implanted foreign bodies, a possible strategy may be to utilize "anti-adhesion" agents in the construction of these devices, thus reducing the generation of a device-associated biofilm infection.

Finally, since a major component of stability and growth in all biofilms appears to be the ECM, this would appear to be a key area for future studies. Possibly developing an "anti-ECM" agent that is able to destroy the ECM and thus expose the persister cells to antimicrobials and the host's immune system. Additional research in this active area will be highly beneficial.

References

1. Lee KW, et al. Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. ISME J. 2014;8(4):894–907.
2. Romling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. J Intern Med. 2012; 272(6):541–61.
3. Pannanusorn S, et al. Characterization of biofilm formation and the role of BCR1 in clinical isolates of Candida parapsilosis. Eukaryot Cell. 2014;13(4):438–51.
4. Hoiby N, et al. The clinical impact of bacterial biofilms. Int J Oral Sci. 2011;3(2):55–65.

5. Sardi Jde C, et al. Highlights in pathogenic fungal biofilms. Rev Iberoam Micol. 2014;31(1):22–9.
6. Teles R, et al. Lessons learned and unlearned in periodontal microbiology. Periodontol 2000. 2013;62(1):95–162.
7. Murphy K, et al. Influence of O polysaccharides on biofilm development and outer membrane vesicle biogenesis in Pseudomonas aeruginosa PAO1. J Bacteriol. 2014;196(7):1306–17.
8. Huertas MG, et al. The Klebsiella pneumoniae yfiRNB operon affects biofilm formation, polysaccharide production and drug susceptibility. Microbiology. 2014;160(Pt 12):2595–606.
9. Chew SC, et al. Dynamic remodeling of microbial biofilms by functionally distinct exopolysaccharides. MBio. 2014;5(4): e01536–14.
10. Moryl M, et al. Effect of nutrient and stress factors on polysaccharides synthesis in Proteus mirabilis biofilm. Acta Biochim Pol. 2014;61(1):133–9.
11. Irie Y, et al. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2012;109(50):20632–6.
12. Ray VA, Driks A, Visick KL. Identification of a novel matrix protein that promotes biofilm maturation in Vibrio fischeri. J Bacteriol. 2014;197(3):518–28.
13. Wu S, et al. Biofilm-specific extracellular matrix proteins of nontypeable Haemophilus influenzae. Pathog Dis. 2014;72(3): 143–60.
14. Zarnowski R, et al. Novel entries in a fungal biofilm matrix encyclopedia. MBio. 2014;5(4):e01333–14.
15. Bales PM, et al. Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens. PLoS One. 2013;8(6):e67950.
16. Cury JA, Koo H. Extraction and purification of total RNA from Streptococcus mutans biofilms. Anal Biochem. 2007;365(2): 208–14.
17. Rajendran R, et al. Extracellular DNA release acts as an antifungal resistance mechanism in mature Aspergillus fumigatus biofilms. Eukaryot Cell. 2013;12(3):420–9.
18. Jechalke S, et al. Analysis of structure, function, and activity of a benzene-degrading microbial community. FEMS Microbiol Ecol. 2013;85(1):14–26.
19. Otto M. Staphylococcal biofilms. Curr Top Microbiol Immunol. 2008;322:207–28.
20. Hung C, et al. Escherichia coli biofilms have an organized and complex extracellular matrix structure. MBio. 2013;4(5): e00645–13.
21. Moorthy S, Watnick PI. Identification of novel stage-specific genetic requirements through whole genome transcription profiling of Vibrio cholerae biofilm development. Mol Microbiol. 2005;57(6):1623–35.
22. Justice SS, et al. Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract pathogenesis. Proc Natl Acad Sci U S A. 2004;101(5):1333–8.
23. Biswas NN, et al. Indole-based novel small molecules for the modulation of bacterial signalling pathways. Org Biomol Chem. 2015;13(3):925–37.
24. Holm A, Vikstrom E. Quorum sensing communication between bacteria and human cells: signals, targets, and functions. Front Plant Sci. 2014;5:309.
25. Mhatre E, Monterrosa RG, Kovacs AT. From environmental signals to regulators: modulation of biofilm development in gram-positive bacteria. J Basic Microbiol. 2014;54(7):616–32.
26. Fazli M, et al. Regulation of biofilm formation in Pseudomonas and Burkholderia species. Environ Microbiol. 2014;16(7): 1961–81.
27. Garg N, Manchanda G, Kumar A. Bacterial quorum sensing: circuits and applications. Antonie Van Leeuwenhoek. 2014;105(2): 289–305.
28. Ferkinghoff-Borg J, Sams T. Size of quorum sensing communities. Mol Biosyst. 2014;10(1):103–9.
29. Pradeep Kumar SS, Easwer HV, Maya Nandkumar A. Multiple drug resistant bacterial biofilms on implanted catheters—a reservoir of infection. J Assoc Physicians India. 2013;61(10):702–7.
30. Weigel LM, et al. High-level vancomycin-resistant Staphylococcus aureus isolates associated with a polymicrobial biofilm. Antimicrob Agents Chemother. 2007;51(1):231–8.
31. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001;9(1):34–9.
32. Domenech M, et al. Biofilm formation avoids complement immunity and phagocytosis of Streptococcus pneumoniae. Infect Immun. 2013;81(7):2606–15.
33. Jabalameli F, et al. Evaluation of biofilm production and characterization of genes encoding type III secretion system among Pseudomonas aeruginosa isolated from burn patients. Burns. 2012;38(8):1192–7.
34. Kharazmi A. Mechanisms involved in the evasion of the host defence by Pseudomonas aeruginosa. Immunol Lett. 1991;30(2): 201–5.
35. Sadowska B, et al. The immunomodulatory activity of Staphylococcus aureus products derived from biofilm and planktonic cultures. Arch Immunol Ther Exp (Warsz). 2013;61(5):413–20.
36. Desai JV, Mitchell AP, Andes DR. Fungal biofilms, drug resistance, and recurrent infection. Cold Spring Harb Perspect Med. 2014. 4(10).
37. Biel MA, et al. Antimicrobial photodynamic therapy treatment of chronic recurrent sinusitis biofilms. Int Forum Allergy Rhinol. 2011;1(5):329–34.
38. Goodman SD, et al. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. Mucosal Immunol. 2011;4(6):625–37.
39. Artigas J, et al. Comparative sensitivity to the fungicide tebuconazole of biofilm and plankton microbial communities in freshwater ecosystems. Sci Total Environ. 2014;468–469:326–36.
40. Mitra S, Sana B, Mukherjee J. Ecological roles and biotechnological applications of marine and intertidal microbial biofilms. Adv Biochem Eng Biotechnol. 2014;146:163–205.
41. Gilan I, Sivan A. Effect of proteases on biofilm formation of the plastic-degrading actinomycete Rhodococcus ruber C208. FEMS Microbiol Lett. 2013;342(1):18–23.
42. Wilhelm L, et al. Microbial biodiversity in glacier-fed streams. ISME J. 2013;7(8):1651–60.
43. Bogino P, et al. Water-limiting conditions alter the structure and biofilm-forming ability of bacterial multispecies communities in the alfalfa rhizosphere. PLoS One. 2013;8(11):e79614.
44. Harriott MM, Noverr MC. Importance of Candida-bacterial polymicrobial biofilms in disease. Trends Microbiol. 2011;19(11): 557–63.
45. Manavathu EK, Vager DL, Vazquez JA. Development and antimicrobial susceptibility studies of in vitro monomicrobial and polymicrobial biofilm models with Aspergillus fumigatus and Pseudomonas aeruginosa. BMC Microbiol. 2014;14:53.
46. Harriott MM, Noverr MC. Ability of Candida albicans mutants to induce Staphylococcus aureus vancomycin resistance during polymicrobial biofilm formation. Antimicrob Agents Chemother. 2010;54(9):3746–55.
47. Harriott MM, Noverr MC. Candida albicans and Staphylococcus aureus form polymicrobial biofilms: effects on antimicrobial resistance. Antimicrob Agents Chemother. 2009;53(9):3914–22.
48. Monteiro DR, et al. Silver colloidal nanoparticle stability: influence on Candida biofilms formed on denture acrylic. Med Mycol. 2014;52(6):627–35.
49. Fonseca E, et al. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. Biofouling. 2014;30(4):447–57.

50. Prazynska M, Gospodarek E. In vitro effect of amphotericin B on Candida albicans, Candida glabrata and Candida parapsilosis biofilm formation. Mycopathologia. 2014;177(1–2):19–27.
51. Pannanusorn S, Fernandez V, Romling U. Prevalence of biofilm formation in clinical isolates of Candida species causing bloodstream infection. Mycoses. 2013;56(3):264–72.
52. Estivill D, et al. Biofilm formation by five species of Candida on three clinical materials. J Microbiol Methods. 2011;86(2):238–42.
53. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nat Rev Microbiol. 2008;6(3):199–210.
54. Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 2007;5(1):48–56.
55. Spoering AL, Lewis K. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol. 2001;183(23):6746–51.
56. Lewis K. Persister cells and the riddle of biofilm survival. Biochemistry (Mosc). 2005;70(2):267–74.
57. Lewis K. Persister cells: molecular mechanisms related to antibiotic tolerance. Handb Exp Pharmacol. 2012;211:121–33.
58. Keren I, et al. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J Bacteriol. 2004;186(24):8172–80.
59. LaFleur MD, Kumamoto CA, Lewis K. Candida albicans biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother. 2006;50(11):3839–46.
60. Mulcahy LR, et al. Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol. 2010;192(23):6191–9.
61. Shah D, et al. Persisters: a distinct physiological state of E. coli. BMC Microbiol. 2006;6:53.
62. Lechner S, Lewis K, Bertram R. Staphylococcus aureus persisters tolerant to bactericidal antibiotics. J Mol Microbiol Biotechnol. 2012;22(4):235–44.
63. Taff HT, et al. Mechanisms of Candida biofilm drug resistance. Future Microbiol. 2013;8(10):1325–37.
64. Conlon BP, et al. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature. 2013;503(7476):365–70.
65. Akers KS, et al. Biofilms and persistent wound infections in United States military trauma patients: a case-control analysis. BMC Infect Dis. 2014;14:190.
66. Staudinger BJ, et al. Conditions associated with the cystic fibrosis defect promote chronic Pseudomonas aeruginosa infection. Am J Respir Crit Care Med. 2014;189(7):812–24.
67. Bjarnsholt T. The role of bacterial biofilms in chronic infections. APMIS Suppl. 2013;136:1–51.
68. Scali C, Kunimoto B. An update on chronic wounds and the role of biofilms. J Cutan Med Surg. 2013;17(6):371–6.
69. Mancl KA, Kirsner RS, Ajdic D. Wound biofilms: lessons learned from oral biofilms. Wound Repair Regen. 2013;21(3):352–62.
70. Amato SM, Brynildsen MP. Nutrient transitions are a source of persisters in Escherichia coli biofilms. PLoS One. 2014;9(3):e93110.
71. Prax M, Bertram R. Metabolic aspects of bacterial persisters. Front Cell Infect Microbiol. 2014;4:148.
72. Lewis K. Persister cells and the paradox of chronic infections. Microbe. 2010;5:429–37.
73. Bigger JW. Treatment of Staphylococcal infections with penicillin. Lancet. 1944;244(6320):497–500.
74. Kristoffersen P, et al. Bacterial toxin-antitoxin gene system as containment control in yeast cells. Appl Environ Microbiol. 2000;66(12):5524–6.
75. Moyed HS, Bertrand KP. hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol. 1983;155(2):768–75.
76. De Groote VN, et al. Novel persistence genes in Pseudomonas aeruginosa identified by high-throughput screening. FEMS Microbiol Lett. 2009;297(1):73–9.
77. Keren I, Mulcahy LR, Lewis K. Persister eradication: lessons from the world of natural products. Methods Enzymol. 2012;517:387–406.
78. Grant SS, et al. Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. Proc Natl Acad Sci U S A. 2012;109(30):12147–52.
79. Allison KR, Brynildsen MP, Collins JJ. Heterogeneous bacterial persisters and engineering approaches to eliminate them. Curr Opin Microbiol. 2011;14(5):593–8.
80. Marques CN, et al. The fatty acid signaling molecule cis-2-decenoic acid increases metabolic activity and reverts persister cells to an antimicrobial-susceptible state. Appl Environ Microbiol. 2014;80(22):6976–91.
81. Barraud N, et al. Mannitol enhances antibiotic sensitivity of persister bacteria in Pseudomonas aeruginosa biofilms. PLoS One. 2013;8(12):e84220.
82. Tripathi A, et al. MazF-induced growth inhibition and persister generation in Escherichia coli. J Biol Chem. 2014;289(7):4191–205.
83. Kasari V, et al. Transcriptional cross-activation between toxin-antitoxin systems of Escherichia coli. BMC Microbiol. 2013;13:45.
84. Mulcahy H, et al. Drosophila melanogaster as an animal model for the study of Pseudomonas aeruginosa biofilm infections in vivo. PLoS Pathog. 2011;7(10):e1002299.
85. Harrison JJ, et al. Persister cells mediate tolerance to metal oxyanions in Escherichia coli. Microbiology. 2005;151(Pt 10):3181–95.
86. Harrison JJ, Turner RJ, Ceri H. Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. Environ Microbiol. 2005;7(7):981–94.
87. Conlon BP. Staphylococcus aureus chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in S. aureus disease. Bioessays. 2014;36(10):991–6.
88. Lechner S, et al. Interplay between population dynamics and drug tolerance of Staphylococcus aureus persister cells. J Mol Microbiol Biotechnol. 2012;22(6):381–91.
89. Singh R, et al. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated Staphylococcus aureus: an in vitro study. J Med Microbiol. 2009;58(Pt 8):1067–73.
90. Van Acker H, et al. Biofilm-grown Burkholderia cepacia complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. PLoS One. 2013;8(3):e58943.
91. Van Acker H, et al. Involvement of toxin-antitoxin modules in Burkholderia cenocepacia biofilm persistence. Pathog Dis. 2014;71(3):326–35.
92. Das T, et al. Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation. PLoS One. 2014;9(3):e91935.
93. Okshevsky M, Meyer RL. The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. Crit Rev Microbiol. 2015;41(3):341–52.
94. Messiaen AS, Nelis H, Coenye T. Investigating the role of matrix components in protection of Burkholderia cepacia complex biofilms against tobramycin. J Cyst Fibros. 2014;13(1):56–62.
95. Ali Mohammed MM, et al. Characterization of extracellular polymeric matrix, and treatment of Fusobacterium nucleatum and Porphyromonas gingivalis biofilms with DNase I and proteinase K. J Oral Microbiol. 2013;5.
96. Conover MS, Mishra M, Deora R. Extracellular DNA is essential for maintaining Bordetella biofilm integrity on abiotic surfaces

and in the upper respiratory tract of mice. PLoS One. 2011;6(2):e16861.
97. Harmsen M, et al. Role of extracellular DNA during biofilm formation by Listeria monocytogenes. Appl Environ Microbiol. 2010;76(7):2271–9.
98. Martins M, et al. Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. Mycopathologia. 2010;169(5):323–31.
99. Vilain S, et al. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. Appl Environ Microbiol. 2009;75(9):2861–8.
100. Hall-Stoodley L, et al. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates. BMC Microbiol. 2008;8:173.
101. Ratjen F, et al. Matrix metalloproteases in BAL fluid of patients with cystic fibrosis and their modulation by treatment with dornase alpha. Thorax. 2002;57(11):930–4.
102. Jiao Y, et al. Identification of biofilm matrix-associated proteins from an acid mine drainage microbial community. Appl Environ Microbiol. 2011;77(15):5230–7.
103. Baillie GS, Douglas LJ. Matrix polymers of Candida biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother. 2000;46(3):397–403.
104. McCrate OA, et al. Sum of the parts: composition and architecture of the bacterial extracellular matrix. J Mol Biol. 2013;425(22):4286–94.
105. Marvasi M, Visscher PT, Casillas Martinez L. Exopolymeric substances (EPS) from Bacillus subtilis: polymers and genes encoding their synthesis. FEMS Microbiol Lett. 2010;313(1):1–9.
106. Chaignon P, et al. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. Appl Microbiol Biotechnol. 2007;75(1):125–32.
107. Caubet R, et al. A radio frequency electric current enhances antibiotic efficacy against bacterial biofilms. Antimicrob Agents Chemother. 2004;48(12):4662–4.
108. Beauvais A, et al. Aspergillus cell wall and biofilm. Mycopathologia. 2014;178(5–6):371–7.
109. Heilmann C. Adhesion mechanisms of staphylococci. Adv Exp Med Biol. 2011;715:105–23.
110. Haagensen JA, et al. In situ detection of horizontal transfer of mobile genetic elements. FEMS Microbiol Ecol. 2002;42(2):261–8.
111. Juhas M. Horizontal gene transfer in human pathogens. Crit Rev Microbiol. 2015;41(1):101–8. doi:10.3109/1040841X.2013.
112. Seitz P, Blokesch M. Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol Rev. 2013;37:336–63. doi:10.1111/j.1574-6976.2012.00353.x.
113. Nadell CD, Xavier JB, Foster KR. The sociobiology of biofilms. FEMS Microbiol Rev. 2009;33:206–24. doi:10.1111/j.1574-6976.2008.00150.x.
114. Maeda S, Ito M, Ando T, Ishimoto Y, Fujisawa Y, Takahashi H, Matsuda A, Sawamura A, Kato S. Horizontal transfer of nonconjugative plasmids in a colony biofilm of Escherichia coli. FEMS Microbiol Lett. 2005;255:115–20.
115. Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: a critical review. Nat Rev Microbiol. 2005;3(9):700–10.
116. Griffith F. The significance of pneumococcal types. J Hyg (Lond). 1928;27(2):113–59.
117. Aspiras MB, Ellen RP, Cvitkovitch DG. ComX activity of Streptococcus mutans growing in biofilms. FEMS Microbiol Lett. 2004;238(1):167–74.
118. Babic A, et al. Direct visualization of horizontal gene transfer. Science. 2008;319(5869):1533–6.
119. Dunny GM, Leonard BA, Hedberg PJ. Pheromone-inducible conjugation in Enterococcus faecalis: interbacterial and host-parasite chemical communication. J Bacteriol. 1995;177(4):871–6.
120. Roberts AP, et al. Transfer of a conjugative transposon, Tn5397 in a model oral biofilm. FEMS Microbiol Lett. 1999;177(1):63–6.
121. Savage VJ, Chopra I, O'Neill AJ. Staphylococcus aureus biofilms promote horizontal transfer of antibiotic resistance. Antimicrob Agents Chemother. 2013;57(4):1968–70.
122. Yang D, et al. Horizontal transfer of antibiotic resistance genes in a membrane bioreactor. J Biotechnol. 2013;167(4):441–7.
123. Ma H, Bryers JD. Non-invasive determination of conjugative transfer of plasmids bearing antibiotic-resistance genes in biofilm-bound bacteria: effects of substrate loading and antibiotic selection. Appl Microbiol Biotechnol. 2013;97(1):317–28.
124. Luo H, Wan K, Wang HH. High-frequency conjugation system facilitates biofilm formation and pAMbeta1 transmission by Lactococcus lactis. Appl Environ Microbiol. 2005;71(6):2970–8.
125. Hobman JL. MerR family transcription activators: similar designs, different specificities. Mol Microbiol. 2007;63(5):1275–8.
126. Chambers JR, et al. BrlR from Pseudomonas aeruginosa is a c-di-GMP-responsive transcription factor. Mol Microbiol. 2014;92(3):471–87.
127. Gupta K, et al. Elevated levels of the second messenger c-di-GMP contribute to antimicrobial resistance of Pseudomonas aeruginosa. Mol Microbiol. 2014;92(3):488–506.
128. Liao J, Schurr MJ, Sauer K. The MerR-like regulator BrlR confers biofilm tolerance by activating multidrug efflux pumps in Pseudomonas aeruginosa biofilms. J Bacteriol. 2013;195(15):3352–63.
129. Liao J, Sauer K. The MerR-like transcriptional regulator BrlR contributes to Pseudomonas aeruginosa biofilm tolerance. J Bacteriol. 2012;194(18):4823–36.
130. Gupta K, et al. Antimicrobial tolerance of Pseudomonas aeruginosa biofilms is activated during an early developmental stage and requires the two-component hybrid SagS. J Bacteriol. 2013;195(21):4975–87.
131. Habash M, Reid G. Microbial biofilms: their development and significance for medical device-related infections. J Clin Pharmacol. 1999;39(9):887–98.
132. Zhang L, Gowardman J, Rickard CM. Impact of microbial attachment on intravascular catheter-related infections. Int J Antimicrob Agents. 2011;38(1):9–15.
133. Veerachamy S, et al. Bacterial adherence and biofilm formation on medical implants: a review. Proc Inst Mech Eng H. 2014;228(10):1083–99.
134. Abdallah M, et al. Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch Microbiol. 2014;196(7):453–72.
135. Goldfinger Y, et al. Biofilm prevention on cochlear implants. Cochlear Implants Int. 2014;15(3):173–8.
136. Donlan RM. Biofilms on central venous catheters: is eradication possible? Curr Top Microbiol Immunol. 2008;322:133–61.
137. Passerini L, et al. Biofilms on indwelling vascular catheters. Crit Care Med. 1992;20(5):665–73.
138. Davis EM, et al. Evidence of extensive diversity in bacterial adherence mechanisms that exploit unanticipated stainless steel surface structural complexity for biofilm formation. Acta Biomater. 2013;9(4):6236–44.
139. Jacqueline C, Caillon J. Impact of bacterial biofilm on the treatment of prosthetic joint infections. J Antimicrob Chemother. 2014;69 Suppl 1:i37–40.
140. Coad BR, et al. Biomaterials surfaces capable of resisting fungal attachment and biofilm formation. Biotechnol Adv. 2014;32(2):296–307.

141. Mishra SK, et al. Detection of biofilm production and antibiotic resistance pattern in clinical isolates from indwelling medical devices. Curr Microbiol. 2015;70(1):128–34.
142. Monteiro DR, et al. The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. Int J Antimicrob Agents. 2009;34(2):103–10.
143. Malik A, Mohammad Z, Ahmad J. The diabetic foot infections: biofilms and antimicrobial resistance. Diabetes Metab Syndr. 2013;7(2):101–7.
144. Cooper RA, Bjarnsholt T, Alhede M. Biofilms in wounds: a review of present knowledge. J Wound Care. 2014;23(11):570–82.
145. Bertesteanu S, et al. Polymicrobial wound infections: pathophysiology and current therapeutic approaches. Int J Pharm. 2014; 463(2):119–26.
146. Watters C, et al. Pseudomonas aeruginosa biofilms perturb wound resolution and antibiotic tolerance in diabetic mice. Med Microbiol Immunol. 2013;202(2):131–41.
147. Percival SL, et al. A review of the scientific evidence for biofilms in wounds. Wound Repair Regen. 2012;20(5):647–57.
148. Oates A, et al. The visualization of biofilms in chronic diabetic foot wounds using routine diagnostic microscopy methods. J Diabetes Res. 2014;2014:153586.
149. Harrison-Balestra C, et al. A wound-isolated Pseudomonas aeruginosa grows a biofilm in vitro within 10 hours and is visualized by light microscopy. Dermatol Surg. 2003;29(6):631–5.
150. James TJ, et al. Antioxidant characteristics of chronic wound fluid. Br J Dermatol. 2001;145(1):185–6.
151. Bowler PG, Davies BJ, Jones SA. Microbial involvement in chronic wound malodour. J Wound Care. 1999;8(5):216–8.
152. Zhao G, et al. Biofilms and inflammation in chronic wounds. Adv Wound Care (New Rochelle). 2013;2(7):389–99.
153. McInnes RL, et al. Contrasting host immuno-inflammatory responses to bacterial challenge within venous and diabetic ulcers. Wound Repair Regen. 2014;22(1):58–69.
154. Nguyen KT, et al. Deficient cytokine expression and neutrophil oxidative burst contribute to impaired cutaneous wound healing in diabetic, biofilm-containing chronic wounds. Wound Repair Regen. 2013;21(6):833–41.
155. Tankersley A, et al. Early effects of Staphylococcus aureus biofilm secreted products on inflammatory responses of human epithelial keratinocytes. J Inflamm (Lond). 2014;11:17.
156. Nibali L, et al. Genetic dysbiosis: the role of microbial insults in chronic inflammatory diseases. J Oral Microbiol. 2014;6.
157. Fuxman Bass JI, et al. Extracellular DNA: a major proinflammatory component of Pseudomonas aeruginosa biofilms. J Immunol. 2010;184(11):6386–95.
158. Benakanakere M, Kinane DF. Innate cellular responses to the periodontal biofilm. Front Oral Biol. 2012;15:41–55.
159. Yucel-Lindberg T, Bage T. Inflammatory mediators in the pathogenesis of periodontitis. Expert Rev Mol Med. 2013;15:e7.

Part III

Bacterial Drug Resistance: Mechanisms

The Importance of β-Lactamases to the Development of New β-Lactams

12

Karen Bush

1 Introduction

β-Lactam antibiotics have been used clinically since World War II and are considered to be among the safest, most efficacious and most widely prescribed antibiotics for the treatment of bacterial infections. Their therapeutic use began with the introduction of benzylpenicillin (penicillin G) during World War II [1, 2] and continues with the development of newer cephalosporins and carbapenems for antibiotic-resistant infections. These agents act by inhibiting bacterial cell wall synthesis, as a result of their strong covalent binding to essential penicillin binding proteins (PBPs) that catalyze the last steps of cell wall formation in both Gram-positive and Gram-negative bacteria [3, 4]. However, resistance to these agents has been a major concern. The discovery and development of new β-lactams has been driven by the continued selection of resistance mechanisms that result in the loss of efficacy of these agents.

Resistance mechanisms associated with β-lactams include modification or acquisition of a low-affinity bacterial target, i.e., a PBP; inactivation of the antibiotic by β-lactamases; and decreased concentration of the β-lactam at the site of the target, due to increased efflux or decreased entry of the drug [5–7]. In Gram-positive bacteria, especially the staphylococci, low-affinity PBPs now represent the most important β-lactam resistance mechanisms [8], in contrast to the initial selection of penicillin-resistant staphylococci due to expression of penicillinases soon after the therapeutic introduction of penicillin G [9, 10]. In Gram-negative bacteria, the appearance of β-lactamases with increased catalytic efficiency for recently introduced β-lactams has remained the major resistance mechanism [11]. The combination of increased β-lactamase production with decreased β-lactam concentrations within the periplasm results in perhaps the most effective β-lactam resistance mechanism [12].

As the expanded spectrum cephalosporins and monobactams entered clinical practice, common β-lactamases with point mutations were identified as extended-spectrum β-lactamases (ESBLs) that could inactivate these enzymes. β-Lactamase inhibitor combinations, along with broad spectrum penicillins, were able to demonstrate efficacy against at least some of these enzymes. Carbapenems, with the broadest spectrum of antimicrobial activity, were stable to inactivation by most β-lactamases, especially the ESBLs. However, widespread use of carbapenems resulted in selection of carbapenem-hydrolyzing enzymes, the serine carbapenemases and the zinc-containing metallo-β-lactamases (the MBLs).

Because the most common β-lactam resistance mechanism, overall, is related to β-lactamase production, it is no coincidence that the introduction of new β-lactam molecules into clinical practice can be correlated with the emergence of new β-lactamases. In this chapter, the origin and hydrolytic action of β-lactamases will be described, together with the most common classification schemes. In addition, the identification of new enzymes will be shown to have a close relationship with recently developed antibacterial drugs and their increased use as therapeutic agents.

2 Hydrolytic Activity

All PBPs and β-lactamases interact with β-lactam antibiotics in reactions that result in the hydrolysis of the antibiotic to form an inactive chemical substance no longer possessing antibacterial activity. The reaction can proceed by at least two separate mechanisms, dependent upon the characteristics of the active site of individual enzymes. All known PBPs react with β-lactams via a conserved active site serine [13]. However, β-lactamases belong to families of enzymes that can utilize either an active site serine or at least one metallo (zinc) ion to mediate hydrolysis [14].

K. Bush, Ph.D. (✉)
Biotechnology Program, Biology Department, Indiana University, Bloomington, IN 47401, USA
e-mail: karbush@indiana.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_12

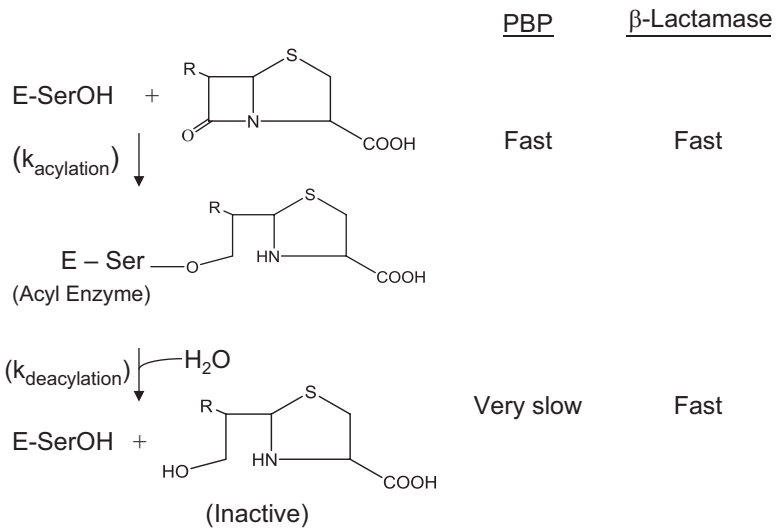


Fig. 12.1 Comparative acylation and deacylation rates for PBPs and serine β-lactamases

PBPs and serine β-lactamases hydrolyze β-lactams by forming an acyl enzyme complex via the active site serine residue (see Fig. 12.1). In this scheme, acylation and deacylation occur at different rates for the two sets of enzymes, with their classification as a PBP or β-lactamase based on the rates at which each step occurs. Thus, for PBPs, acylation may be rapid, but deacylation must be quite slow to allow the enzyme to remain inactivated during at least one cell division cycle [15, 16]. For β-lactamases, both acylation and deacylation are rapid, with k_{cat} values approaching the limit for a diffusion-controlled reaction [17–19].

3 β-Lactamase Origins

Much speculation abounds concerning the origin of β-lactamases. They have been reported to be a part of the bacterial armamentarium for centuries long before the introduction of β-lactams into clinical practice [20], with claims of β-lactamase identification in bacterial samples analyzed from soil clinging to plants from the seventeenth century [21]. Recent studies identified DNA fragments that encoded β-lactamase-like sequences from 30,000-year-old permafrost sediments in Canada [22]. Although most of the newer β-lactamases are plasmid-encoded, many bacteria have β-lactamase genes incorporated into their chromosomes, thus endowing them with a form of permanence as they are passed from one generation to the next. It has been reported that the appearance of β-lactamase genes on plasmids was a fairly recent occurrence. Studies of culture collections from 1917 to 1954 showed that the same conjugative plasmids existed in the older strains, but β-lactam-inactivating activities were not associated with these plasmids [23, 24]. From these studies, Datta and Hughes concluded that plasmid-encoded resistance determinants were introduced by transposons that accumulated in previously existing plasmids. However, Hall and Barlow estimated that the plasmid-encoded β-lactamases may have existed millions of years ago [25].

Serine β-lactamases most likely evolved from the PBPs, as there are many notable similarities between the two sets of enzymes. Not only do they catalyze the same enzymatic reactions using conserved amino acids, but they also exhibit very similar three-dimensional structures [14]. Even the metallo-β-lactamases appear to be folded in a spatial pattern that resembles the PBPs and serine β-lactamases.

In the few organisms that do not produce traditional β-lactamases, notably *Streptococcus pneumoniae* [26] and *Helicobacter pylori* [27], resistant PBPs may play that role through a more rapid deacylation reaction than for other PBPs. This has been reported for *S. pneumoniae* where resistant PBP2x variants demonstrate from 70- to 110-fold increases in deacylation rates compared to the corresponding PBP from a susceptible strain [28, 29]. In amoxicillin-resistant *H. pylori*, several surrogate β-lactam-hydrolyzing enzymes have been identified: (a) a mutant form of PBP 1A [27] and (b) HpcB, an unusual cysteine-rich protein that may play a role as a PBP from a new structural class [30].

Because β-lactams are prevalent in soil samples that contain β-lactam-producing actinomyces and bacteria [31, 32], it is an obvious suggestion that β-lactamases exist in bacteria to provide an ecological advantage to the β-lactamase-producing cells [33]. A soil bacterium that can outcompete its bacterial neighbors by destroying potent β-lactams secreted into the soil would have a distinct evolutionary advantage [34]. Notably, many of the first "penicillinases" that were described in the literature in the 1940s were from soil organisms, e.g., *Nocardia* spp., *Streptomyces* spp., and *Bacillus* spp. [35].

However, others argue that β-lactams in the soil would not diffuse far enough to be a threat to surrounding bacteria [36]. Bacteria generally conserve resources for only the most critical functions to ensure survival. Thus, when bacteria produce large amounts of β-lactamase in preference to other proteins, there must be a reason other than protection against natural predators. Investigators such as A. Medeiros believe that β-lactamases instead have a major, but poorly understood, role in bacterial physiology [37], possibly by serving to regulate cell growth. Although this latter argument cannot be dismissed lightly, the proliferation of β-lactams in soil isolates suggests that a protection mechanism may have been an important selecting factor in bacterial physiology.

4 Classification Schemes

Classification schemes for β-lactamases have been described since 1970 when eight β-lactamases were separated into functional categories [38]. For the most part, these schemes have focused on differences in enzymes that appear in Gram-negative bacteria where increased numbers of both chromosomal and plasmid-encoded enzymes contribute to resistance. There has been less interest in the β-lactamases in Gram-positive bacteria, primarily because the enzymes in Gram-positive bacteria that contribute to clinical resistance have been mainly the staphylococcal penicillinases, a rather homogenous set of enzymes that have also appeared sporadically in enterococci [39, 40], and the β-lactamases in the Gram-positive bacilli that have been studied more as academic curiosities than as contributors to therapeutic failures [41, 42].

When the heterogeneity of β-lactamases was investigated in the 1960s and 1970s, enzymes were differentiated on the basis of their functional characteristics. Some of the earliest attempts to classify these enzymes were described by Sawai et al. [43] who included the concept of "species specific" β-lactamases, and Jack and Richmond [38] who evaluated functional characteristics such as hydrolysis profiles of penicillins and cephalosporins and sensitivity to inhibitors. Others built upon this approach for β-lactamase classification, resulting in the widely accepted schemes of Richmond and Sykes [44] and, later, Bush, Jacoby, and Medeiros [45], with an updated version of the latter scheme published in 2010 [46]. At the time the first functional schemes were being proposed, no β-lactamases had been fully characterized with respect to amino acid sequence. By 1980, the sequences of four enzymes had been substantially determined after long and tedious processes of protein digestions and sequencing of many small peptide fragments [47]. In 1978, the first β-lactamase sequence was reported as the result of nucleotide sequencing of a bla_{TEM} gene, a breakthrough for molecular biologists [48].

Technological advances associated with nucleotide sequencing marked a major change in the approach to the characterization of β-lactamases. Initially, new enzymes had been characterized on the basis of substrate profiles, inhibitor properties, and isoelectric points [44]. Only a select set of representative enzymes that could be purified in high quantities were analyzed to determine their amino acid sequences [47]. However, once it became almost effortless to obtain a nucleotide sequence for a new β-lactamase gene, the in-depth enzymology of β-lactamases was relegated to only a few groups in the world. Thus, today many β-lactamases have been characterized only on the basis of gene sequences, and frequently, but not always, on the basis of elevated MIC values for selected β-lactam antibiotics. More than 2000 unique β-lactamase sequences have now been recorded in the literature, or in compilations of gene bank data [49–51], but only a small number of new β-lactamases are being characterized for their enzymatic properties.

Molecular classifications for Class A and Class B β-lactamases were initially proposed by Ambler on the basis of the four amino acid sequences available in 1980 [47]. There are now four major molecular classes of β-lactamases. Classes A, C, and D include β-lactamases with an active site serine [52, 53], whereas class B β-lactamases include zinc at their active site [54]. In Table 12.1, the most commonly used molecular and functional classification schemes are aligned. Although the functional classification schemes were first proposed in the absence of many sequences, the structure–function relationships predicted in 1988 appear to remain valid [34]. Thus, the molecular class C enzymes with larger molecular sizes than the other serine β-lactamases [45] continue to be identified with elevated rates of cephalosporin hydrolysis. All zinc β-lactamases in class B exhibit the ability to hydrolyze carbapenems, but do not hydrolyze monobactams effectively [55]. Although class A and D β-lactamases encompass a broad heterogeneity in their functional properties, they can be readily broken into functional subgroups based on substrate and inhibitor profiles.

5 Historical Development of β-Lactam Antibiotics

Fleming's fortuitous discovery of the antibiotic activity of penicillin heralded a new therapeutic approach to the treatment of infectious disease. Although penicillin was not commercialized until after World War II, the knowledge that natural environmental microbes could produce antibacterial activities led many to examine additional sources for new structural classes of antibiotics. Academic investigators such as Waksman at Rutgers University [56], as well as natural product scientists at most of the pharmaceutical companies [57], utilized vast resources to examine natural products for

Table 12.1 Alignment of molecular and functional β-lactamase classification schemes (based on [45–47])

				Enzyme characteristics	
Active site	Molecular class	Functional class	Typical enzymes	Typical substrates	Inhibitors[a]
Serine	A	2a	Staphylococcal penicillinases	Penicillins	CA, TZB
		2b	TEM-1, SHV-1	Penicillins, narrow-spectrum cephalosporins	CA, TZB
		2be	ESBLs[b] (TEM, SHV, CTX-M families)	Penicillins, cephalosporins, monobactams (aztreonam)	CA, TZB
		2br	TEM-IRT enzymes, SHV-10	Penicillins, narrow-spectrum cephalosporins	TZB active
					Resistant to CA
		2ber	TEM-50	Penicillins, cephalosporins, aztreonam	None
		2c	PSE-1, CARB-3	Penicillins, including carbenicillin	CA
		2ce	RTG-4	Penicillins, including carbenicillin; cefepime	CA, TZB
		2e	CepA, *Proteus* and *Bacteroides* cephalosporinases	Cephalosporins, including expanded-spectrum cephalosporins	CA
		2f	SME, IMI/NMC and KPC families	Penicillins, cephalosporins, carbapenems	(CA, TZB)[c]
Zinc	B	3a	CcrA, IMP, NDM and VIM families	Penicillins, cephalosporins, carbapenems, but not aztreonam	EDTA
		3b	L1, CAU-1, FEZ-1	Carbapenems	EDTA
Serine	C	1	AmpC, Chromosomal and plasmid-encoded cephalosporinases	Cephalosporins	Aztreonam, cloxacillin
		1e	GC1, CMY-37	Increased hydrolysis of expanded-spectrum cephalosporins	Aztreonam, cloxacillin
Serine	D	2d	OXA-1, OXA-10	Penicillins, including cloxacillin/oxacillin	(CA)[c]
		2de	OXA-ESBLs	Penicillins including cloxacillin/oxacillin; cephalosporins except cephamycins	(CA)[c]
		2df	OXA-23, OXA-48	Penicillins, including cloxacillin/oxacillin; carbapenems	(CA)[c]

[a]CA, clavulanic acid; TXB, tazobactam, EDTA, ethylenediaminetetraacetic acid
[b]Extended-spectrum β-lactamase
[c]Variable, dependent upon specific enzyme

the production of novel compounds capable of killing bacteria. During this period of intense investigation beginning in the 1940s and continuing for at least 50 years, β-lactam structures were among the most prevalent compound class identified in any antibiotic screening program (K. Bush, personal communication). Most of these programs relied on extracts of soil samples to provide their new antibiotics, and pharmaceutical microbiologists devised clever screening techniques to identify new compounds from these extracts for antibiotic production [58]. In these studies, a variety of soil-dwelling microorganisms, including fungi, actinomyces, and bacteria, was shown to produce new β-lactams. In fact, it was possible to identify specific microenvironments that could serve as rich sources of these new molecules, such as leaf litter in New Jersey that provided multiple bacterial sources of monobactams [58].

As a result of the natural occurrence of β-lactams, these natural products in the environment had already served as a natural selection for various families of β-lactamases. It is easy to envision how penicillins in common molds selected for penicillinases in the Gram-positive bacilli and cocci that existed in the same ecological space [9], and how cephalosporins produced by *Cephalosporium* spp. [59] served to apply pressure on the soil-dwelling pseudomonads to maintain their chromosomal AmpC cephalosporinases. Carbapenems and olivanic acids produced by the streptomyces [60] encouraged the production of metallo-β-lactamases by organisms such as the Gram-positive bacilli and anaerobes.

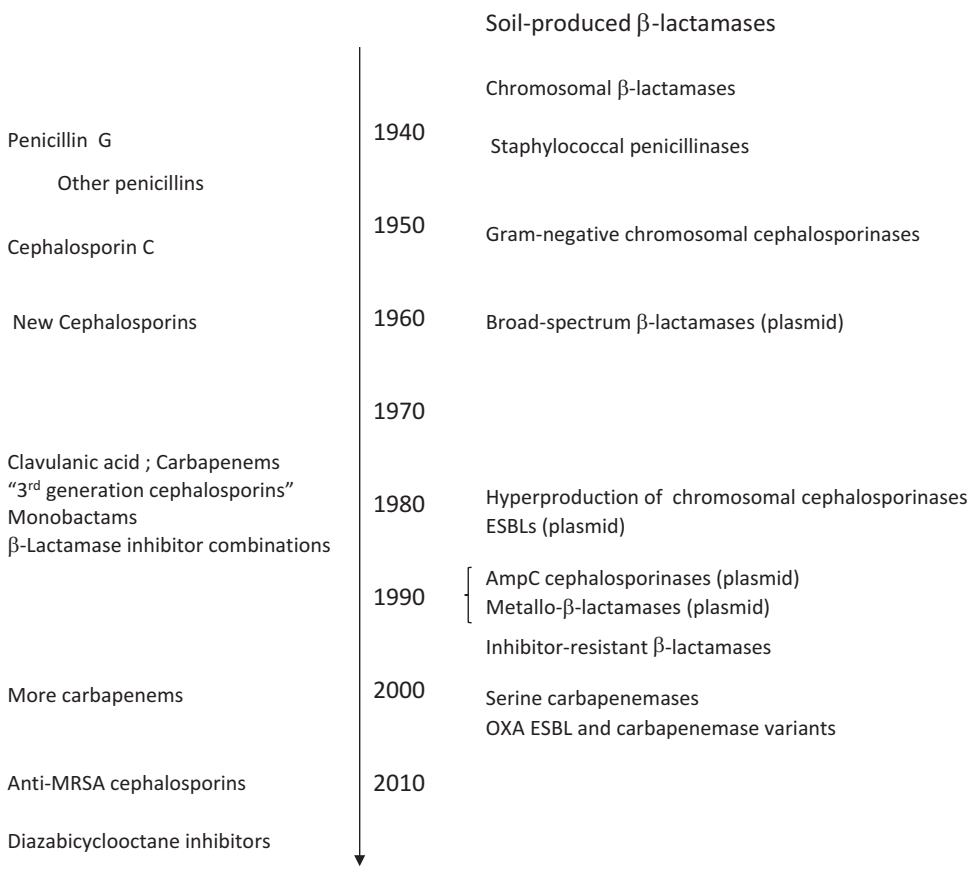


Fig. 12.2 Correlation of β-lactamase identification with the therapeutic introduction of new β-lactam antibiotics

However, as shown in Fig. 12.2, the major driving force for the plethora of β-lactamases has been the introduction and widespread clinical use of β-lactams, both natural and (semi-)synthetic [37]. In the 1940s, following the introduction of penicillin, the production of penicillinases in staphylococci increased from <10 % to almost 60 % in one British hospital over a 5 year period [9, 10]. What makes this even more interesting is that this was the result of collateral damage: as penicillin was used extensively to treat streptococcal infections, it was concurrently selecting for penicillinase production in commensal staphylococci.

After cephalosporin C was identified as a modifiable chemical entity in the mid-1950s [59], the introduction of penicillinase-stable cephalosporins led to the emergence of Gram-negative bacteria that produced species-specific cephalosporinases capable of hydrolyzing these new molecules. The result was the continued introduction of even more cephalosporins with chemical substitutions designed to render them stable to β-lactamase hydrolysis. The result was the identification of broad-spectrum β-lactamases such as TEM-1 that appeared in Greece in 1962 [61], together with organisms that produced high levels of chromosomal cephalosporinases [62].

The mid-1970s and early 1980s resulted in an explosion of new β-lactams from natural product sources, with the identification of the structurally unique clavulanic acid [63], the carbapenems [60, 64], the cephamycins [65], and monobactams [32, 66], and the introduction of the synthetic β-lactamase inhibitors [67, 68]. These classes of β-lactams were developed into new antimicrobial agents that could circumvent the most common β-lactamases that were appearing among clinical isolates. Screening of new β-lactams, whether antimicrobial agents themselves or enzyme inhibitors, frequently included testing against an RTEM β-lactamase and an AmpC cephalosporinase, usually the *Enterobacter cloacae* P99 enzyme because it was produced in high quantities and could be readily purified for enzymatic studies [63, 69]. In addition, the K1 β-lactamase from *Klebsiella oxytoca* (or, as it was known in the 1970s, *Klebsiella pneumoniae*) was a part of many initial screening panels [63, 70], perhaps because this enzyme served as a precursor to the then unknown ESBLs, with the capability of hydrolyzing some of the oxyimino-substituted cephalosporins and monobactams. With this screening panel, pharmaceutical investigators could discriminate among various cephalosporins and monobactams according to their potential lability to hydrolysis or vulnerability to inhibition. As a result, a variety of expanded-spectrum cephalosporins such as cefotaxime, ceftazidime, and ceftriaxone were developed, as well as the monobactam aztreonam, the carbapenem imipenem and the β-lactamase inhibitors clavulanic acid and sulbactam.

By the mid-1980s, it appeared that organisms producing all known β-lactamases of clinical importance could be treated with one of these newer agents, or with a combination product that incorporated a β-lactamase inhibitor and a labile penicillin. However, insidious plasmids bearing resistance determinants for β-lactamases also became loaded with genes conveying resistance to a multiplicity of antibiotic classes. Thus, new β-lactamases did not need to be selected only by β-lactams if their genes were linked to resistance determinants for other drugs.

Following the introduction of the expanded-spectrum cephalosporins and the monobactam aztreonam, the emerging resistance mechanism that was anticipated was selection of β-lactamase hyperproduction in the Enterobacteriaceae [71]. Class A plasmid-encoded TEM β-lactamases were appearing with strong promoters, leading to high enzyme levels that could not be inhibited by the inhibitor combinations [72]. More importantly, it was predicted that high levels of AmpC cephalosporinases coupled with porin mutations, would be the major factor leading to cephalosporin resistance [73]. Many investigators weighed the various contributions of β-lactamases induction, selection of derepressed mutants, and decreased permeability as they affected susceptibility to the new β-lactams [12, 71, 74, 75]. Although these Enterobacteriaceae began to be associated with clinical failures of agents such as cefoxitin or cefamandole, an unexpected resistance mechanism rapidly emerged in the late 1980s: the selection of mutant class A β-lactamases with the ability to hydrolyze the previously stable extended spectrum cephalosporins and monobactams [76, 77].

ESBLs, the *E*xtended *S*pectrum *β-L*actamases, were first identified in Europe [76, 78], followed by their appearance in the United States [79–81]. These enzymes initially arose as a result of point mutations in the TEM and SHV broad-spectrum penicillinases, with no more than two mutations necessary to confer high level resistance to cephalosporins such as cefotaxime and ceftazidime (and the monobactam aztreonam). Some enzymes such as TEM-3 and TEM-5 exhibited a preference for either cefotaxime or ceftazidime [77, 82], while others such as TEM-26 were promiscuous and readily hydrolyzed both sets of substrates [83]. Although substrate specificities varied among all these enzymes, virtually all class A ESBLs remained susceptible to inhibition by the β-lactamase inhibitors clavulanic acid and tazobactam [45]. In a number of ESBL epidemiological studies, the appearance of these enzymes was directly associated with the recent prior use of expanded spectrum cephalosporins such as cefotaxime or ceftazidime [77, 81, 83]. Concurrently with the proliferation of plasmid-encoded ESBLs, there appeared plasmid-borne AmpC-type cephalosporinases such as MIR-1 [84] and ACT-1 [85], presumably selected by the same cephalosporins as the ESBLs. As a result, the carbapenem class became an attractive alternative, especially in institutions with major ESBL problems [81, 84, 85]. Three new carbapenems were approved between 1997 and 2007, i.e., meropenem, ertapenem, and doripenem, in an effort to provide therapeutic options for the treatment of cephalosporin- and monobactam-resistant Gram-negative pathogens. This has succeeded in increasing the selective pressure from the carbapenems.

By the mid-1990s plasmid-encoded class B metallo-β-lactamases (MBLs) began to appear outside Japan where they had first been described in 1990 in *Bacteroides fragilis* [86] and in *Pseudomonas aeruginosa* in 1991 [87], probably selected by widespread use of imipenem [88]. Today three major families of MBLs have emerged, with the IMP and VIM families appearing in abundance in diverse geographic areas such as southern Europe, South America, and Asia [87–90] but only rarely in North America [91, 92]. The recently identified NDM family of MBLs that originated in the India-Pakistan region is gaining a foothold in many parts of the world [93]. In addition to the MBLs, serine carbapenemases, particularly the KPC-2 and KPC-3 β-lactamases, have caused serious clinical issues, particularly in the United States, Israel, and southern Europe [94].

As an alternative to synthesizing β-lactams that were stable to hydrolysis, combinations of penicillins were developed with β-lactamase inhibitors to treat infections caused by many class A (serine) β-lactamases, including ESBLs [36, 68]. The first of these were clavulanic acid combinations, followed by sulbactam combinations in the early to mid-1980s. In a somewhat delayed response to these combinations, inhibitor-resistant class A β-lactamases were first reported in 1994 [95] when a set of TEM variants was described from clinical isolates that demonstrated unexpected resistance to clavulanic acid, yet retaining antimicrobial activity against common cephalosporins [96]. These enzymes have not yet posed a major problem globally, and remain generally confined to Europe, with infrequent reporting of their presence in North America [97, 98]. It appears that the use of a β-lactam combination rather than a single agent has provided a greater hurdle for resistance selection.

6 Emergence of β-Lactamase Families

6.1 Gram-Positive Bacteria

In Gram-positive bacteria, β-lactamase-mediated resistance is important only among the staphylococci, where penicillinase production became the first emergent resistance mechanism [9]. Decades after penicillin entered clinical practice, a second, even more far-reaching β-lactam resistance mechanism evolved, but only after the introduction of the cephalosporins. This latter resistance in methicillin-resistant *S. aureus* (MRSA), due to the introduction of a new penicillin-binding

protein, PBP 2a (or, PBP 2′), is most often combined with penicillinase production, with co-regulation of the two proteins in many strains, indicating that the β-lactamase is still an important commodity [99]. Until recently, MRSA was considered to be untreatable by β-lactam antibiotics, but the penicillinase-stable ceftaroline, a cephalosporin with tight binding to PBP 2a [100], was recently shown to be effective in the treatment of infections caused by MRSA [101].

For a few years β-lactamase production was reported in the enterococci [39, 102], but these strains seem to have become less prevalent [103]. Upon close examination, the enterococcal β-lactamase appeared to have been introduced intact from the staphylococci [39], and probably did not provide a major ecological advantage to the producing organism.

Among the Gram-positive bacilli, multiple β-lactamases have been identified, with both zinc and serine β-lactamases appearing as chromosomal enzymes in a single strain. The most studied set of enzymes include the class A penicillinase and class B metallo-enzyme from *Bacillus cereus* [42], with counterparts to these enzymes appearing in *Bacillus anthracis* [104, 105]. It is interesting that these organisms are most frequently found as soil organisms, again supporting an association between soil-produced β-lactams and β-lactamases.

6.2 Gram-Negative Bacteria

In spite of the widespread use of penicillin as an agent to treat Gram-positive infections in the 1940s, the first literature citation referring to β-lactamase production was associated with a penicillinase from *E. coli* [1]. As more β-lactamases were identified, investigators assumed that species-specific β-lactamases were the rule [35, 43]. Hence the naming of enzymes by simple names referring to their producing organism, e.g., K1 or KOXY from *Klebsiella oxytoca* (previously, *K. pneumoniae*), or AER from *Aeromonas* spp. [51]. This hypothesis was supported by the identification of what appeared to be species-specific chromosomal cephalosporinases among the Enterobacteriaceae and *P. aeruginosa*. However, as an increasing number of plasmid-encoded β-lactamases were identified among the Gram-negative spectrum, it became evident that multiple enzymes could survive among these organisms, both of chromosomal and of plasmidic origin [44]. In some recent clinical isolates, as many as eight different β-lactam-hydrolyzing enzymes were identified from multidrug-resistant Klebsiellae [85, 97, 106], with multiple enzymes often encoded on the same plasmid [85, 107].

Although the species-specific concept was retained for chromosomally encoded β-lactamases for many years, even this idea was challenged with the identification of MIR-1, the plasmid-encoded cephalosporinase in *K. pneumoniae* that appeared to originate from an *Enterobacter cloacae* AmpC enzyme [84]. To date, over 200 plasmid-encoded AmpC-related cephalosporinases have been identified [50, 51, 107], with their sequences clustering in families. Plasmid-encoded AmpC families originating from chromosomal genes from *Citrobacter freundii*, *Enterobacter* spp., *M. morganii*, *H. alvei*, or *Aeromonas* have all been shown to share >90 % homology among the individual members of their respective clusters [107].

For a number of years, the predominant families of ESBLs arose from the TEM and SHV β-lactamases such that in mid-2014 there were >215 TEM variants and >185 SHV mutant enzymes [50]. However, it was not long before the OXA family of enzymes began to emerge, derived from the third most common family of plasmid-encoded β-lactamases from epidemiological evaluations of Gram-negative bacteria in the late 1970s and early 1980s, prior to the introduction of the later generation of cephalosporins. In fact, seven variants within the OXA family had already been described by 1985 [108]. Today the OXA family is the largest of all the β-lactamase families possessing perhaps the most diverse substrate profiles, with carbapenemases such as OXA-48 and OXA-23 identified as chromosomal enzymes in *Acinetobacter baumannii* [49, 109]. By the early 1990s new families of ESBLs were identified (Table 12.2), with the most notable being the CTX-M family of enzymes [113]. CTX-M

Table 12.2 Introduction and proliferation of major ESBL families

Enzyme family	First report of parent enzyme	Location	First ESBL report	Location	Number of enzymes in family in 2014	Reference
TEM	1962	Greece	1987	France	219[a]	[50, 61, 77]
OXA	1967	Japan	1993	Turkey	426[b]	[50, 110, 111]
SHV	1972	France	1983	Germany	189[c]	[50, 76, 112]
CTX-M	1988	Germany	1988	Germany	160	[50, 113]
PER	1991	France	1991	France	8	[50, 114]

[a]Not all enzymes in the family can be considered to be ESBLs. Included are inhibitor-resistant TEM enzymes (IRTs) and complex-mutant TEM variants (CMTs) with characteristics of both ESBLs and IRTs

[b]Not all enzymes in the family can be considered to be ESBLs. Included are oxacillinases with diverse hydrolytic properties, and carbapenemases

[c]Not all enzymes in the family can be considered to be ESBLs. Included are inhibitor-resistant SHV variants

β-lactamases have become the predominant ESBL throughout the world [20, 115–118]. This enzyme family is apparently derived from chromosomal β-lactamases produced by *Kluyvera* spp. [119, 120], some of which have reduced hydrolysis rates for ceftazidime compared to cefotaxime. However, the two most common CTX-M enzymes, CTX-M-14 and CTX-M-15, confer resistance to both cephalosporins in many enteric bacteria [121].

Some of the more worrisome β-lactamases include recently identified plasmid-encoded carbapenem-hydrolyzing enzymes, in both the serine and the metallo-β-lactamase families. Many of these enzymes not only inactivate carbapenems, but also hydrolyze all other β-lactams, with the exception of aztreonam which is stable to hydrolysis by the metallo-enzymes. Although the serine carbapenemases, such as the KPC enzymes, are inhibited by the classical β-lactamase inhibitors in isolated enzyme assays, commercially available penicillin-inhibitor combinations are generally ineffective when tested in whole cells [97]. Most disturbing are the increasing numbers of plasmid-encoded ultra-broad-spectrum enzymes such as the KPC enzymes and the VIM, IMP, and NDM metallo-β-lactamases spreading throughout the world, resulting in the use of colistin, possibly combined with tigecycline for enteric bacteria, as empiric therapy for infections caused by pan-resistant Gram-negative bacteria [122, 123]. The recent regulatory approval of the β-lactamase inhibitor combination ceftazidime-avibactam (see below), however, provides the potential for treatment of infections caused by organisms with serine carbapenemases [124].

7 Future Directions

Many β-lactams have been developed for commercial use, based on their abilities to treat infections caused by the most important human pathogens known at the time. However, bacteria have consistently demonstrated their survival tactics over time, and have successfully counteracted the multiplicity of attempts by pharmaceutical companies to decimate their populations. If β-lactams are to remain within our antibacterial armamentarium, therefore, it will be essential to devise new agents stable to all known β-lactamases, or to conceive and implement a new approach to β-lactamase inhibition. Although unremitting attempts have been made by synthetic chemists to circumvent β-lactamase resistance mechanisms, perhaps the most successful agents to date have been the β-lactamase inhibitor-penicillin combinations. On a somewhat optimistic note, new β-lactamase inhibitor combinations have recently been approved for use or are in late stage clinical development. Ceftolozane-tazobactam with potent anti-pseudomonal activity was approved in late 2014 [125], and is also effective against many CTX-M-producing enteric bacteria. Ceftazidime in combination with avibactam, a structurally novel non-β-lactam diazabicyclooctane inhibitor of class A (including KPCs), class C, and some class D β-lactamases, was approved by the FDA in early 2015 [124] on the basis of Phase 2 clinical trials. Some other combinations in advanced therapeutic trials include (1) avibactam combined with ceftaroline or aztreonam, (2) relebactam (MK-7655), structurally and functionally related to avibactam, combined with imipenem (+-cilastatin), and (3) vaborbactam (RPX7009), a novel boronic acid inhibitor of many serine β-lactamases including KPCs, combined with meropenem [126]. These approaches provide some hope that enzymes such as the ESBLs and serine carbapenemases may be dealt with sufficiently for the immediate future. As has been demonstrated quite convincingly in the past, however, these measures will only buy us time before the next β-lactamase-related calamity emerges.

Bibliography

1. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. Nature. 1940;146:837.
2. Selwyn S. The discovery and evolution of the penicillins and cephalosporins. In: The Beta-Lactam Antibiotics: penicillins and cephalosporins in perspective. London: Hodder and Stoughton; 1980. pp. 1–45.
3. Spratt BG. Penicillin-binding proteins and the future of β-lactam antibiotics. J Gen Microbiol. 1983;129:1247–60.
4. Tipper DJ, Strominger JL. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci U S A. 1965;54:1133–41.
5. Li X-Z, Ma D, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob Agents Chemother. 1994;38:1742–52.
6. Rice LB. Successful interventions for gram-negative resistance to extended-spectrum beta-lactam antiobiotics. Pharmacotherapy. 1999;19(8 Pt 2):120S–8S.
7. Zimmermann W, Rosselet A. Function of outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob Agents Chemother. 1977;12:368–72.
8. Rossi L, Tonin E, Cheng YR, Fontana R. Regulation of penicillin-binding protein activity: description of a methicillin-inducible penicillin-binding protein in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1985;27(5):828–31.
9. Kirby WMM. Bacteriostatic and lytic actions of penicillin on sensitive and resistant staphylococci. J Clin Invest. 1945;24:165–9.
10. Medeiros AA. ß-lactamases. Br Med Bul. 1984;40(1):18–27.
11. Matagne A, Misselyn-Baudin A-M, Joris B, Erpicum T, Graniwer B, Frere J-M. The diversity of the catalytic properties of class A β-lactamases. Biochem J. 1990;265:131–46.
12. Bush K, Tanaka SK, Bonner DP, Sykes RB. Resistance caused by decreased penetration of ß-lactam antibiotics into *Enterobacter cloacae*. Antimicrob Agents Chemother. 1985;27(4):555–60.
13. Spratt BG, Cromie KD. Penicillin-binding proteins of gram-negative bacteria. Rev Infect Dis. 1988;10:699–711.
14. Massova I, Mobashery S. Kinship and diversification of bacterial penicillin-binding proteins and β-lactamases. Antimicrob Agents Chemother. 1998;42(1):1–17.
15. Chambers HF, Miick C. Characterization of penicillin-binding protein 2 *Staphylococcus aureus* of *Staphylococcus aureus*:

deacylation reaction and identification of two penicillin-binding peptides. Antimicrob Agents Chemother. 1992;36(3):656–61.
16. Tuomanen E, Schwartz J. Penicillin-binding protein 7 and its relationship to lysis of nongrowing Escherichia coli. J Bacteriol. 1987;169(11):4912–5.
17. Fisher JF, Knowles JR. Bacterial resistance to β-lactams: the β-lactamases. Ann Rep Med Chem. 1978;13:239–48.
18. Hardy LW, Kirsch JF. Diffusion-limited component of reactions catalyzed by *Bacillus cereus* beta-lactamase I. Biochemistry. 1984;23(6):1275–82.
19. Christensen H, Martin MT, Waley SG. β-lactamases as fully efficient enzymes. Determination of all the rate constants in the acyl-enzyme mechanism. Biochem J. 1990;266:853–61.
20. Jacoby G, Bush K. Beta-Lactam resistance in the 21st century. In: White DG, Alekshun MN, McDermott PF, editors. Frontiers in antimicrobial resistance: a tribute to Stuart B Levy. Washington, DC: ASM Press; 2005. p. 53–65.
21. Pollock MR. Origin and function of penicillinase: a problem in biochemical evolution. Br Med J. 1967;4:71–7.
22. D'Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, et al. Antibiotic resistance is ancient. Nature. 2011;477(7365): 457–61.
23. Datta N, Hughes VM. Plasmids of the same Inc groups in enterobacteria before and after the medical use of antibiotics. Nature. 1983;306:616–7.
24. Hughes VM, Datta N. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature. 1983;302:725–6.
25. Hall BG, Barlow M. Evolution of the serine beta-lactamases: past, present and future. Drug Resist Updat. 2004;7(2):111–23.
26. Bush K. Beta-lactamases of increasing clinical importance. Curr Pharm Des. 1999;5:839–45.
27. Kwon DH, Dore MP, Kim JJ, Kato M, Lee M, Wu JY, et al. High-level beta-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob Agents Chemother. 2003;47:2169–78.
28. Lu WP, Kincaid E, Sun Y, Bauer MD. Kinetics of beta-lactam interactions with penicillin-susceptible and -resistant penicillin-binding protein 2x proteins from *Streptococcus pneumoniae*. Involvement of acylation and deacylation in beta-lactam resistance. J Biol Chem. 2001;276(34):31494–501.
29. Chesnel L, Zapun A, Mouz N, Dideberg O, Vernet T. Increase of the deacylation rate of PBP2x from Streptococcus pneumoniae by single point mutations mimicking the class A beta-lactamases. Eur J Biochem. 2002;269(6):1678–83.
30. Luthy L, Grutter MG, Mittl PRE. The crystal structure of Helicobacter pylori cysteine-rich protein B reveals a novel fold for a penicillin-binding protein. J Biol Chem. 2002;277(12): 10187–93.
31. Hill P. The production of penicillins in soils and seeds by penicillium chrysogenum and the role of penicillin -lactamase in the ecology of soil bacillus. J Gen Microbiol. 1972;70(2):243–52.
32. Sykes RB, Cimarusti CM, Bonner DP, Bush K, Floyd DM, Georgopapadakou NH, et al. Moncyclic β-lactam antibiotics produced by bacteria. Nature. 1981;291:489–91.
33. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. Science. 2006;311(5759):374–7.
34. Bush K. Recent developments in β-lactamase research and their implications for the future. Rev Infect Dis. 1988;10:681–90. 739–43.
35. Hamilton-Miller JMT. An historical introduction to beta-lactamases. In: Hamilton-Miller JMT, Smith JT, editors. Beta-lactamases. London: Academic; 1979. p. 1–16.
36. Bush K. The evolution of β-lactamases. In: Chadwick DJ, Goode J, editors. Antibiotic resistance: origins, evolution, selection and spread. Ciba Foundation Symposium. vol. 207. Chichester: Wiley; 1997. pp. 152–66.
37. Medeiros AA. Evolution and dissemination of β-lactamases accelerated by generations of beta-lactam antibiotics. Clin Infect Dis. 1997;24 Suppl 1:S19–45.
38. Jack GW, Richmond MH. Comparative amino acid contents of purified β-lactamases from enteric bacteria. FEBS Lett. 1970; 12:30–2.
39. Zscheck KK, Murray BE. Nucleotide sequence of the beta-lactamase gene from *Enterococcus faecalis* HH22 and its similarity to staphylococcal beta-lactamase genes. Antimicrob Agents Chemother. 1991;35(9):1736–40.
40. Voladri RKR, Tummuru MKR, Kernodle DS. Structure-function relationships among wild-type variants of *Staphylococcus aureus* β-lactamase: importance of amino acids 128 and 216. J Bacteriol. 1996;178:7248–53.
41. Pollock MR. Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical physicochemical and physiological comparison. Biochem J. 1965;94:666–75.
42. Kuwabara S, Abraham EP. Some properties of two extracellular β-lactamases from *Bacillus cereus* 569/H. Biochem J. 1967; 103:27c–30.
43. Sawai T, Mitsuhashi S, Yamagishi S. Drug resistance of enteric bacteria. XIV. Comparison of β-lactamases in gram-negative rod bacteria resistant to α-aminobenzylpenicillin. Jpn J Microbiol. 1968;12:423–34.
44. Richmond MH, Sykes RB. The β-lactamases of gram-negative bacteria and their possible physiological role. In: Rose AH, Tempest DW, editors. Advances in microbial physiology, vol. 9. New York: Academic; 1973. p. 31–88.
45. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 1995;39:1211–33.
46. Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrob Agents Chemother. 2010;54:969–76.
47. Ambler RP. The structure of β-lactamases. Philos Trans R Soc Lond B Biol Sci. 1980;289:321–31.
48. Sutcliffe JG. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc Natl Acad Sci U S A. 1978;75:3737–41.
49. Bush K. Proliferation and significance of clinically relevant β-lactamases. Ann N Y Acad Sci. 2013;1277:84–90.
50. Jacoby GA, Bush K. β-Lactamase classification and amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant enzymes. 2014. http://www.lahey.org/Studies/. Accessed 17 Sep 2014–3 Oct 2014.
51. Jacoby GA. Beta-lactamase nomenclature. Antimicrob Agents Chemother. 2006;50(4):1123–9.
52. Jaurin B, Grundstrom T. *ampC* cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β-lactamases of the penicillinase type. Proc Natl Acad Sci U S A. 1981;78:4897–901.
53. Huovinen P, Huovinen S, Jacoby GA. Sequence of PSE-2 beta-lactamase. Antimicrob Agents Chemother. 1988;32:134–6.
54. Ambler RP, Daniel M, Fleming J, Hermoso JM, Pang C, Waley SG. The amino acid sequence of the zinc-requiring beta-lactamase II from the bacterium Bacillus cereus 569. FEBS Lett. 1985;189(2):207–11.
55. Rasmussen BA, Bush K. Carbapenem-hydrolyzing β-lactamases. Antimicrob Agents Chemother. 1997;41(2):223–32.
56. Waksman SA. A quarter-century of the antibiotic era. Antimicrob Agents Chemother. 1965;5:9–19.
57. Silver L, Bostian K. Screening of natural products for antimicrobial agents. Eur J Clin Microbiol Infect Dis. 1990;9(7): 455–61.
58. Wells JS, Hunter JC, Astle GL, Sherwood JC, Ricca CM, Trejo WH, et al. Distribution of β-lactam and β-lactone producing bacteria in nature. J Antibiot (Tokyo). 1982;35:814–21.

59. Abraham EP. Cephalosporins 1945–1986. Drugs. 1987;34 Suppl 2:1–14.
60. Butterworth D, Cole M, Hanscomb G, Rolinson GN. Olivanic acids, a family of beta-lactam antibiotics with beta-lactamase inhibitory properties produced by *Streptomyces* species. I. Detection, properties and fermentation studies. J Antibiot (Tokyo). 1979;32:287–94.
61. Datta N, Kontomichalou P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature. 1965;208: 239–41.
62. Kabins SA, Sweeny HM, Cohen S. Resistance to cephalosporin *in vivo* associated with increased cephalosporinase production. Ann Intern Med. 1966;65:1271–7.
63. Reading C, Cole M. Clavulanic acid: a beta-lactamase inhibitor from *Streptomyces clavuligerus*. Antimicrob Agents Chemother. 1977;11:852–7.
64. Kahan JS, Kahan FM, Goegelman R, Currie SA, Jackson M, Stapley EO, et al. Thienamycin, a new beta-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J Antibiot (Tokyo). 1979;32:1–12.
65. Stapley EO, Birnbaum J, Miller AK, Wallick H, Hendlin D, Woodruff HB. Cefoxitin and cephamycins: microbiological studies. Rev Infect Dis. 1979;1:73–89.
66. Imada A, Kitano K, Kintaka K, Muroi M, Asai M. Sulfazecin and isosulfazecin, novel beta-lactam antibiotics of bacterial origin. Nature. 1981;289(5798):590–1.
67. English AR, Retsema JA, Girard AE, Lynch JE, Barth WE. CP-45.899, a beta-lactamase inhibitor that extends the antibacterial spectrum of beta-lactams: initial bacteriological characterization. Antimicrob Agents Chemother. 1978;14:414–9.
68. Aronoff SC, Jacobs MR, Johenning S, Yamabe S. Comparative activities of the β-lactamase inhibitors YTR 830, sodium clavulanate, and sulbactam combined with amoxicillin or ampicillin. Antimicrob Agents Chemother. 1984;26:580–2.
69. Sykes RB, Bonner DP, Bush K, Georgopapadakou NH. Azthreonam (SQ 26,776), a synthetic monobactam specifically active against aerobic gram-negative bacteria. Antimicrob Agents Chemother. 1982;21(1):85–92.
70. Bush K, Bonner DP, Sykes RB. Izumenolide—a novel beta-lactamase inhibitor produced by *Micromonospora*. J Antibiot (Tokyo). 1980;33:1262–9.
71. Gootz TD, Sanders CC, Goering RV. Resistance to cefamandole: derepression of beta-lactamases by cefoxitin and mutation in *Enterobacter cloacae*. J Infect Dis. 1982;146:34–42.
72. Thomson KS, Weber DA, Sanders CC, Sanders Jr WE. β-lactamase production in members of the family Enterobacteriaceae and resistance to β-lactam-enzyme inhibitor combinations. Antimicrob Agents Chemother. 1990;34:622–7.
73. Neu HC. What do beta-lactamases mean for clinical efficacy? Infection. 1983;11 Suppl 2:S74–80.
74. Vu H, Nikaido H. Role of β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive *Enterobacter cloacae* strain to expanded-spectrum β-lactams. Antimicrob Agents Chemother. 1985;27:393–8.
75. Then RL, Angehrn P. Trapping of nonhydrolyzable cephalosporins by cephalosporinases in *Enterobacter cloacae* and *Pseudomonas aeruginosa* as a possible resistance mechanism. Antimicrob Agents Chemother. 1982;21(5):711–7.
76. Kliebe C, Nies BA, Meyer JF, Tolxdorff-Neutzling RM, Wiedemann B. Evolution of plasmid-coded resistance to broad spectrum cephalosporins. Antimicrob Agents Chemother. 1985; 28:302–7.
77. Sirot D, Sirot J, Labia R, Morand A, Courvalin P, Darfeuille-Michaud A, et al. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. J Antimicrob Chemother. 1987;20:323–34.
78. Knothe H, Shah P, Krcmery V, Antal M, Mitsuhashi S. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection. 1983;11:315–7.
79. Jacoby GA, Medeiros AA, O'Brien TF, Pinto ME, Jiang H. Broad-spectrum, transmissible β-lactamases. N Engl J Med. 1988; 319:723–4.
80. Quinn JP, Miyashiro D, Sahm D, Flamm R, Bush K. Novel plasmid-mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 1989;33:1451–6.
81. Meyer KS, Urban C, Eagan JA, Berger BJ, Rahal JJ. Nosocomial outbreak of *Klebsiella* infection resistant to late-generation cephalosporins. Ann Int Med. 1993;119:353–8.
82. Sirot J, Labia R, Thabaut A. *Klebsiella pneumoniae* strains more resistant to ceftazidime than to other third-generation cephalosporins. J Antimicrob Chemother. 1987;20:611–2.
83. Naumovski L, Quinn JP, Miyashiro D, Patel M, Bush K, Singer SB, et al. Outbreak of ceftazidime resistance due to a novel extended-spectrum β-lactamase in isolates from cancer patients. Antimicrob Agents Chemother. 1992;36(9):1991–6.
84. Papanicolaou G, Medeiros AA, Jacoby GA. Novel plasmid-mediated β-lactamase (MIR-1) Conferring resistance to oxyimino- and α-methoxy ß-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 1990;34:2200–9.
85. Bradford PA, Urban C, Mariano N, Projan SJ, Rahal JJ, Bush K. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. Antimicrob Agents Chemother. 1997;41:563–9.
86. Yamaoka K, Watanabe K, Muto Y, Katoh N, Ueno K, Tally FP. R-Plasmid mediated transfer of β-lactam resistance in *Bacteroides fragilis*. J Antibiot. 1990;43:1302–6.
87. Watanabe M, Iyobe S, Inoue M, Mitsuhashi S. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1991;35:147–51.
88. Minami S, Akama M, Araki H, Watanabe Y, Narita H, Iyobe S, et al. Imipenem and cephem resistant *Pseudomonas aeruginosa* carrying plasmids coding for class B β-lactamase. J Antimicrob Chemother. 1996;37:433–44.
89. Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-beta-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother. 1999;43(7):1584–90.
90. Livermore DM. The impact of carbapenemases on antimicrobial development and therapy. Curr Opin Investig Drugs. 2002;3(2): 218–24.
91. Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2011;17(10): 1791–8.
92. Lolans K, Queenan AM, Bush K, Sahud A, Quinn JP. First nosocomial outbreak of *Pseudomonas aeruginosa* producing an integron-borne metallo-beta-lactamase (VIM-2) in the United States. Antimicrob Agents Chemother. 2005;49(8):3538–40.
93. Bushnell G, Mitrani-Gold F, Mundy LM. Emergence of New Delhi metallo-beta-lactamase type 1-producing enterobacteriaceae and non-enterobacteriaceae: global case detection and bacterial surveillance. Int J Infect Dis. 2013;17(5):e325–33.
94. Canton R, Akova M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, et al. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect. 2012;18(5):413–31.

95. Belaaouaj A, Lapoumeroulie C, Canica MM, Vedel G, Nevot P, Krishnamoorthy R, et al. Nucleotide sequences of the genes coding for the TEM-like β-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol Lett. 1994;120: 75–80.
96. Chaibi EB, Sirot D, Paul G, Labia R. Inhibitor-resistant TEM β-lactamases: phenotypic, genetic and biochemical characteristics. J Antimicrob Chemother. 1999;43:447–58.
97. Bradford PA, Bratu S, Urban C, Visalli M, Mariano N, Landman D, et al. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 beta-lactamases in New York City. Clin Infect Dis. 2004;39(1):55–60.
98. Kaye KS, Gold HS, Schwaber MJ, Venkataraman L, Qi Y, De Girolami PC, et al. Variety of beta-lactamases produced by amoxicillin-clavulanate-resistant *Escherichia coli* isolated in the northeastern United States. Antimicrob Agents Chemother. 2004; 48(5):1520–5.
99. Berger-Bachi B. Genetic basis of methicillin resistance in Staphylococcus aureus. Cell Mol Life Sci. 1999;56(9-10): 764–70.
100. Kosowska-Shick K, McGhee PL, Appelbaum PC. Affinity of ceftaroline and other beta-lactams for penicillin-binding proteins from *Staphylococcus aureus* and *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2010;54(5):1670–7.
101. Corey GR, Wilcox M, Talbot GH, Friedland HD, Baculik T, Witherell GW, et al. Integrated analysis of CANVAS 1 and 2: phase 3, multicenter, randomized, double-blind studies to evaluate the safety and efficacy of ceftaroline versus vancomycin plus aztreonam in complicated skin and skin-structure infection. Clin Infect Dis. 2010;51(6):641–50.
102. Murray BE, Mederski-Samaroj B. Transferable beta-lactamase. A new mechanism for in vitro penicillin resistance in Streptococcus faecalis. J Clin Invest. 1983;72(3):1168–71.
103. Ono S, Muratani T, Matsumoto T. Mechanisms of resistance to imipenem and ampicillin in *Enterococcus faecalis*. Antimicrob Agents Chemother. 2005;49(7):2954–8.
104. Chen Y, Succi J, Tenover FC, Koehler TM. Beta-lactamase genes of the penicillin-susceptible *Bacillus anthracis* Sterne strain. J Bacteriol. 2003;185(3):823–30.
105. Materon IC, Queenan AM, Koehler TM, Bush K, Palzkill T. Biochemical characterization of beta-lactamases Bla1 and Bla2 from *Bacillus anthracis*. Antimicrob Agents Chemother. 2003; 47(6):2040–2.
106. Moland ES, Hong SG, Thomson KS, Larone DH, Hanson ND. A *Klebsiella pneumoniae* isolate producing at least eight different beta-lactamases including an AmpC and KPC beta-lactamase. Antimicrob Agents Chemother. 2007;51:800–1.
107. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type beta-lactamases. Antimicrob Agents Chemother. 2002;46(1): 1–11.
108. Medeiros AA, Cohenford M, Jacoby GA. Five novel plasmid-determined β-lactamases. Antimicrob Agents Chemother. 1985; 27:715–9.
109. Livermore DM. Fourteen years in resistance. Int J Antimicrob Agents. 2012;39:283–94.
110. Egawa R, Sawai T, Mitsuhashi S. Drug resistance of enteric bacteria. XII. Unique substrate specificity of penicillinase produced by R-factor. Jpn J Microbiol. 1967;11:179–86.
111. Hall LMC, Livermore DM, Gur D, Akova M, Akalin HE. OXA-11, an extended spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1993;37:1637–44.
112. Pitton JS. Mechanisms of bacterial resistance to antibiotics. In: Adrina RH, editor. Reviews of physiology, vol. 65. Berlin: Springer; 1972. p. 15–93.
113. Bauernfeind A, Grimm H, Schweighart S. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infection. 1990;18(5):294–8.
114. Nordmann P, Ronco E, Naas T, Duport C, Michel-Briand Y, Labia R. Characterization of a novel extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1993;37:962–9.
115. Castanheira M, Farrell SE, Krause KM, Jones RN, Sader HS. Contemporary diversity of β-lactamases among *Enterobacteriaceae* in the nine U.S. census regions and ceftazidime-avibactam activity tested against isolates producing the most prevalent β-lactamase groups. Antimicrob Agents Chemother. 2014;58(2):833–8.
116. Izdebski R, Baraniak A, Fiett J, Adler A, Kazma M, Salomon J, et al. Clonal structure, extended-spectrum beta-lactamases, and acquired AmpC-type cephalosporinases of *Escherichia coli* populations colonizing patients in rehabilitation centers in four countries. Antimicrob Agents Chemother. 2013;57(1):309–16.
117. Zubair M, Malik A, Ahmad J. Study of plasmid-mediated extended-spectrum beta-lactamase-producing strains of enterobacteriaceae, isolated from diabetic foot infections in a North Indian tertiary-care hospital. Diabetes Technol Ther. 2012; 14(4):315–24.
118. Walther-Rasmussen J, Hoiby N. Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum β-lactamases. Can J Microbiol. 2004;50:137–65.
119. Oliver A, Perez-Diaz JC, Coque TM, Baquero F, Canton R. Nucleotide sequence and characterization of a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-10) isolated in Spain. Antimicrob Agents Chemother. 2001;45(2):616–20.
120. Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A. Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother. 2002;46(9):3045–9.
121. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother. 2004;48: 1–14.
122. Miriagou V, Tzelepi E, Daikos GL, Tassios PT, Tzouvelekis LS. Panresistance in VIM-1-producing *Klebsiella pneumoniae*. J Antimicrob Chemother. 2005;55(5):810–1.
123. Helfand MS, Bonomo RA. Current challenges in antimicrobial chemotherapy: the impact of extended-spectrum beta-lactamases and metallo-beta-lactamases on the treatment of resistant Gram-negative pathogens. Curr Opin Pharmacol. 2005;5(5):452–8.
124. Winkler ML, Papp-Wallace KM, Taracila MA, Bonomo RA. Avibactam and inhibitor-resistant SHV β-lactamases. Antimicrob Agents Chemother. 2015;59:3700–9.
125. Chan JF-W, Yuen K-Y. A new ASPECT for complicated urinary tract infections. Lancet. 2015;385:1920–2.
126. Drawz SM, Papp-Wallace KM, Bonomo RA. New beta-lactamase inhibitors: a therapeutic renaissance in an MDR world. Antimicrob Agents Chemother. 2014;58:1835–46.

Penicillin-Binding Proteins and β-Lactam Resistance

13

André Zapun, Pauline Macheboeuf, and Thierry Vernet

1 Introduction

Resistance to β-lactams has risen in a variety of ways. In this chapter, we consider those cases where resistance results from the expression of targets and the penicillin-binding proteins that have a "low affinity" for the drugs. In the last three decades, different resistance mechanisms that involve the PBPs have been uncovered in important human pathogens such as *Staphylococcus aureus*, *Enterococci*, *Streptococcus pneumoniae,* and *Neisseria*.

2 What Are PBPs?

Penicillin-binding proteins (PBPs) are the targets of β-lactam antibiotics. These enzymes catalyze the last steps in the polymerization of the peptidoglycan, the major constituent of the cell wall. The peptidoglycan, or murein, is a giant molecule, which constitutes a molecular mesh surrounding the plasma membrane. Chains of tandemly repeated disaccharides form the glycan strands that are linked to each other by short peptide bridges. The discoveries of the PBPs and of the cross-bridging mechanism were intimately intertwined. Based on studies of the effect of penicillin on peptidoglycan synthesis, it was concluded that cross-linking of the glycan chains resulted from a transpeptidation reaction, which is inhibited by β-lactams [1, 2]. The first PBPs were isolated a few years later by covalent affinity chromatography on penicillin-substituted resin [3]. Some of these PBPs were DD-carboxypeptidases or endopeptidases rather than transpeptidases. In the following four decades, PBPs have been the subject of intense research, particularly regarding their role in the resistance to β-lactams of some important pathogens such as *Staphylococcus aureus*, enterococci, *Streptococcus pneumoniae*, and more recently *Neisseria* and others such as *Pseudomonas aeruginosa* and *Acinetobacter baumanii*.

PBPs are characterized by the presence of a penicillin-binding domain, which harbors three specific motifs: SXXK, (S/Y)XN, and (K/H)(S/T)G. This signature is common to the ASPRE protein family (for Active-site Serine Penicillin Recognizing Enzymes), which also includes the class A and C β-lactamases. The topology of these β-lactamases is shared with the penicillin-binding domain of the PBPs [4, 5]. The penicillin-binding domain is characterized by an active site cleft between an α-helical sub-domain and an α/β-sub-domain, which consists of a 5-stranded β-sheet covered by a C-terminal α-helix. Following the topological nomenclature devised for β-lactamases [4, 6], the first motif SXXK is on the N-terminus of helix α2 of the helical sub-domain, on the bottom of the active site groove, in the standard representation. The third KTG motif on strand β3 of the α/β sub-domain is located on the right side of the active site. Note that this strand is termed β3 as a result of the connectivity of the polypeptide chain, although it forms the margin of the 5-stranded β-sheet. The second SXN motif is on the left side of the active site, on a loop between helix 4 and 5 of the helical sub-domain (Fig. 13.1).

The serine of the SXXK motif is central to the catalytic mechanism, which is thought to occur in the following manner (Fig. 13.2). The Oγ of the serine performs a nucleophilic attack on the carbonyl of the penultimate D-Ala amino acid of the stem peptide, which results in the departure of the last D-Ala amino acid and the formation of a covalent acyl-enzyme complex between the "donor" stem peptide and the protein. The carbonyl of the D-Ala amino acid, now forming an ester linkage with the active site serine, then undergoes a nucleophilic attack from a primary amine linked in various ways to the third residue of a second "acceptor" stem peptide. This second reaction forms a peptide bond between the two stem peptides and regenerates a free active site serine. What was just described is the catalysis of transpeptidation

A. Zapun, Ph.D. (✉) • T. Vernet, Ph.D. • P. Macheboeuf
Institute for Structural Biology, CNRS, CEA, Université Grenoble Alpes, Grenoble, France
e-mail: andre.zapun@ibs.fr

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_13

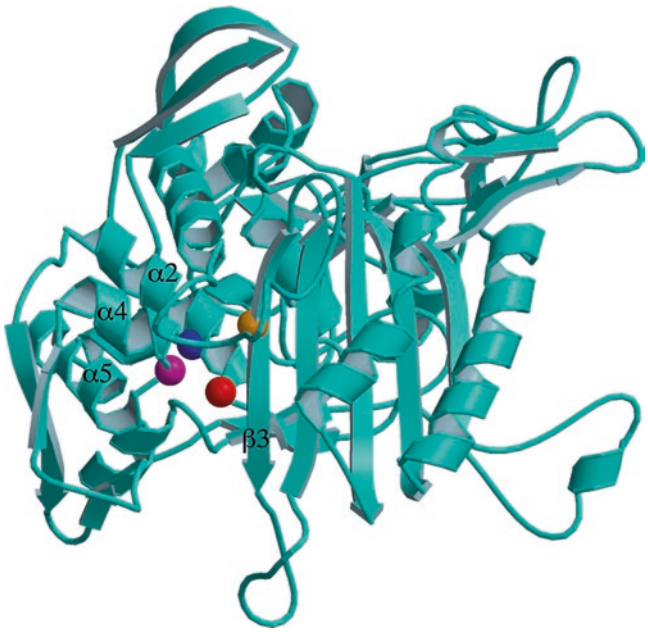


Fig. 13.1 Topology of the penicillin-binding domain. The example presented is the transpeptidase domain of *S. pneumoniae* PBP2x. The positions of the serine and lysine of the first SXXK motif are shown by *red* and *blue spheres*, respectively. The serine of the second SXN motif is indicated by a *purple sphere*. The lysine of the third KTG motif is shown in *yellow*. The elements of secondary structure, which bear the catalytic motifs, are indicated with the standard nomenclature

(Fig. 13.2a). In the case of DD-caboxypeptidases, the acyl-enzyme intermediate is hydrolyzed (Fig. 13.2b).

β-Lactams resemble the D-Ala-D-Ala dipeptide in an elongated conformation (Fig. 13.3). More than the similarity of linked atoms, it is the distribution of three electrostatic negative wells that accounts for the resemblance. With PBPs, β-lactams act as suicide inhibitors. The active site serine attacks the carbonyl of the β-lactam ring, resulting in the opening of the ring and formation of a covalent acyl-enzyme complex. This complex is hydrolyzed very slowly, thus effectively preventing the active-site serine from engaging in further productive reactions. β-Lactamases differ in that they react with β-lactams rather than with D-Ala-D-Ala dipeptides, and that hydrolysis of the acyl-enzyme complex is extremely fast, thus releasing an active enzyme and an inactive compound.

The reaction of PBPs and serine β-lactamases with β-lactams can be described kinetically as follows (Fig. 13.4). A non-covalent complex EI is formed between the enzyme E and the inhibitor I, with the dissociation constant K_d, from which acylation proceeds to form the covalent complex EI* with the rate k_2. EI* is finally hydrolyzed with the rate k_3 to regenerate the enzyme E and an inactivated product P. The rate described by k_3 is extremely rapid with β-lactamases, whereas it is negligible for PBPs on the time scale of a bacterial generation. The following nomenclature will be used throughout this review. The rate constants k_2 and k_3 describe the acylation and deacylation reactions, respectively. The second order rate constant k_2/K_d will be referred to as the efficiency of acylation, which allows calculation of the overall acylation rate at a given concentration of antibiotic. Note that the inhibitory potency of a particular β-lactam for a PBP is given by the c_{50}, which is the antibiotic concentration resulting in the inhibition of half the PBP molecules at steady state (i.e., when the acylation and deacylation reactions proceed at the same rate). The value of c_{50} is equal to the ratio $k_3/(k_2/K_d)$. In this review as in the literature in general, PBPs are referred to as being (or having) high- or low-"affinity" for β-lactams. This "affinity" implicitly refers to the c_{50}, and should not be confused with the strength of a non-covalent interaction, which can be described by an association-dissociation equilibrium with a K_d constant, such as the formation of the pre-acylation complex.

Despite the availability of several crystal structures of PBPs and β-lactamases, and detailed kinetic studies, the enzymatic mechanism is still a matter of debate. Several scenarios have been proposed that involve various residues of the conserved catalytic motifs and the carboxylate of the antibiotic. It is likely that the precise mechanisms differ between various ASPRE enzymes, and even for a single protein between different β-lactams [7].

3 Classification of PBPs

PBPs are commonly classified in three groups according to their molecular weight and domain structure: high molecular weight PBPs, which fall in two broad families called class A and B, and low molecular weight PBPs that constitute class C [8]. Note that the nomenclature of the PBPs is particularly confusing as it is historically based on the observed electrophoretic mobility. Thus there is no necessary functional or genetic relationship between homonymic PBPs of various organisms.

Class A PBPs comprise a single transmembrane segment, sometimes preceded by a short N-terminal cytoplasmic region, and two extracellular domains. The first extracellular domain carries the glycosyltransferase activity that is responsible for the polymerization of the glycan strands. The glycosyltransferase activity has been demonstrated for various purified recombinant class A PBPs including *Escherichia coli* PBP1b and PBP1a [9–13] and *S. pneumoniae* PBP1a, PBP1b, and PBP2a [14, 15]. The glycosyltransferase activity is inhibited by the glycopeptide antibiotic moenomycin [16], which is not used in therapy due to poor pharmacokinetic properties. As the focus of this review is on β-lactam resistance, the glycosyltransferase domain of the class A PBPs will not be discussed further. The C-terminal region of class A PBPs constitutes the penicillin-binding domain that cata-

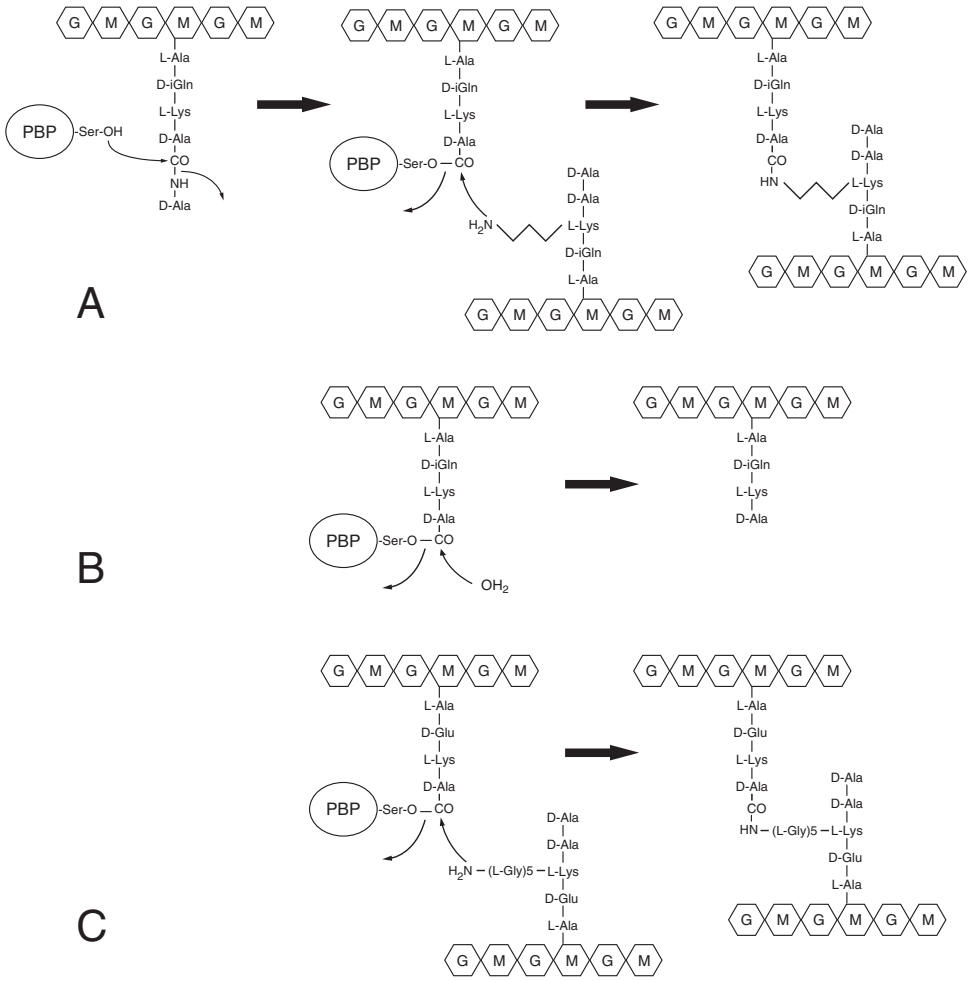


Fig. 13.2 (**a**) Catalysis of transpeptidation. Fragments of glycan strands are represented by chains of hexagones standing for the hexoses N-acetyl glucosamine (G) and N-acetyl muramic acid (M). The "donor" pentapeptide is depicted on the *upper* glycan strand, whereas the "acceptor" is attached here on the *lower* strand. The peptides shown are those from *Streptococcus pneumoniae*. The second and third amino acids may differ in various species. (**b**) Reaction catalyzed by DD-carboxypeptidase PBPs. With such proteins, the acyl-enzyme intermediate is hydrolyzed. (**c**) Transpeptidation reaction scheme in *Staphylococcus aureus*. Note that in many instances, including in *S. pneumoniae*, various intervening amino acids are attached to the third residue of the acceptor peptide, and provide the free amine that attacks the acyl-enzyme intermediate. Such stem peptides are called "branched"

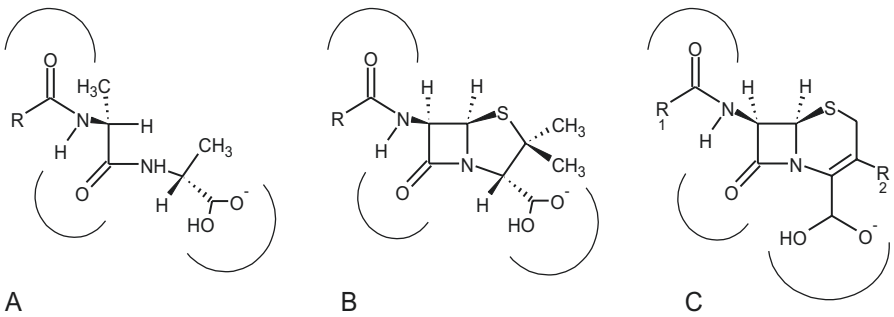


Fig. 13.3 Structural similarity between β-lactams and the natural substrate of the PBPs. (**a**) N-acyl-D-Alanyl-D-Alanine peptide. (**b**) Penicillin backbone. (**c**) Cephalosporin backbone. The regions of negative electrostatic potential are indicated by *arcs*

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P$$

$$K_d = k_{-1}/k_1$$

Fig. 13.4 Kinetic scheme of the reaction between a PBP (E) and a β-lactam (I). EI is a preacylation non-covalent complex. EI* is the covalent acyl-enzyme complex. P is the open inactivated product

lyzes transpeptidation, thus bridging adjacent glycan strands. Demonstration of the transpeptidase activity in vitro with purified recombinant proteins has been achieved for *E. coli* PBP1b and PBP1a [9, 10, 13], and *S. pneumoniae* PBP1a and PBP2a [15].

Class B PBPs consists of a transmembrane anchor, a domain of unknown function, and a transpeptidase penicillin-binding domain. The transpeptidase activity of *E. coli* PBP2

and *S. pneumoniae* PBP2b and PBP2x has been demonstrated in vitro with recombinant enzymes [15, 17]. The transmembrane segment and non-penicillin-binding domain are certainly involved in proper cellular targeting through probable interactions with other proteins, as demonstrated in the case of *E. coli* PBP3 [18].

Class C low molecular weight PBPs constitute the third group. These consist mainly of a penicillin-binding domain with a small additional C-terminal domain, which is anchored to the plasma membrane either through a transmembrane segment or an amphipathic helix presumably lying onto the lipid bilayer [19]. Low molecular PBPs have either demonstrated DD-carboxypeptidase or DD-endopeptidase activities [20–23].

4 Physiological Function of the PBPs

The cellular function of some PBPs has been inferred from various lines of evidence, but our knowledge remains sketchy. One type of data is the phenotype of mutant strains, or of cells treated with β-lactams that are specific of particular PBPs. The second type of result is the cellular localization of various PBPs, determined by immunofluorescence or fusion with the green fluorescent protein. Thus, various class A and B PBPs are involved in peptidoglycan synthesis during cell enlargement, cell division, or sporulation. In *E. coli*, for example, the class B PBP3 plays a role in division [24], whereas the class B PBP2 is involved in cell elongation and the onset of the division [25–27]. Less is known of the specific role of *E. coli* class A PBPs, as they show some degree of functional redundancy. PBP1a likely works in concert with the class B PBP2, as the latter stimulates the former in vitro [17]. The septal class B PBP3 on the other hand certainly functions together with PBP1b [28]. In *S. pneumoniae*, and similarly shaped streptococci, enterococci, and lactococci, there certainly are two machineries of peptidoglycan synthesis [29, 30], with the class B PBP2x and PBP2b participating to septal and peripheral cell wall synthesis, respectively. The respective function of the class A PBPs remains unknown in *S. pneumoniae*, although PBP1a remains co-localized with PBP2b throughout the cell cycle, rather than with septal PBP2x [31]. *S. aureus* is a spherically shaped coccus, whose division appears to produce the entirety of the new hemisphere of the daughter cell, in a process that involves its single class A PBP2 [32]. The relative role of the two class B PBP1 and PBP3 in *S. aureus* is not clear.

In summary, peptidoglycan synthesis occurs in different phases, sometimes at different locations, depending on the morphology of the organism considered, with different participating PBPs. The class B PBP strictly involved in cell division can be generally identified by sequence comparisons with well-characterized examples and by the localization of its gene in a cluster coding for division proteins [33]. The specific cellular function of the other class B and class A PBPs is more difficult to determine without dedicated genetic and localization studies.

5 PBP-Based β-Lactam Resistance

Inhibition of PBPs produces an imbalance in cell wall metabolism resulting in lysis or growth inhibition. The link between PBP inhibition and the biological outcome, lysis or growth arrest, remains poorly understood (e.g., *Escherichia coli* [34], *Staphylococcus aureus* [35], *Enterococcus hirae* [36]). Despite our ignorance of the detailed physiological consequences of β-lactam treatment, various means of resistance have been uncovered and investigated. Resistance to β-lactams was found to arise from decreased permeability of the outer membrane, export of the antibiotics by efflux pumps (these two mechanisms are restricted to gram-negative bacteria), degradation of the antibiotic by β-lactamases, or utilization of PBPs with low-affinity for the β-lactams. The following sections will be devoted to the PBPs of organisms that exploit this latter strategy.

6 *Staphylococcus aureus*

After the spread of *S. aureus* strains that were resistant to penicillin through the acquisition of a β-lactamase, the semisynthetic β-lactam methicillin was introduced, which was not degraded by β-lactamases known at the time. A methicillin resistant clinical strain was isolated soon afterwards [37]. The so-called MRSA (methicillin resistant *S. aureus*) strains are particularly dangerous in that they exhibit wide resistance to virtually all β-lactams, often associated with co-resistance to other classes of antibiotics. MRSA strains were initially found in hospitals causing difficult to treat nosocomial infections (HA-MRSA, for hospital-acquired). Distinct strains, more virulent, appeared in the community in the 1990s (CA-MRSA, for community-acquired). CA-MRSA are now also commonly found in hospital settings [38]. Vancomycin, a glycopeptide antibiotic, has long been used as a last resort weapon to fight MRSA strains. However, strains exhibiting both high methicillin and vancomycin resistance have appeared repeatedly [39, 40]. Strains non-susceptible to several novel antibiotics such as linezolid, daptomycin, or mupirocin have also appeared (e.g., [41]).

The wide spectrum β-lactam resistance of MRSA strains results from the expression, in addition to the four native PBPs, of a fifth PBP termed PBP2a or PBP2′ with low affinity for the antibiotics [42, 43]. No correlation, however, could be detected between the level of resistance and the

amount of PBP2a expressed [44]. PBP2a is the product of the *mecA* gene whose transcription is controlled by the *mecI* and *mecR1* regulatory elements. MecI is a DNA-binding protein that represses *mecA* transcription [45]. By analogy with the homologous BlaI and BlaR1 system that controls the expression of the β-lactamase BlaZ, the Mec system is thought to function in the following manner. MecR1 is a signal-transduction protein with an extracellular penicillin-binding domain that senses the presence of β-lactams in the medium, and activates its cytoplasmic domain. The intracellular domain of MecR1 is a protease that undergoes activation through autocatalytic cleavage. In parallel, β-lactams inhibiting PBPs would cause a change in peptidoglycan turnover. Peptidoglycan fragments imported in the cytoplasm would then be cleaved by MecR1 to generate a dipeptide that binds to MecI to alleviate the repression of *mecA* [46]. The *mecA* gene and its regulatory system are found on a large mobile genetic element called the staphylococcal cassette chromosome *mec* that integrates at a unique site in the chromosome [47]. Several variants of the cassette have been found that include in addition to the *mec* genes, several genes encoding resistance to other types of antibacterial agents. A thorough presentation of the current understanding of these genetic elements and their history can be found elsewhere [38, 48, 49].

Interestingly, the intact *mec* system does not confer resistance, as the expression of PBP2a is normally well repressed. Only few β-lactams, not including methicillin, can alleviate this repression. Mutations, for example, in *mecI* or in the *mecA* operator region, lead to de-repression of *mecA*. Even so, strains with unrestricted expression of PBP2a exhibit methicillin resistance only in a small subpopulation (at a frequency of 10^{-4}–10^{-6}), when maintained without β-lactam selective pressure. Following exposure to β-lactams, a homogenous resistant population is selected. When the antibiotic selective pressure is removed, heterogeneity is rapidly restored, with only a small subpopulation retaining resistance. These observations indicate that the functioning of PBP2a in cell wall synthesis bears a cost that is best avoided in the absence of β-lactams. The nature of the genetic determinants of homogenous high methicillin resistance in wild strains is complex but certainly involves the stringent stress response [50].

PBP2a is of class B and therefore lacks the glycosyltransferase activity that is also required for peptidoglycan synthesis. Although PBP2a supports all the transpeptidase activity when this activity is inhibited by β-lactams in the four native high molecular weight PBPs, the presence of the class A PBP2 with an active glycosyltransferase domain is nevertheless required [51, 52].

More recently, further complexity has been revealed. The transpeptidase activity either of PBP2 or from the low molecular weight PBP4 was found to contribute to the resistance provided by PBP2a. PBP4 is a small PBP with both transpeptidase and carboxypeptidase, as well as β-lactamase activity in vitro, whose structure was solved [53]. Recent works have shed light on the required cooperation of PBP4, in addition to PBP2, with PBP2a to express β-lactam resistance. A laboratory mutant strain selected for resistance to ceftizoxime was found to have a single substitution in PBP2. Disruption of *pbpD* (encoding PBP4) decreased this resistance, along with a diminished degree of peptidoglycan cross-linking. To the contrary, expression of PBP2a restored resistance and high levels of peptidoglycan cross-linking [54]. In a CA-MRSA strain, deletion of PBP4 resulted in a loss of resistance, decreased cross-linking of the peptidoglycan, and lower expression of PBP2. In that strain, overexpression of PBP2 or PBP2a did not revert the loss of resistance [55]. Wall teichoic acids appear to play an important role in this cooperation between PBPs. Staphylococcal wall teichoic acids are long polyol phosphate polymers anchored to the peptidoglycan. The disruption of *tarO*, a gene required for the initiation of wall teichoic acids synthesis, decreased the β-lactam resistance and peptidoglycan cross-linking in HA- and CA-MRSA strains [56]. Ticlopidine, a compound that inhibits TarO, had a similar effect. Most interestingly, ticlopidine had a synergistic antibacterial effect with cefuroxime, which targets PBP2 [56]. This observation was explained by the fact that wall teichoic acids are required for the proper localization of PBP4 [57]. The combined use of two β-lactams that target PBP2 and PBP4 also had a synergistic effect that overcame the resistance provided by PBP2a [55, 56].

The feature of wall teichoic acid required for β-lactam resistance in MRSA strains is the attachment of β-O-GlcNAc moieties by the enzyme TarS. Disruption of *tarS* prevents the resistance of MRSA while preserving other functions of the wall teichoic acids [58].

Other genes have been found to be necessary for the full expression of the resistance conferred by PBP2a. Over 30 of these auxiliary genes, often termed *fem* (for factor essential for methicillin resistance, or *aux* for auxiliary), have been identified [59]. Several *fem* genes are involved in cell wall metabolism, other genes participate to regulatory or putative sensory functions. How they cooperate to allow the *mecA*-based resistance is a complex and unresolved issue. The *femAB* operon, for example, adds the second to fifth glycine residues to the peptidoglycan precursor to form the pentaglycine branch that serves afterwards as the cross-bridge of staphylococcal peptidoglycan [60, 61] (Fig. 13.2c). A trivial conclusion would be that PBP2a has a specific requirement for "acceptor" peptides with a pentaglycine branch. This expectation turned out to be naïve, for PBP2a can confer resistance to *Enterococcus faecalis* and *faecium*, which lack *femAB* and have the alternative peptidoglycan cross-bridges $(Ala)_2$ and D-Asx, respectively [62]. Note that MRSA strains,

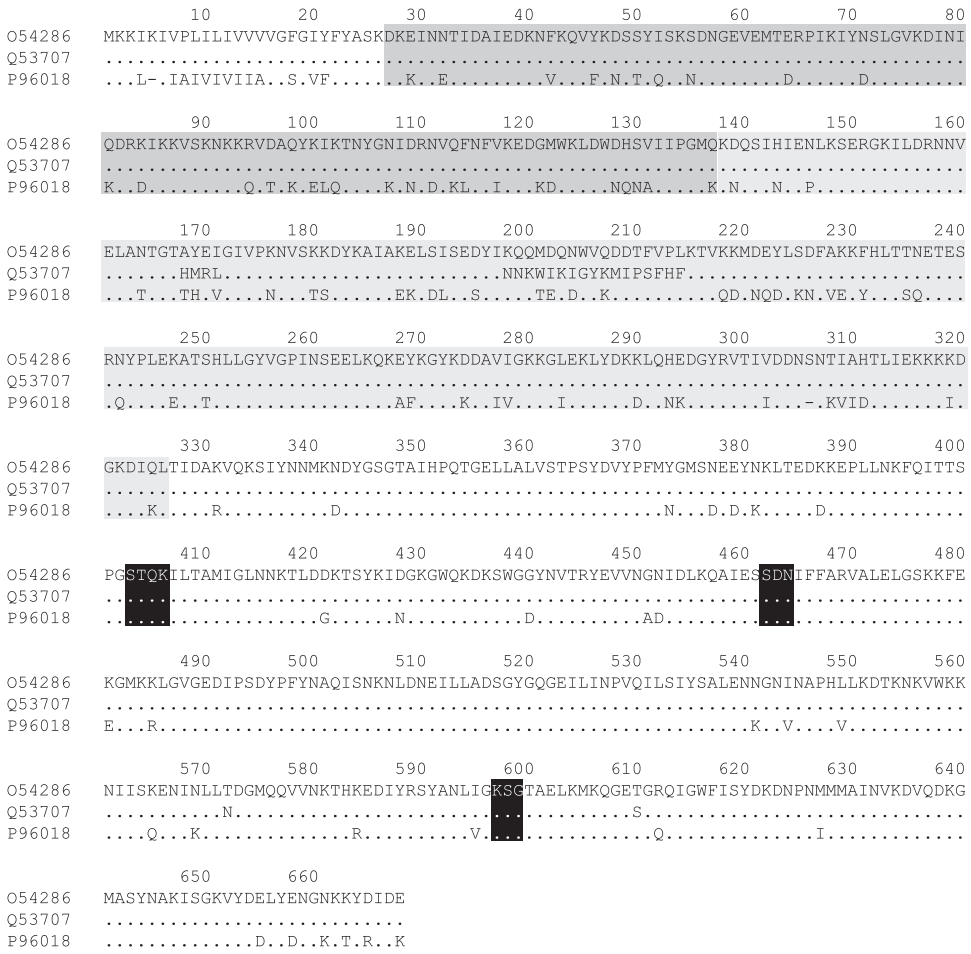


Fig. 13.5 Sequence alignment of staphylococcal PBP2a sequences (designated by their Uniprot accession numbers). The # P96018 sequence is from *S. sciuri*. The N-terminal domain is *shaded. Dark shading* indicates the extension specific to the subgroup of class B PBPs that includes PBP2a. The catalytic motifs are in *black boxes*

containing the *mecA* gene, that are oxacillin resistant were found to have mutations in their *femXAB* genes [63].

Another gene involved in MRSA resistance to β-lactams is *fmtA* [64]. FmtA is homologous to PBPs and β-lactamases. FmtA reacts slowly with β-lactams, has a weak DD-carboxypeptidase activity, and binds to wall teichoic acid [65, 66].

PBP2a belongs to a subgroup of class B PBPs characterized by the presence of an insertion of about 100 residues following the transmembrane anchor (Fig. 13.5). This group also includes chromosomally encoded PBP5 from *Enterococcus faecium*, *E. hirae*, and *E. faecalis*, and plasmid encoded PBP3 from *Enterococcus hirae*, which are all low affinity PBPs involved in some degree of β-lactam resistance (see below). There are other members of this subgroup of PBPs in *Bacillus subtilis* and related species, in *Listeria monocytogenes* and *innocua* and in *Clostridium acetobutylicum*, although these do not appear to confer reduced susceptibility to β-lactams. A close *mecA* homologue (*pbpD* encoding PBP4) has been found both in susceptible and resistant *Staphylococcus sciuri* strains [67–69]. The expression of *S. sciuri* PBP4 in *S. aureus* generated a MRSA strain in the lab [67]. A recent phylogenetic analysis pin-pointed *S. fleurettii pbpD* as a likely ancestor of *mecA* [70]. The *mec* system may thus have spread from a closely related staphylococcal species, not only to *S. aureus*, but also to *S. epidermitis*, *S. haemolyticus*, *S. hominis,* and *S. simulans* [71]. The sequence of this most commonly found PBP2a is nearly identical in all strains, with over 97 % identity between *S. aureus* and *S. fleurettii*.

Recently, an alternative *mec* system was uncovered in some β-lactam-resistant isolates, where PBP2a exhibits only 63 % sequence identity with the common PBP2a [72, 73]. This variant encoded by *mecC* has a better affinity for oxacillin than for cefoxitin, in contrast to the standard PBP2a, which "prefers" cephalosporins to penicillins. This difference of affinity for the various β-lactams is mirrored in the resistance conferred by the two variants [74].

The reaction of PBP2a with β-lactams is extremely slow. The acylation efficiency of PBP2a by penicillin G, characterized by the second order rate constant k_2/K_d of approximately 15 $M^{-1}s^{-1}$, is roughly 500-, 800-, 900-, and 20-fold smaller than that of the native PBP1, PBP2, PBP3, and PBP4 from *S. aureus*, respectively [75–77]. When compared to PBP2x from the susceptible *S. pneumoniae* strain R6, PBP2a is acylated three to four orders of magnitude more slowly [77–79].

With such a poor acylation efficiency [76, 77], the acylation rate of PBP2a at therapeutic concentrations of β-lactams is negligible compared to the bacterial generation time ($t_{1/2}$ for acylation greater than 1 h with 10 μM of penicillin).

The low efficiency of acylation appears to result both from a poor "true" affinity of PBP2a for the β-lactams, with dissociation constants (K_d) of the pre-acylation complex in the millimolar range, and extremely slow acylation rates (k_2) ranging from 0.2 to 0.001 s^{-1} [77, 80]. Although published values differ for various β-lactams and means of measurement, the acylation rate k_2 of PBP2a by penicillin G, for example, is three orders of magnitude slower than that of the susceptible PBP2x from *S. pneumoniae* [77, 78].

The structure of a soluble form of PBP2a without its transmembrane anchor has been solved to a resolution of 1.8 Å [81]. The N-terminal non-penicillin-binding domain (residues 27–328) is bilobal, with the first lobe (27–138) formed by the subgroup specific extension. The transpeptidase domain shares its overall fold with other PBPs. The N-terminal domain confers a rather elongated shape to the whole molecule with the active site reaching approximately 100 Å from the membrane anchor.

In the absence of bound antibiotic, the active site of PBP2a appears to be rather closed with the active site S403 poorly positioned for a nucleophilic attack and a twisting of strand β3 that is required to accommodate the N-terminus of helix α2 and the active site S403. The structures of PBP2a with covalently bound nitrocefin, methicillin, and penicillin G revealed a tilt of the whole helical subdomain with respect to the α/β-subdomain (2.3° with nitrocefin, O. Dideberg, personal communication). This rotation opens the active site and is accompanied by a substantial local rearrangement of the active site (Fig. 13.6). The Oγ of S403 is displaced by 1.8 Å (with nitrocefin), whereas the strand β3 is straightened. It has been argued that this conformational rearrangement is costly and impedes acylation. The 20-fold slower acylation by methicillin (k_2=0.008 s^{-1}) compared to penicillin G (k_2=0.2 s^{-1}) has likewise been rationalized on the basis that bound methicillin is translated along the active site cleft relative to bound penicillin G. This relative displacement increases the distance between the putative proton donor (S462) of the second catalytic motif and the nitrogen group of the opening β-lactam ring. Although possible, these explanations rely on the assumption that the conformations of the acyl-enzyme intermediates are relevant to the transition states of the acylation reaction. However, it must be remembered that there is a complete absence of correlation between the efficiency of acylation (k_2/K_d) and the strength of the non-covalent interaction between the covalently bound antibiotic and the PBP, as demonstrated with *E. coli* PBP5 [82]. Therefore, analysis of the complementarity of bound open antibiotics may bear little relevance to the understanding of the acylation process.

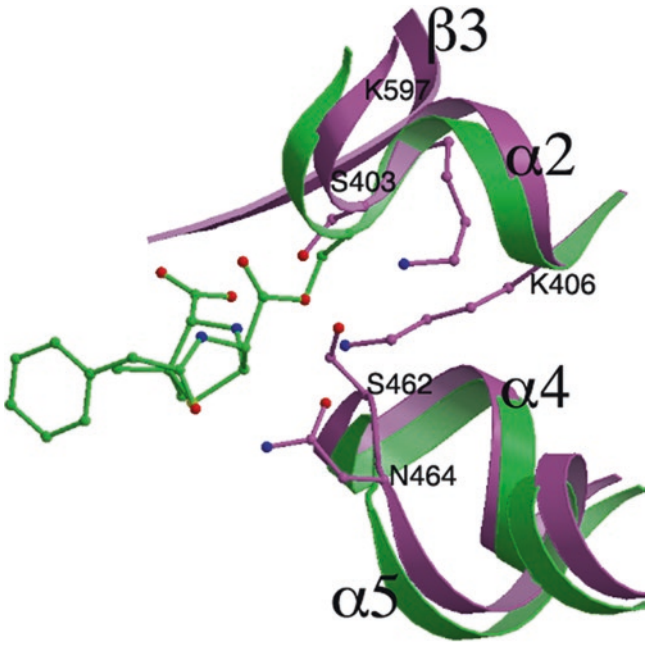


Fig. 13.6 Superposition of the active site of *S. aureus* PBP2a without (*purple*) and with (*green*) bound penicillin (shown in *balls* and *sticks*). The first motif on helix α2 and the second motif between α4 and α5 are moved away from strand β3, which bears the third catalytic motif

However, support for a significant rearrangement of PBP2a upon acylation was provided by circular dichroism spectra showing a diminishing helix content with rates consistent with the acylation kinetics [80, 83]. Binding of peptidoglycan fragments also caused conformational changes detected by circular dichroism [84]. As binding of peptidoglycan mimics also led to an increase of the reaction rate with β-lactams, it was proposed that the catalytic activity of PBP2a, and possibly of PBPs in general, might be stimulated by peptidoglycan [84].

Ceftaroline, a cephalosporine, showed great efficacy against MRSA and was found to acylate PBP2a, 25-fold better than ceftobiprole [85]. The crystal structure of PBP2a with ceftaroline showed two antibiotic molecules [86]. One molecule formed the expected acyl-enzyme within the active site, whereas a second molecule of ceftaroline was found non-covalently bound in a cleft between two subdomains of the N-terminal region, 60 Å from the active site. It was proposed that binding at this distant site triggers the opening of the active site through a long distance allosteric interaction mediated by a chain of salt bridges [86, 87]. Ceftaroline at the allosteric site may mimic the binding of peptidoglycan.

However, several clinical strains have already been isolated with a reduced susceptibility to ceftaroline. Two substitutions in the allosteric domains (N146K and E150K) were found in distinct strains [88]. The combination of both mutations was shown to decrease three- to fourfold the acylation

efficiency by ceftaroline. Examination of the structure of PBP2a single and double mutants suggests that the salt bridge network that mediates the allosteric communication between the active site and the non-covalent binding site is affected [87].

Mildly β-lactam-resistant strains of *S. aureus* have also been isolated that lack both *mecA* and β-lactamases. There are good indications that the resistance of these strains is due to modified native PBPs. Alterations of penicillin binding by PBP1, PBP2, PBP3, PBP4, and elevated amount of PBP4 were observed in such strains [89–92]. The acylation rate of PBP1 and PBP2 was decreased, and the deacylation rates increased [75]. The kinetic modifications result from point mutations, as demonstrated for PBP2 [93]. A tenfold decrease of the acylation efficiency results from the double substitution S569A and A576S. Another variant with the A450D and A462V substitution surrounding the SXN motif and the Q629D mutation has a k_2/K_d lowered 20-fold [93]. A laboratory mutant selected with ceftizoxime has the single substitution P458L close to the SXN catalytic motif [54]. The mutations found in PBP4 E183A and F241R are close to the active site [90].

Thus, *Staphylococcus aureus* has been found to resist β-lactams in three ways, by using β-lactamases to degrade the antibiotic, by lowering the affinity of its endogenous PBPs for β-lactams or increasing their expression, and most dangerously through the recruitment of an additional PBP that is unaffected by β-lactams.

To combat MRSA, the search of new drugs acting on PBPs has been pursued. New alkylboronates compounds were explored as inhibitors of PBPs and showed promising antibacterial activity against MRSA while binding to PBP1 and PBP2a [94]. Likewise, new sulfonamide and anthranilic acid derivatives constitute promising leads for the development of new drugs as they were found to have antibacterial activity and inhibit PBP2a in vitro [95]. Other compounds that exhibit interesting properties regarding the PBPs of MRSA are the epicatechin gallate, which reduces resistance presumably by delocalizing PBP2 [96], and spermine, which is antibacterial against MRSA in synergy with β-lactams [97].

6.1 Enterococci

The intrinsic resistance to β-lactams is a characteristic of enterococci. Isolates of *Enterococcus faecalis* typically exhibit MICs for penicillin of 2–8 mg/L [98], and *E. faecium* of 16–32 mg/L [99]. These two species, which cause important human health problems, have been the subject of intense molecular studies over the past three decades, together with *E. hirae*, which is more of a concern in veterinary medicine.

Enterococci morphologically resemble streptococci, which may be related to the fact that they share the same set of three class A and two class B high molecular weight PBPs [100]. However, the intrinsic moderate resistance to β-lactams results from the presence of an additional sixth high molecular weight PBP, which takes over the transpeptidase function of the other PBPs when these are inhibited by the antibiotics [101, 102]. This was concluded from three lines of evidence in early studies of an *E. hirae* strain and several derivatives (initially identified as *Streptococcus faecium* ATCC 9790). Firstly, it was found that one of the high molecular weight PBPs (PBP5) had a much lower affinity for penicillin, and spontaneous mutants with greater resistance had elevated amounts of this PBP [101]. Secondly, a mutant hypersensitive to penicillin was found to lack PBP5 expression [102]. Finally, saturation of PBP5 with β-lactams led to bacterial death [103].

Subsequent and parallel studies uncovered the same mechanism underlying intrinsic β-lactam resistance in *E. faecium* [104, 105] and *E. faecalis* [106]. The wide range of elevated levels of resistance exhibited by clinical isolates of *E. faecium* was found to arise from two mechanisms: increased expression of PBP5 and mutations of PBP5 that further decrease its affinity for β-lactams [107–109]. Strains with intermediate level of resistance (MIC for ampicillin of 8 mg/ml) appear to rely mainly on the first mechanism, while extremely resistant strains (MIC for ampicillin of up to 512 mg/L) appear to combine both overexpression and reduced affinity [107, 109] or use only the latter mechanism [110, 111]. Note that the exclusive use of the PBP5 transpeptidase, when the others are inhibited by β-lactams, does not modify the composition of the peptidoglycan cross-bridges [104].

Although the gene *pbp5* is endogenous to enterococci, alleles encoding low-affinity enzymes can be transferred from strain to strain [112]. A peculiar strain of *E. hirae* (S185) was found to express, in addition to its chromosomally encoded PBP5, a second PBP with low β-lactam affinity. This related but plasmid-encoded PBP is termed PBP3r [113, 114].

When the genes encoding PBP5 from various *E. faecium* clinical isolates were sequenced (Fig. 13.7, Table 13.1), several point mutations were found to be correlated with a low affinity for β-lactams and high resistance [110, 111, 115]. However, as clinical isolates are not isogenic, assessment of the effect of various PBP5 sequences awaited their introduction in a single strain. When three PBP5 sequences originating from strains with MICs for ampicillin of 2, 24, and 512 mg/L were introduced in a strain with no PBP5 expression (MIC ampicillin of 0.03 mg/L), the resulting strains had MICs of 6, 12, and 20 mg/L, respectively [104]. These results demonstrate that variants of PBP5 indeed confer different MICs, but that this effect is strongly modulated by other

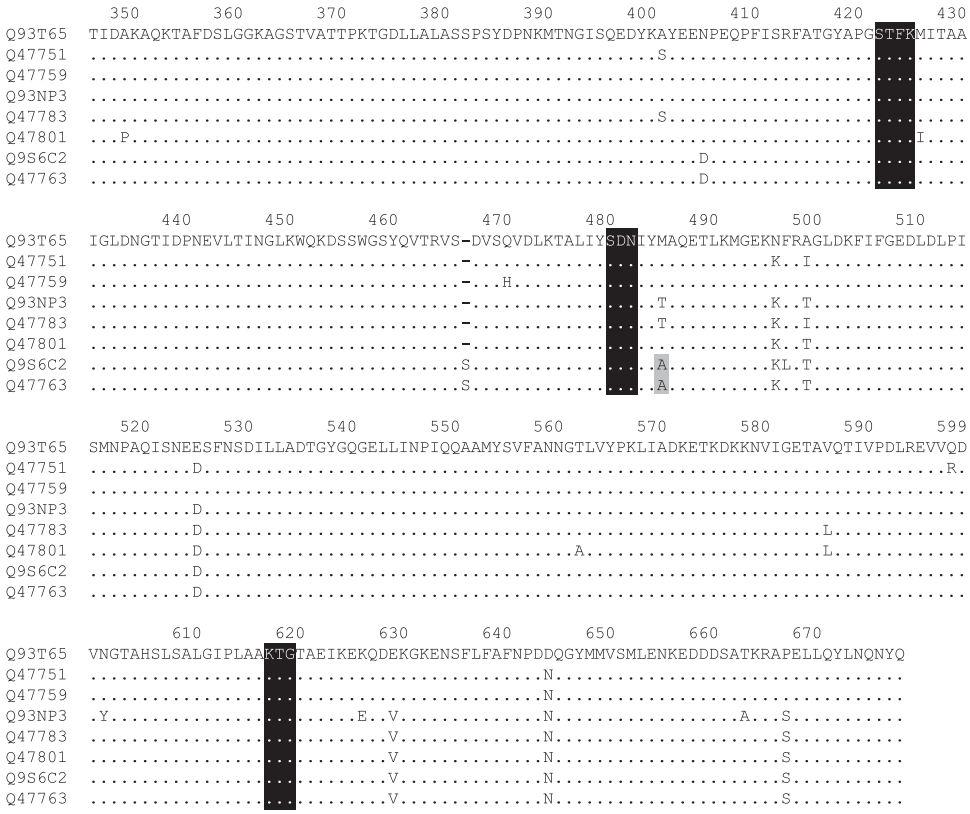


Fig. 13.7 Alignment of publicly available sequences of *E. faecium* PBP5 transpeptidase domain. Sequences are ordered according to the MIC of their originating strain (see Table 13.1). Catalytic motifs are *blackened*. The M485S substitution that was investigated and shown to increase resistance is highlighted in *gray*

Table 13.1 Characteristics of *E. faecium* strains and their PBP5, for which sequences are publicly available

Uniprot #	Strain	MIC (mg/L)	MIC (mg/L) in isogenic strains[a]	Expression level	k_2/K_d ($s^{-1}M^{-1}$)[b]
Q93T65	BM4107	2 (Amp)[a]	6	→[a]	
Q47751	D366	16 (Pen)[b]		↗[b]	17
Q47759	D63	5 (Pen)[b]		→[b]	24
	D63r	70 (Pen)[b]		↗↗[b]	20
Q93NP3	D344	24 (Amp)[a]	12	↗↗[b]	17
		64 (Pen)[b]			
Q47783	EFM-1	90 (Pen)[b]		→[b]	1.5
Q47801	9439	128 (Amp)[d]		↗[c]	
Q9S6C2	C68	256 (Amp)[e]			
Q47763	H80721	512 (Amp)[a]	20	→[b]	<1.3
		512 (Pen)[b]			

[a][104]
[b][111]
[c][107]
[d][115]
[e][124]

unknown factors. Sequencing of *pbp5* and MIC determination of numerous clinical strains also showed that overexpression and substitutions cannot account entirely of the various levels of resistance, and that other contributing factors exist [108, 116, 117].

The particular mutation M485A was hypothesized to have a very important effect as it was found in two highly resistant strains and is located close to the second catalytic motif SXN482 [111]. When introduced individually, this mutation caused only a modest increase of resistance, when compared to the resistance of the clinical strains that harbor this substitution [104, 111]. However, in an isogenic background, the M485A substitution accounted for most of the difference of resistance conferred by two PBP5 variants that otherwise differed at seven positions in the transpeptidase domain [104]. Substitutions M485A/T, A/I499T, E629V and

the introduction of an additional Ser466′ or Asp466′ were found to be significantly associated with ampicillin resistance [108]. Some of these mutations investigated individually caused modest effects: I499T, E629V and the introduction of an additional Ser466′ [118]. When combined with other mutations, in particular M485A, the additional Ser466′ increased the MIC for penicillin nearly threefold [118]. This study also found that various substitutions had different effects on the MICs of different β-lactams.

Enterococcal PBP5 belongs to the same subgroup of class B PBPs as the acquired *S. aureus* PBP2a, with an insertion of about 120 residues following the transmembrane helix. The crystal structure of *E. faecium* PBP5 bound to penicillin was solved to a resolution of 2.4 Å [119]. The originating strain (D63r) had a MIC for penicillin of 70 mg/L that appears to result solely from overproduction of the same PBP5 found in the parental strain (D63), which has the basal MIC of 5 mg/L [111]. Therefore, the structure is that of a "wild-type" PBP5, without substitutions that further decrease the affinity for β-lactams. The efficiency of acylation of D63r *E. faecium* PBP5 defined by the second order rate constant k_2/K_d = 20 $M^{-1}s^{-1}$ is similar to that of *S. aureus* PBP2a, that is 2–3 orders of magnitude slower than that of a "regular" high-affinity PBP [111]. A study of the reaction of a variety of β-lactams with a soluble form of PBP5 showed that the acylation is sensitive to the various substituents of the drug, thus offering some scope for improving β-lactams against enterococci [120].

As no structure was obtained in the absence of antibiotic, no comment could be made regarding a possible rearrangement upon acylation, although the authors speculate that some loop residues, which are conserved in this subgroup of PBPs (residues 461–465), may have been pushed aside to allow antibiotic binding [119]. Another proposal is that S480 of the second catalytic motif may not be appropriately positioned to act as the proton donor for the nitrogen of the opening β-lactam ring [119], much as proposed in the case of *S. aureus* PBP2a and methicillin [81]. The important role of the substitution of M485 by Ala or Thr in the expression of high resistance [104, 110, 111] was rationalized as follows. The side chain of M485 lies behind K425 of the first catalytic site, which may be involved in the proton abstraction of the catalytic S422. Smaller residues in position 485 may result in greater conformational freedom of K425 and thus hinder acylation. The same argument might apply to the M426I substitution found in a highly resistant strain [115]. The addition of a second serine after S466 that is found in a PBP5 with an extremely low efficiency of acylation [111] was tentatively explained by a reinforcement of the steric hindrance due to the rigid loop 451–466 [119].

PBP5, as a class B PBP, does not support the necessary glycosyltransferase activity for peptidoglycan synthesis, although it can take over all the required transpeptidase activity. Deletion studies in *E. faecalis* and *E. faecium* have demonstrated that for expression of resistance, the glycosyltransferase activity must be provided by at least one of the two class A PBPs encoded by *ponA* or *pbpF* [100, 121]. The third class A PBP encoded by *pbpZ* is not required.

Although the high resistance of many enterococcal clinical strains results from their greater amount of PBP5, the reasons underlying this overexpression are still unclear. An open-reading frame upstream of the gene encoding PBP5 is truncated in an *E. hirae* strain overproducing PBP5. This finding suggested that this gene might be a PBP5 synthesis repressor (*psr*) [122]. However, subsequent tests of this hypothesis in *E. hirae* using isogenic strains have ruled out a role of *psr* in the regulation of PBP5 expression [123]. Similarly, no role for *psr* was found in PBP5 expression in *E. faecium* [124] or *E. faecalis* [106].

Four isolates of *E. faecalis* were found to exhibit high resistance to ampicillin and imipenem without overexpression of PBP5. Instead, the resistance is due to two substitutions, P520S and Y605H, in PBP4 (the orthologue of streptococcal PBP2x) [125].

In addition to the modes of resistance presented above, the plasmid-borne expression of β-lactamases has been documented in some clinical strains of *E. faecalis*, and less frequently in *E. faecium* [126]. Although not (yet?) found in clinical isolates, an intriguing mechanism of β-lactam resistance was selected in laboratory strains of *E. faecium* [127–130]. These mutants bypass altogether the need for PBPs. A β-lactam insensitive L,D-transpeptidase is responsible for cross-linking of the peptidoglycan, generating L-Lys-D-Asx-L-Lys instead of D-Ala-D-Asx-L-Lys bridges. However, increased resistance does not result from higher L,D-transpeptidase activity, but from a greater amount of precursor that lacks the terminal D-Ala. This elevated amount of truncated precursor is due to the cytoplasmic overexpression of a β-lactam insensitive D,D-carboxypeptidase [129, 130]. This precursor cannot be a "donor" substrate for the PBPs but is adequate for the L,D-transpeptidase activity. If ever found in clinical isolates, this mechanism would spell the end of β-lactam-based therapy for enterococci, as it completely obviates the transpeptidase function of the PBPs.

7 *Streptococcus pneumoniae*

Expression of a β-lactamase or of an additional low-affinity PBP has never been reported in pneumococcus. Instead, β-lactam-resistant strains of *S. pneumoniae* always harbor modified versions of their own PBPs that are inefficiently acylated by β-lactams [131, 132].

Once electrophoretic techniques were good enough to resolve the six PBPs from *S. pneumoniae*, it became apparent that PBP1a, PBP2b, PBP2x, and sometimes PBP2a were

altered in resistant clinical isolates. These modified PBPs bound less radiolabeled antibiotic, whereas the affinity of PBP1b and PBP3 was unchanged [133]. Sequencing revealed that mosaic genes encode PBP2b [134], PBP2x [133], and PBP1a [135] in resistant clinical strains. Mosaicity is the product of recombination events between different alleles within a species or between homologous genes of related species. *S. pneumoniae* as a naturally competent organism is particularly apt to this type of genomic plasticity [136].

Mosaic sequences of *pbp* genes are very difficult to classify and organize. Comparison of nucleotide sequences originating from susceptible strains show that they exhibit the same level of polymorphism as other loci, with less than 1 % of differences leading to one or two amino acid substitutions over the protein length [133, 134]. In contrast, mosaic *pbp* genes show blocks of sequences that differ from non-mosaic alleles by about 14–23 % (PBP2b [134, 137]; PBP1a [135]; PBP2x [133]). The diverging blocks span various lengths of the region coding for the transpeptidase domain or even most of the extracellular domain. The degree of difference compared to the normal level of intraspecies polymorphism suggested that the diverging sequence blocks originate from other streptococcal species [133, 134]. Parallel examination of various mosaic *pbp* genes showed that multiple sources of homologous DNA had been tapped by pneumococcal strains to survive antibiotic selection [133, 134, 138]. Evidence of multiple recombination events in the history of individual *pbp* alleles further complicates the analysis, although favored sites of recombination can be identified [138].

The origin of the sequence blocks found in mosaic *pbp* genes remains largely mysterious with the possible following exceptions for *pbp2x*. Fragments of the *pbp2x* sequences of two penicillin-susceptible strains of the commensal *Streptococcus mitis* and *Streptococcus oralis* could be identified in many alleles encoding PBP2x from resistant pneumococci [138, 139]. Although large fragments of these *S. oralis* and *S. mitispbp2x* sequences can be recognized in resistant strains of *S. pneumoniae*, the identity in these blocks is not perfect. In one instance, the comparison of numerous sequences from strains of *S. oralis*, *S. mitis,* and *S. pneumoniae* permitted the identification of a *pbp2x* gene in a susceptible *S. mitis* strain as the origin of a large sequence fragment spanning the whole transpeptidase domain in numerous resistant strains [139]. Comparison of this sequence fragment between the originating strain and resistant strains allowed the identification of several substitutions likely important for the diminished affinity for β-lactams [139]. This observation supports the following scenario for the emergence of pneumococcal resistance. Commensal streptococci sharing the same niche, such as *S. oralis* and *S. mitis*, have acquired resistance through point mutations selected by repeated exposure to β-lactam treatment for various ailments. Fragments of genes encoding PBPs with reduced affinity were subsequently exchanged between closely related streptococcal species, including *S. pneumoniae*, and selected by antibiotic pressure [139, 140]. The recognition of these multiple horizontal gene transfers in commensal streptococci and pneumococcus has led to the concept of global gene pool of altered *pbp* sequences for β-lactam resistance [141].

Since *S. pneumoniae* can easily exchange genetic material, closely related strains can differ in capsular biosynthetic genes (hence serotype) and *pbp* genes. Conversely, identical *pbp* alleles or capsular biosynthetic genes can be found in unrelated strains [142, 143]. Nevertheless, despite the complications that horizontal gene transfers bring to the definition of pneumococcal lineage, it appears from numerous studies that the worldwide spread of pneumococcal β-lactam resistance results from the dispersion of a limited number of successful clones [144, 145].

Besides mosaicity resulting from inter- and intraspecies homologous recombination, point mutations in *pbp* genes directly in *S. pneumoniae* have also certainly contributed to the resistance phenomenon. A case in point is the T550A substitution in PBP2x that confers resistance to cephalosporins but susceptibility to penicillin. This substitution was found in the laboratory upon selection with cefpodoxime or cefotaxime [146–148], as well as in PBP2x from clinical isolates where it was caused by a mutation within either a mosaic [149] or a "virgin" *pbp2x* gene [150].

Selection in the laboratory has demonstrated that PBP2x and PBP2b are the primary resistance determinants for cefotaxime (a cephalosporin) and piperacillin (a penicillin), respectively [146, 151]. This could naively be interpreted as PBP2x and PBP2b being the essential PBPs most reactive towards cefotaxime and piperacillin, respectively. Indeed, cefotaxime does not react with PBP2b [152]. However, PBP2x is also the most reactive PBP with piperacillin [151]. This paradox points to our deep lack of understanding of the physiological functions of the PBPs.

Surprisingly, the amino acid substitutions selected in the laboratory do not match those found in clinical isolates, with the exception of the aforementioned T550A in PBP2x [146–148, 153] and T446A in PBP2b [146]. This discrepancy may simply reflect the limited sampling. Alternatively, the most useful substitutions may be different in the molecular context of the native PBPs from *S. pneumoniae*, as selected in the laboratory, or in the PBPs from the commensal streptococcal species where they were likely originally selected in their host.

Like the laboratory point mutants, transfer of *pbp2x* genes from clinical resistant isolates to a susceptible strain can confer a moderate level of resistance to cephalosporins and most penicillins [133, 150, 154–159]. Introduction of mosaic *pbp2b* genes can be selected by a modest reduction of the susceptibility to piperacillin [160]. Increased resistance to

penicillins is achieved upon transfer of both mosaic *pbp2x* and *pbp2b* genes [155, 156, 158, 159]. Higher level of resistance to cephalosporins and penicillins results from the additional introduction of a mosaic *pbp1a* gene [154, 156, 158, 159, 161]. A high level of resistance restricted to the cephalosporins is obtained following transformation of a susceptible strain with mosaic *pbp2x* and *pbp1a* [149, 154, 158, 159, 162].

These experimental findings are mirrored in clinical strains [163, 164]. Most resistant clinical isolates harbor three mosaic *pbp* genes encoding PBP1a, PBP2b, and PBP2x (e.g., [156, 165–169]). However, some weakly resistant strains have mosaic alleles only of *pbp2x* and *pbp2b* (e.g., [165, 168, 170]). At least one example was found of a clinical strain with barely reduced susceptibility to penicillin that has only *pbp2x* modified [168]. Some isolates with cephalosporin resistance, yet susceptible to penicillin, were found to have mosaic *pbp2x* and *pbp1a* while retaining a "virgin" *pbp2b* [171, 172]. A resistant strain with a slightly reduced susceptibility of penicillin was reported with a modified *pbp1a*, but original *pbp2x* and *pbp2b* genes [173].

The identification of amino acid substitutions that are relevant to the reduction of affinity of a particular PBP is a difficult task. Due to the process of recombination, superfluous substitutions have been imported together with the ones that provide antibiotic resistance (the "hitchhiking" effect). Indeed, even genes neighboring *pbp2b* or *pbp1a* have been incidentally modified through recombination of large DNA fragments [174, 175]. Nevertheless, a number of likely important substitutions were proposed based on their presence in many resistant strains, absence in susceptible strains, or their proximity to the catalytic motifs. The role of some of these substitutions was probed by detailed genetic, enzymatic, and structural studies, as presented below. A statistical analysis of substitutions found in over 300 sequences each of PBP1a, PBP2b, and PBP1a was carried out but with a couple of exceptions, the substitutions proposed to have been under positive selection by amoxicillin did not match experimental results [176]. It is possible that mosaicity confounds this type of statistical approach.

The reaction of PBPs with β-lactams occurs readily in vitro. By measuring the decrease in intrinsic fluorescence of a recombinant soluble form of PBP2x upon antibiotic binding, the overall acylation efficiency defined by the second order rate constant k_2/K_d was determined to be between 60,000 and 110,000 $M^{-1}s^{-1}$ for penicillin and about twice as fast for cefotaxime [79, 177–179]. The deacylation rate k_3 measured in different ways (recovery of enzymatic activity, loss of bound radiolabeled penicillin, mass spectrometry) is between 0.8 and 5 s^{-1} for penicillin and somewhat slower for cefotaxime [78, 177–179]. The very fast acylation and slow deacylation reactions result in a concentration of antibiotic at which half the enzyme is acylated at steady state (c_{50}) that lies in the micromolar range. This value of c_{50} is consistent with MIC of susceptible strains [78, 154]. Attempts have been made to delineate the dissociation constant of the non-covalent preacylation complex K_d and the rate of acylation k_2 with penicillin. One study found a K_d of 0.9 mM and a k_2 of 180 s^{-1} [78], whereas a second study reported a K_d of 20 mM and a k_2 of 1600 s^{-1} [180]. The published data lend more credence to the latter higher numbers. Thus penicillin has a very poor "true" affinity for PBP2x, and this finding presumably applies to β-lactams and PBPs in general. The efficacy of β-lactams against susceptible bacteria does not result from a particularly good fit of the antibiotic to its target (K_d), but rather from the extremely high rate of acylation (k_2).

The crystal structure of PBP2x from the susceptible strain R6, truncated of its cytoplasmic and transmembrane regions, was solved to a resolution of 2.4 Å [5, 181]. The extracellular part of PBP2x consists of a transpeptidase domain with the common fold of the ASPRE proteins (residues 266–616), flanked by an elongated N-terminal domain (residues 49–265) and a small globular C-terminal domain (residues 617–750) composed of two so-called PASTA subdomains. The N-terminal domain is shaped like a pair of sugar tongs with a hole of about 10 Å in diameter [5]. The function of this domain remains unknown although it was proposed to interact with other protein partners. Alternatively, this domain may recognize some chemical motif of the peptidoglycan. When all the amino acid substitutions found in different mosaic sequences of PBP2x are mapped onto the crystal structure (*i.e.*, 30 of the 217 positions of the N-terminal domain), they are all distributed on the outer surface of the domain and none is found within the hole. The conservation of the residues forming the inner surface of the hole supports the idea that the sugar tong serves to grasp an unknown partner [182]. The function of the C-terminal PASTA domains, which are found only in the class B PBPs involved in the division of some Gram-positive bacteria, is largely unknown, although they play a role in the localization of PBP2x and cell shape maintenance [183, 184].

The main feature of the transpeptidase domain, with respect to other known structures of the ASPRE family, is the presence of a very long groove, at the center of which is found the active site. Modeling showed that this cleft can accommodate two molecules (NAG-NAM)-L-Ala-D-Glu-L--Lys-D-Ala, one of which is covalently bound to the active site serine, and the other providing the Nζ of its L-Lys ready to complete the transpeptidation. Both disaccharide moieties can sit in the larger valleys at both ends of the groove [5].

Regarding the precise mechanism of acylation by antibiotics, the crystal structure of PBP2x and a number of theoretical studies have left the question open (*e.g.*, [7]). The conservation of the hydrogen bonding pattern involving residues of the three catalytic motifs SXXK, SXN, and KTG in PBP2x and the TEM-1 β-lactamase suggests that the acylation

mechanisms are similar [7, 181]. The pH dependence of the acylation rate is consistent with a model where a residue with a pK_a of 4.9 functions as a base to help deprotonate the active site serine, a group with a pK_a of 7.6 triggers upon deprotonation a rearrangement to a less reactive conformation, and a residue with a pK_a of 9.9 is hydrogen bonded in its protonated form to the free carboxylate of the substrate [180]. The base was proposed to be K340 of the first motif with the unusual pKa of 4.9. T550, which binds the carboxylate of the antibiotic [181], would have the pKa of 9.9. Investigation of solvent isotope effects on the rate of acylation suggested a complex process partially rate-limited by the chemistry (the proton exchanges) and by solvation and/or conformational rearrangement [180].

Based upon sequence comparisons and the proximity to the catalytic motifs, the substitutions most likely to impart some resistance include T338A, T338G, T338P, T338S, and M339F found within the SXXK motif [79, 149, 150, 158, 185], H394Y and M400T that surround the SXN motif [149, 151, 167, 168], and L546V, T550A, and Q552E, which are close to the KTG motif [149, 150, 165, 186]. The effect of some of these substitutions has been characterized in details as discussed below. The stretch 595–600 was changed from YSGIQL to LSTPWF in some highly resistant strains to penicillins and cephalosporins, including ceftriaxone [187, 188]. These mutations do not appear randomly in sequences, but some families can be recognized.

Examination of approximately 100 publicly available sequences of the transpeptidase domain of PBP2x reveals three broad families (Fig. 13.8). One family contains non-mosaic sequences that are very similar to the PBP2x from the reference susceptible strain R6. The mosaicity complicates the picture of the two other families and the grouping would differ for various sequence blocks. Nevertheless, the emerging pattern suggests that two main mechanisms have been selected that reduce the affinity of PBP2x for the antibiotics [157]. Figure 13.9 shows the distribution of the substitutions in the structure of the transpeptidase domain of PBP2x from two resistant isolates, representing two modes of reducing the affinity for β-lactams.

One family of sequences is characterized by the T338A substitution. About 30 other substitutions in the transpeptidase domain accompany this defining mutation, although no mutation is consistently found together with T338A, and never found in the absence of the T338A mutation. The side chain of T338 is pointing away from the active site cavity and is hydrogen bonded to a buried water molecule. It has been proposed that suppression of the hydrogen bonding by replacement of T338 can lead to destabilization of the active site due to the loss of the water molecule [79]. Introduction of the sole T338A mutation in PBP2x from the susceptible strain R6 reduces its efficiency of acylation by penicillin by a factor of two [79], which is not enough to be selected following transformation into a susceptible strain [154]. Reversion of the substitution in the related PBP2x from resistant strains Sp328 and 4790 increases the acylation efficiency sixfold [79, 154].

A subset of sequences that contain the T338A mutation also have the adjacent M339F substitution. These sequences are from strains with particularly high levels of resistance [149, 150, 154, 163, 165, 167]. PBP2x molecules from such isolates have an efficiency of acylation by penicillin reduced more than 1000-fold [78, 154]. Most of this reduction is due to a slower rate of acylation (k_2 decreased 300-fold), although a weaker pre-acylation binding (K_d fourfold higher) also contributes to the overall extremely poor affinity of the PBP2x with the double T338A/M339F [78, 154]. In addition, these PBP2x variants have significantly faster deacylation kinetics (k_3 increased 40- to 70-fold), an effect mostly due to the M339F substitution [154, 178]. The slow acylation and fast deacylation combine to elevate the c_{50} (concentration of antibiotic resulting in the steady-state acylation of half the enzyme) by four to five orders of magnitude [78, 154].

The M339F mutation alone, introduced in the reference R6 PBP2x, reduces the efficiency of acylation by penicillin by sixfold and is sufficient to confer a measurable level of resistance [154]. Combination of the M339F and T338A mutations produces a greater effect. The structure of the latter double mutant has been solved to a resolution of 2.4 Å. The salient feature of the mutated active site is the reorientation of the hydroxyl of the catalytic S337 that is now pointing away from the active site center and is hydrogen bonded to the main chain nitrogen of T550 instead of to K340 [154] (Fig. 13.10). The active site serine 337 may exist in an equilibrium between two rotamers, only one of which can be acylated. Mutations such as M339F, by subtly altering the active site, may shift the equilibrium towards the unproductive rotamer. Note that this effect could be restricted to the reaction with β-lactams if binding of the physiological substrates favors a conformation that offsets the effect of the mutations.

The detailed studies of a few mutations fell short of explaining the reduction of affinity measured for PBP2x from clinical resistant isolates. The individual reversions of the 41 mutations of the PBP2x transpeptidase domain from a highly resistant strain, studied by in vitro kinetic and in vivo phenotypic characterization, revealed the importance of four substitutions, in positions 371, 384, 400, and 605, in addition to those in position 338 and 339 [189] (Fig. 13.11). The combined reversion of the 6 substitutions nearly restored the normal rapid rate of acylation by β-lactams. Conversely, introduction of 5 combined mutations diminished the reactivity towards β-lactams almost to the level of the original PBP2x with 41 substitutions. These effects measured in vitro were mirrored by the expected phenotypic consequences in vivo. A conceptually similar study in vivo with a different

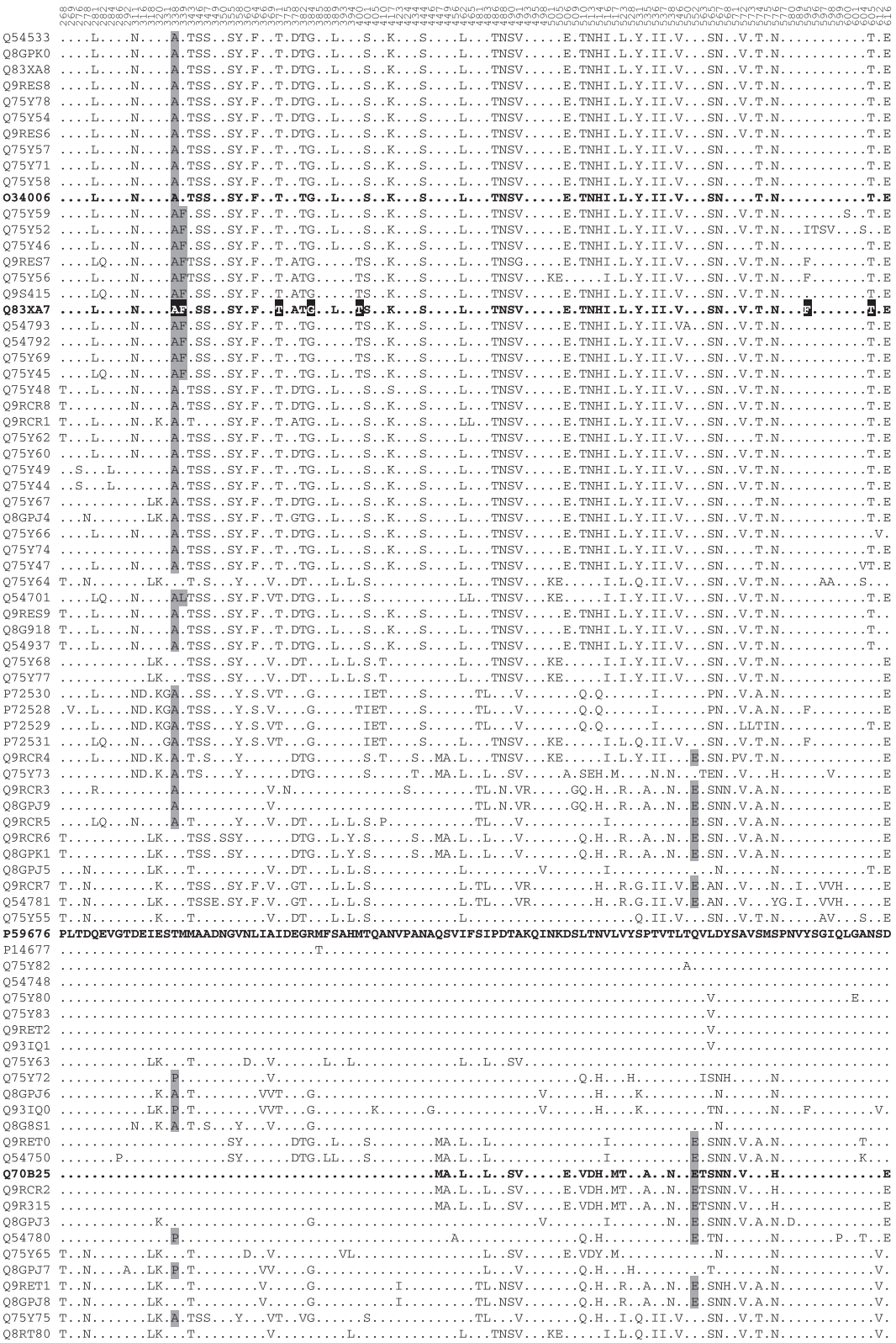


Fig. 13.8 Alignment of PBP2x transpeptidase domain sequences (aligned and clustered with CLUSTALW). Only positions where at least one sequence differs from the R6 reference (Uniprot accession number #P59676) are shown. Substitutions at position 338, 339, and 552 are highlighted in *gray*. Although the mosaicity confounds effort to classify unambiguously these sequences, this representation allows to visualize that sequences characterized by a mutation in position 338 (denoted by the *thick black line* on the *right* of the alignment) differ substantially from sequences with the Q552E substitution (denoted by the *thin black* on the *right* of the alignment), although a few sequences harbor both mutations. The absence of line on the *right* of the alignment denotes non-mosaic sequences or sequences with few substitutions. The crystal structure of the high affinity PBP2x from strains R6 (# P59676), as well as the two low affinity proteins from strains Sp328 (# O34006) and 5259 (# Q70B25) have been solved, revealing two modes of reducing the affinity for β-lactams. The corresponding sequences are in *bold* characters. Also in *bold* is PBP2x from strain 5204 (# Q83KA7) with substitutions experimentally identified as contributing to the resistance highlighted in *black*

PBP2x also identified the I371T and R384G substitutions as central for the reduced acylation rate [190]. Strikingly, substitutions at positions 338, 339, 371, 384, and 605 were also identified by the sequence comparison study of ancestral *S. mitis* and resistant *S. pneumoniae* sequences [139]. The positions 364 and 389 proposed as important for resistance by the sequence comparison study were not confirmed by the detailed in vitro study [139, 189].

Resolution of the structure of PBP2x from the resistant strain Sp328, which belongs to the family defined by the T338A substitution, has confirmed the absence of the buried water molecule [182]. The most striking feature of Sp328 PBP2x is the great flexibility of the loop spanning residues 365–394. This instability extends in part to the SXN motif in positions 395–397, with S395 being somewhat displaced. Thus, the 60-fold reduction of the acylation efficiency by cefotaxime, for example, is due to a distortion of the active site [79, 154]. The 365–394 segment forms one side of the groove leading to the active site. The flexibility of this region generates a more accessible "open" active site that may better accommodate alternative physiological substrates with branched stem peptides [182]. The destabilization of the 365–394 region was shown to result from the I371T and R384G mutations [189].

Fig. 13.9 Distribution of the amino acid substitutions (*red*) in the PBP2x transpeptidase domain from *S. pneumoniae* strains Sp328 (sequence # O34006) and 5259 (# Q70B25), with respect to PBP2x from strain R6

A second family of PBP2x molecules from resistant strains can be defined by the presence of the Q552E substitution. Introduction of this single substitution in PBP2x reduces about fourfold the efficiency of acylation and confers a modest level of resistance to the recipient R6 strain [153, 157, 186]. The structure of a PBP2x from a clinical strain that possess the Q552E substitution has been solved to a resolution of 3 Å. This PBP2x has an efficiency of acylation reduced more than 15-fold [157]. The only significant difference found in comparison to the structure of R6 PBP2x is the displacement of strand β3, which carries the KTG motif [157] (Fig. 13.12). This displacement of 0.5 Å narrows the active site, and is reminiscent of the closed conformation of PBP2a from *S. aureus*, which is thought to cause the low efficiency of acylation of this enzyme by coupling the reaction to a major structural rearrangement [81]. In addition to

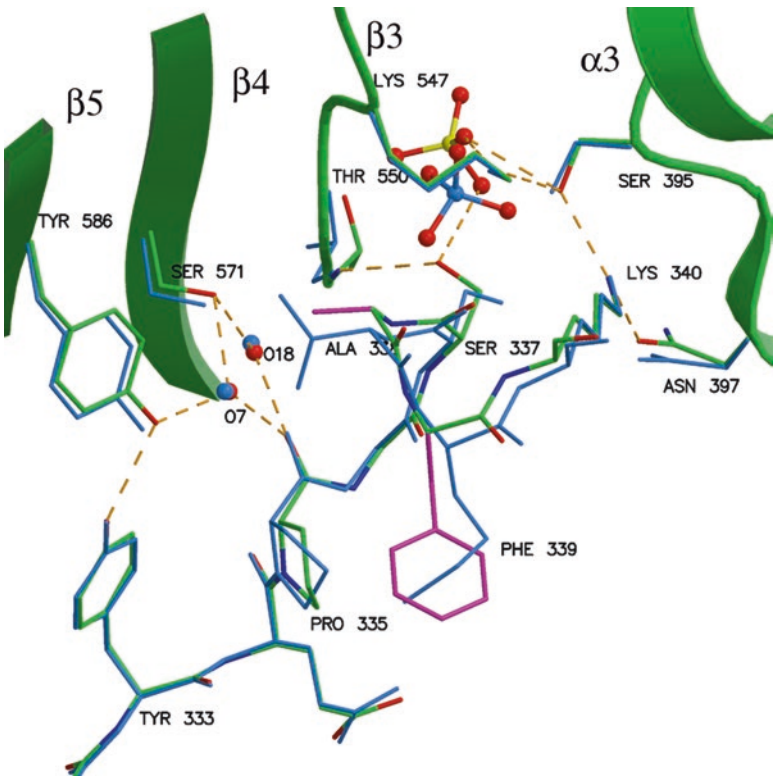


Fig. 13.10 Superposition of the active site of wild-type R6 PBP2x (*blue*) and of the double mutant T338A/M339F (*green* carbon atoms and side chains of the mutated residues in *purple*). Note that the hydroxyl of the catalytic S337 is pointing in opposite directions

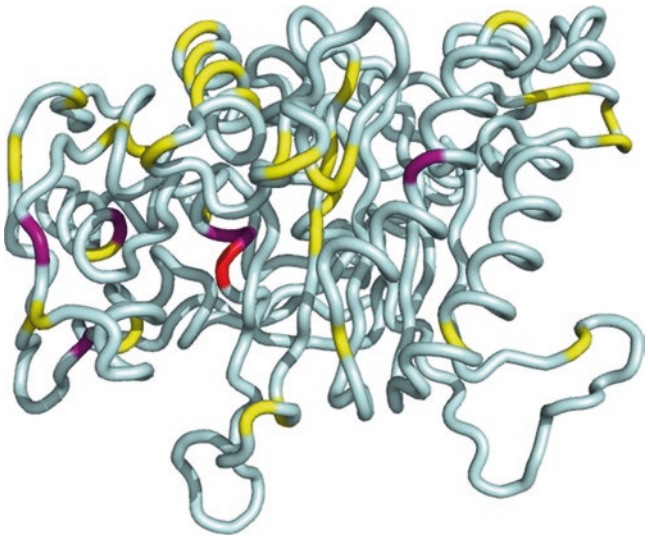


Fig. 13.11 Structure of *S. pneumoniae* PBP2x transpeptidase domain, showing the distribution of six substitutions (*purple*) that were shown do contribute to resistance in strain 5204, out of 41 in the transpeptidase domain (in *yellow* and *purple*). The active site Ser337 in *red*

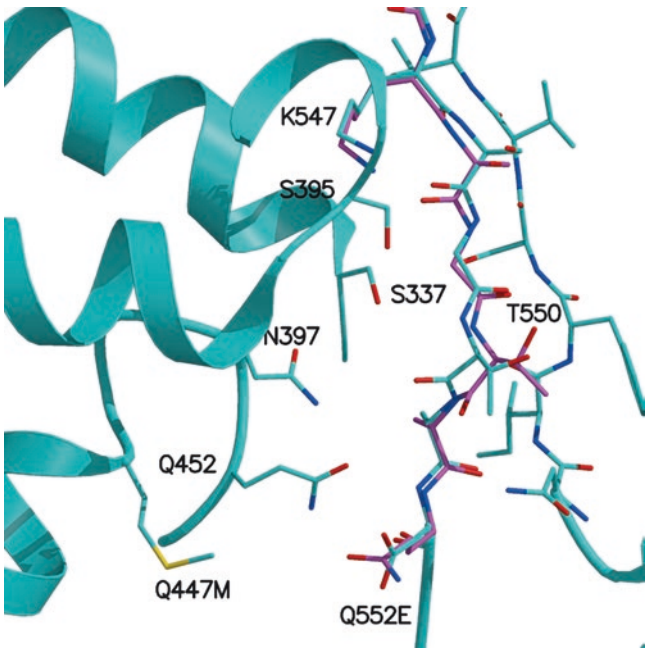


Fig. 13.12 Structure of the PBP2x active site from strain 5259 (*cyan*). The position of strand β3 from R6 PBP2x is shown in *purple*. Note the slight closure of the active site from 5259 PBP2x

this conformational effect, the introduction of a negative charge in position 552 greatly affects the entry of the active site and does not favor binding of β-lactams, which are negatively charged [157].

Consequently, it appears that two distinct mechanisms have been selected that reduce the reactivity of PBP2x towards β-lactams. One mechanism primarily affects the chemistry of the active site S337, whereas the second mechanism hinders acylation by requiring an opening of the active site [154, 157]. These two mechanisms may be a reflection of two major sources of exogenous genetic material that have been incorporated in strains of *S. pneumoniae*. Note that a few sequences of PBP2x have both T338A and Q552E substitutions and may thus combine the effect of both mechanisms.

Another significant substitution is T550A, which confers resistance to cephalosporins only, in both laboratory and clinical strains [146, 148–150]. When the T550A point mutation occurs within a mosaic PBP2x, which contains the T338A/M339F double mutation, it further increases the resistance to cephalosporins, while it almost abolishes resistance to penicillin [149]. This effect is mirrored in the acylation efficiency of a T550A point mutant of R6 PBP2x, which is decreased 20-fold towards cefotaxime and unaffected towards penicillin [186]. This effect has been rationalized by the abolition of the hydrogen bond between T550 and the carboxylate that is attached to the six-member ring of second- and third-generation cephalosporins [181].

PBP2b, the other class B PBP from *S. pneumoniae*, has not been subjected to the same thorough investigations, although high-resolution structures are now available, both from the drug-susceptible strain R6 and from the highly resistant strain 5204, which displays 58 substitutions, including 44 in the transpeptidase domain [191].

Over 140 nonredundant sequences spanning the transpeptidase domain (314–680) are available. Two substitutions, T446A or T446S and E476G are most often found in PBP2b from clinical resistant strains. The probable importance of these two substitutions was pointed out in numerous studies [137, 155, 163, 165]. The T446A mutation, which is immediately adjacent to the SXN motif, is also selected by piperacillin in the laboratory [146]. T446A is the only substitution that has been characterized biochemically [160] and it reduces the affinity for penicillin by 60 %. The crystal structure showed that the side chain of Thr446 is involved in several polar and hydrophobic contacts that could stabilize a loop between α4 and α 5 helices [191]. The destabilization of this loop could hamper drug binding.

The affinity of various PBP2b molecules from clinical isolates with 6–43 mutations in addition to T446A is reduced by 90–99 % [160]. In addition to the mutations in positions 446 and 476, some PBP2b sequences are distinguished by other salient features such as the S412P, N422Y, T426K, and Q427L substitutions that change the polar character and charge of the entry of the active site [191]. In a distinct set of sequences the substitution of six adjacent residues at position 427–432 impact the same region on one side of the active site cleft [134, 137]. Also in the same region of the structure, three related PBP2b sequences from Japanese isolates are

noteworthy by the insertion of three residues (SWY) after position 422 [192]. This is one of two occurrences of a change in the number of residues in a mosaic PBP. The other case was found in PBP1a (see below). In all other cases, the total length of the proteins and the position of the catalytic motifs are fully conserved, despite extensive sequence remodeling.

The recent emergence of strains that show a particularly high resistance to amoxicillin, relative to other β-lactams, appears to result from a set of ten substitutions in the region 591–640 surrounding the third catalytic motif KTG [156, 193, 194]. Some of these substitutions destabilize the loop between strands β3 and β4. This loop forming the other side of the active site furrow could not be resolved between residue 619 and 629 in the structure of PBP2b from the resistant strain 5204 due to its flexibility [191]. Seven related sequences from Korean clinical strains show a substitution within the third catalytic motif KTG, which is changed to KSG [195]. In contrast to PBP2x and PBP1a where mutations within the first catalytic motif are commonplace, a single case was reported of a V388A substitution within the SVVK motif [196].

Several strains have five adjacent substitutions spanning residues 565–569 in a β-strand that extends to make contacts with the N-terminal pedestal domain, on the side of the transpeptidase domain opposite the active site. The importance of these substitutions for the resistance is not known, but a correlation was proposed with the presence of a particular allele of *murM*, a gene required for the synthesis of branched stem-peptides that are alternative substrates of the transpeptidation catalyzed by PBPs and required for resistance (see below) [193].

PBP1a may be considered clinically as the most important and troublesome PBP. Indeed, the resistance potentially provided by mosaic PBP2x and PBP2b is capped by the presence of a "virgin" PBP1a, which still warrants some efficacy to β-lactam therapy. High level of resistance depends on a modified PBP1a. The crystal structure of the transpeptidase domain is now available at the resolution of 2.6 Å for PBP1a from the susceptible strain R6 [197], and at 1.9 Å for that from the highly resistant strain 5204 [198]. The acylation efficiency of PBP1a from the susceptible strain R6 was measured to be about 70,000 $M^{-1}s^{-1}$ for penicillin and the deacylation rate constant to be about 10^{-5} s^{-1} [199]. These values are of the same magnitude as those reported for PBP2x. Over 50 PBP1a sequences are publicly available. The T471A substitution within the first catalytic motif, analogous to the T338A mutation in PBP2x, is commonly found in PBP1a sequences from resistant strains [159, 165, 167, 168, 200]. Reversion of this substitution reduced but did not abrogate the resistance that PBP1a confers in addition to PBP2x and PBP2b [159]. The T371A substitution diminishes the efficiency of acylation of R6-PBP1a by cefotaxime (2.4-fold), and particularly by penicillin G (26-fold). Conversely, reversion of the substitution at position 371 in 5204-PBP1a from A to T increases the k_2/K (1.8- and 4.8-fold for cefotaxime and penicillin G, respectively) [198]. Some mosaic sequences lack the T471A mutation, including PBP1a from a highly resistant Hungarian isolate (MIC for penicillin of 16 mg/L) [201]. In the structure from the low-affinity PBP1a, the hydrogen bond network within the active site is markedly altered and the hydroxyl group of the active Ser370 points in another direction than in the structure from a susceptible strain [198], as observed with PBP2x.

Another remarkable feature is the mutation of a stretch of four residues (TSQF to NTGY) at position 574–577, which is observed in all the mosaic sequences. Amino acids at positions 574–577 belong to a loop between strands β3 and β4, which form the side of a tunnel at the entrance of the catalytic cleft. This loop is flexible and not visible in the structure of PBP1a from strain 5204 [198], much like in PBP2b [191]. Moreover, this wall has a hydrophobic character conferred by Phe577, which is certainly changed in the mutant [197]. The impact of the TSQF(574–577)NTGY mutations on the acylation efficiency of R6-PBP1a is greater than that of T371A, with decreases of 5.5- and 49-fold for cefotaxime and penicillin G, respectively. The introduction of reciprocal mutations (NTGY(574–577)TSQF) in 5204-PBP1a increases the k_2/K_D by factors of 2.5 and 1.7, respectively [198]. Reversion of this set of substitutions decreased the additional resistance conferred by PBP1a [201]. A similar effect of the reversion was found for the L539W substitution, although the sequence in which the experiment was performed is the only one that presents this particular mutation [201].

The combined effect of the two sets of mutations at positions 371 and 574–577 is greater than the individual mutations, both in R6-PBP1a and in 5204-PBP1a.

Although PBP2x, PBP2b, and PBP1a are the major PBPs responsible for the resistance of *S. pneumoniae*, a number of studies have hinted at the possible involvement of various other PBPs. Transfer of a high level of resistance from a strain of *S. mitis* to a laboratory strain of *S. pneumoniae* was shown to require transfer of the genes encoding the five high molecular weight PBPs [202]. A point mutation in the low molecular weight PBP3 was found to contribute to the resistance of a strain selected on cefotaxime in the laboratory [147]. In contrast to these laboratory experiments, examination of the PBPs from clinical isolates failed to reveal significant modification of PBP1b or PBP3 [163, 203]. Early studies, which examined various strains through the labeling of PBPs with radioactive penicillin, found several instances where binding to PBP2a was diminished in resistant strains [133, 141]. Also, transfer in the laboratory of resistance from a *S. mitis* strain to *S. pneumoniae* involved modification of PBP2x, PBP2b, PBP1a, and PBP2a, but not of PBP1b and PBP3 [158]. Various combinations of point mutations,

including silent ones, were observed in some PBP2a sequences, suggesting events of intraspecies recombination [204]. The role of PBP2a in β-lactam resistance is now firmly established in at least one instance [205]. A strain isolated from an AIDS patient was found to harbor a mosaic PBP2a in addition to mosaic PBP2x, PBP2b, and PBP1a. Transformation experiments demonstrated that this PBP2a variant is indeed responsible for an elevated resistance to various β-lactams. The sequence shows 25 substitutions including 12 within the transpeptidase domain. The absence of crystal structure precludes a detailed analysis, but it is noteworthy that the threonine following the catalytic serine is replaced by an alanine, like in numerous variants of PBP2x and PBP1a.

Both class B PBPs, PBP2x and PBP2b, are essential in *S. pneumoniae*, which is consistent with the selection of variants of these proteins by β-lactams [196]. PBP1b and PBP3 are not essential [206, 207], which again is consistent with the fact that these proteins are not involved in the resistance process. PBP1a and PBP2a are not essential individually, but one of them must be present and functional [206, 208]. The fact that PBP1a, rather than PBP2a, is the main target of antibiotic selective pressure may be due to PBP2a having a low intrinsic affinity for β-lactams [209].

Are the substitutions in PBPs hampering their function in building the cell wall of *S. pneumoniae*? In France, the reduction of antibiotic consumption following a public awareness campaign launched in 2005 was correlated with a drop in the proportion of isolated strains that were resistant [210]. This observation suggests that substitutions providing resistance to β-lactams may entail a fitness cost. Indeed, in a mouse model of pneumococcal colonization, strains harboring a mosaic *pbp2x* were outcompeted by a susceptible strain in the absence of β-lactam challenge [211]. Fitness was further reduced by the introduction of *pbp2b* and *pbp2x* mosaic alleles. Another study found that transformation of a susceptible strain with mosaic *pbp2b* genes reduced fitness. However, further transformation with some mosaic *pbp1a* and *pbp2x* genes restored fitness [212]. This latter result suggests that substitutions in the different PBPs may not be independent from each other.

The fitness cost of having mosaic PBPs could be due to altered enzymatic properties, or to their less than optimal insertion into functional complexes. This second explanation was first hinted at by the fact that when using a *pbp2x* gene from a resistant *S. oralis* to transform a susceptible *S. pneumoniae*, the sequence being transferred was restricted to the immediate vicinity of the important codon [146]. That some combinations of the different mosaic PBPs result in fitter cells support the hypothesis of direct interaction between PBPs [212]. PBPs also interact with a variety of proteins involved in cell wall building and morphogenesis. Thus, substitutions affecting these interactions rather than the reactivity with the β-lactams could also potentially affect resistance. Two substitutions selected in vitro by cefotaxime in the last rounds leading to the highest resistance levels where found to map in PBP2x at the interface between the transpeptidase domains and the C-terminal domain of unknown function [213]. This domain of PBP2x consists of two so-called PASTA modules that have been proposed to play a regulatory role and bind to peptidoglycan motifs or β-lactams [214]. Such modules are also found in a Ser/Thr-kinase that plays a morphogenetic function in *S. pneumoniae* and interacts with PBP2x [183].

A puzzling discovery was made, which is directly related to PBP-based β-lactam resistance. Clinical resistant isolates have an abnormal peptidoglycan structure with an elevated proportion of cross-bridges that involve branched stem-peptides [215]. Instead of having the L-Lys of the "acceptor" peptide cross-linked directly to the D-Ala of the "donor" peptide, there are intervening L-Ala-L-Ala or L-Ala-L-Ser dipeptides. The genetic determinants of this cell wall abnormality could nevertheless be separated from the resistance determinants (the mosaic *pbp* genes) [216]. The genes responsible for the synthesis of branched precursors were found to constitute the *murMN* operon [217], also known as the *fibAB* operon [218]. Mosaic *murM* genes often increase the resistance level conferred by a set of mosaic *pbp* genes [172, 217]. A naïve explanation is that mosaic PBPs prefer branched substrates. However, deletion of *murM* abolishes the resistance but does not impact on the growth rate in the absence of antibiotic challenge [217], demonstrating that mosaic PBPs can efficiently use linear precursors. The situation is reminiscent of the role of the *femAB* operon in *S. aureus*, which is required for expression of *mecA*-based resistance, while the *mecA*-encoded PBP2a can nevertheless function with alternative substrates produced in the absence of *femAB* [61, 62]. It has been proposed that branched stem-peptides may be superior competitors against β-lactams for the active site of some PBPs of resistant strains, or that they may be involved in some signaling function of cell wall metabolism, or that they play a particular role in the integrity of the peptidoglycan, a role that becomes critical when some PBPs are inhibited by antibiotics [217].

Besides MurM, other unknown factors modulate β-lactam resistance. Indeed, five clinical isolates with significantly different levels of resistance were found to have the same MurM allele and strictly identical sequence of their penicillin-binding domains, for the six PBPs [204]. For example, the two-component regulatory system CiaRH appears to be required for the cells to tolerate altered PBP2x, both point mutants and mosaic [219]. Although much is known about the biochemistry of the PBPs, the MurM and CiaRH complications highlight our limited understanding of the physiological function of the PBPs in cell wall metabolism, both in the absence and in the presence of antibiotics.

It is of note that new cephalosporins have been introduced recently that exhibit good antibacterial activity against *S. pneumoniae*, strains resistant to previous molecules. Ceftobiprole is active against strains with mosaic *pbp1a*, *pbp2b,* and *pbp2x* that are resistant to cefotaxime, ceftriaxone, penicillin, and amoxicillin [220]. Ceftaroline is a more recent cephalosporin that also shows great efficacy against *S. pneumoniae*, including resistant strains with altered PBPs [85, 221–223]. In contrast to other cephalosporins, ceftaroline also binds to PBP2b, including variants with the common T446A substitution, and substitution in the C-terminal part associated with amoxicillin resistance [85].

Lactivicins are compounds that form a covalent adduct with the active site serine of PBPs. Some lactivicins show antibacterial activity and acyl-enzyme complexes with PBP1b have been studied structurally by crystallography [224]. However, PBPs with a lower reactivity with β-lactams, such as PBP2x from strain 5204, also exhibit a decreased reactivity towards lactivicins [224].

In the future, novel non-β-lactam inhibitors should be developed that are active against resistant *S. pneumoniae*, in particular if these molecules are screened directly for their binding to altered PBPs with low affinity for β-lactams. A sulfonamide derivative and an anthranilic acid derivative were thus found that inhibit PBP2x from the resistant strain 5204 and are antibacterial against a variety of Gram-positive organisms [95].

7.1 Neisseria

Neisseria meningitidis and *Neisseria gonorrhoeae* are pathogens that have acquired reduced susceptibility to penicillin via two routes. The modification of at least one chromosomally encoded PBP will be discussed below. Alternatively, production of a plasmid-encoded β-lactamase is common in *N. gonorrhoeae* (*e.g.*, [225]), while it is rare in *N. meningitidis* [226].

Neisseria species contain only three PBPs called PBP1, PBP2, and PBP3, which are, respectively, of class A, of class B, and a low molecular weight carboxypeptidase of class C. Gonococcal strains with reduced susceptibility to β-lactams that do not express a β-lactamase were found to exhibit reduced labeling of PBP2 and PBP1 with radiolabeled penicillin [227]. Reduced labeling of PBP2 was observed in meningococci [228]. PBP2 is encoded by the *penA* gene, which is often mosaic in resistant strains of *N. gonorrhoeae* [229] and *N. meningitidis* [230]. A major European study determined a partial nucleotide sequence of *penA* from 1670 meningococcal strains isolated over six decades. A total of 139 alleles were uncovered, including 38 very similar sequences from susceptible strains and 101 highly diverse sequences from strains with a diminished susceptibility to penicillin [231]. A Swedish study revealed similar findings [232]. At the protein sequence level in *N. gonorrhoeae*, 50 different full sequences have been reported which are commonly ascribed a roman numeral [233–240] (Fig. 13.13). Although no global clonal expansion was detected, some clones can be highly successful locally. In a study at a clinic for sexually transmitted diseases in the Netherlands, a single strain harboring the XXXIV was identified in 53 of 128 cefotaxime resistant isolates [241].

Like *S. pneumoniae*, *Neisseria* species are naturally competent organisms and horizontal gene transfers are common [242]. The mechanism of acquisition of non-plasmidic resistance in *Neisseria* is therefore similar to that of *S. pneumoniae*. Indeed, horizontal transfer of a *penA* alleles conferring resistance was observed during co-cultivation of drug-susceptible and resistant gonococcal strains [243]. Additional point mutations were found to arise during this experiment [243].

The origin of the foreign sequence fragments that are found in the *penA* gene of clinical resistant gonococci and meningococci has been investigated in some depth. Several commensal species, such as *Neisseria flavescens*, *Neisseria cinerea*, or *Neisseria perflava*, appear to have each contributed sequence blocks to *penA* genes from resistant strains [230, 244–246]. An analysis of a 402 bp-fragment of the *penA14* allele encoding the C-terminus of PBP2 from *N. meningitidis* showed it to be mosaic with likely contribution from *N. flavesens*, *N. cinerea*, *N. mucosa,* and *N. perflava* [231]. *N. flavescens* isolates recovered from the pre-antibiotic era have relatively high penicillin MICs and a PBP2 with an intrinsic low affinity for penicillin [245]. Transfer in the laboratory of the *penA* gene from such *N. flavescens* isolates could indeed confer some resistance to *N. meningitidis* [245]. In contrast, *N. cinerea* is not naturally resistant, and accordingly, no resistance was achieved in *N. meningitidis* upon transfer of the *penA* gene from this species [245]. In some instances, PenA sequences with few substitutions such as sequence XIII from *N. gonorrhoeae* have been considered as non-mosaic, by contrast with sequences that have many substitution (such as sequence X) [247]. However, nucleotide sequence alignments reveal that the short modified segment spanning residue 504–516 in sequence XIII may have originated from *N. perflava*.

PBP2 sequences of *N. cinerea* origin found in resistant meningococci have an additional aspartic acid following D345, which is not present in the susceptible *N. cinerea* strains [245]. This insertion was also found in PBP2 sequences from many resistant gonococcal strains. Site-directed mutagenesis has demonstrated that this insertion is sufficient to decrease the reactivity of PBP2 for β-lactams and to confer some resistance to *N. gonorrhoeae* [248]. A clinical resistant strain was later discovered that only has this additional aspartic acid [235]. The consequences of this insertion have been investigated in some depth (see below).

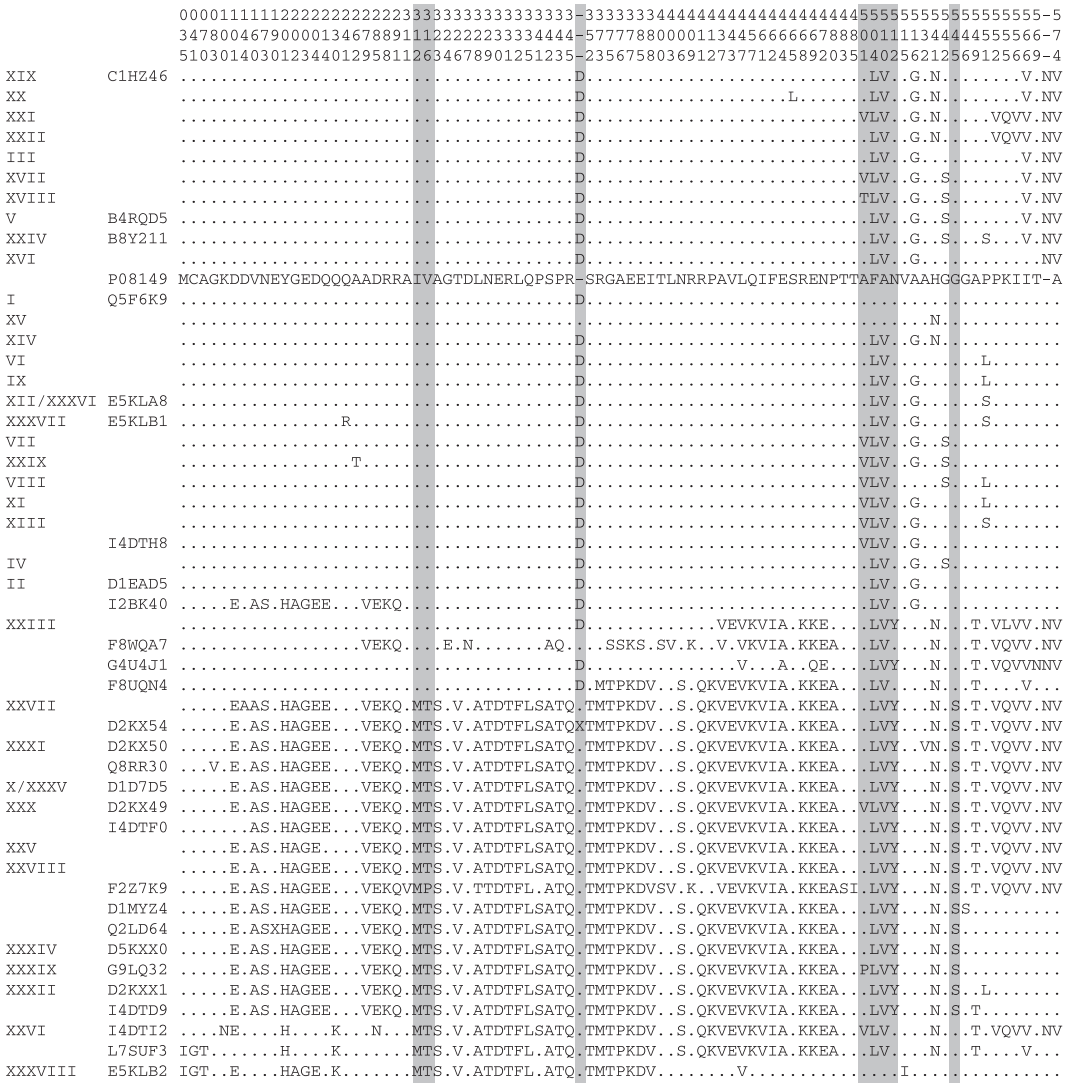


Fig. 13.13 Alignment of PBP2 sequences from *N. gonorrhoeae* (aligned and clustered with CLUSTALW). The Roman numeral nomenclature and the Uniprot accession number are given on the *left*. Only positions where at least one sequence differs from the reference (Uniprot accession number #P08149) are shown. Substitutions shown experimentally to contribute to resistance are highlighted in *gray*

The crystal structure of gonococcal PBP2 from a penicillin-susceptible strain was solved to a resolution of 2.4 Å [249]. Asp345 is involved in a hydrogen bond network with the second catalytic motif SSN363. Although no structure with the additional adjacent D345a residue could be obtained, it was proposed that the additional acidic residue likely interferes with this hydrogen bonding network, causing a fivefold decrease in the acylation efficiency [250]. However, only an additional Asp was tolerated in vivo and maintained viability. The structural comparison of different PBP structures have emphasized the importance of the hydrogen bonding of the second catalytic motif [250]. Indeed, the middle serine of the SSN motif of pneumococcal PBP2x is also hydrogen bonded to an aspartate similarly located as gonococcal Asp345 on the β2a-β2d hair-pin loop. In PBP2a from *S. aureus*, the middle residue of the SXN motif is an asparate that makes a salt bridge to a lysine in the β2a-β2d hair-pin loop. In *Mycobacterium tuberculosis* PBPA, this linkage is provided by a covalent disulfide bond [250] (Fig. 13.14).

PBP2 with sequence XII (XXXVI), which harbors four substitutions (F504L, A510V, A516G, and P551S) in addition to the D345a insertion, showed a 16-fold reduction in acylation efficiency (k_2/K_D). The structure of PBP2 with these four substitutions, but without the D345a insertion, a sequence not found in clinical strain, was also solved and showed very little structural modification, although the four substitution caused a fivefold reduction of the acylation efficiency, mostly due to the F504L and P551S substitutions, and a drop in thermal stability [249].

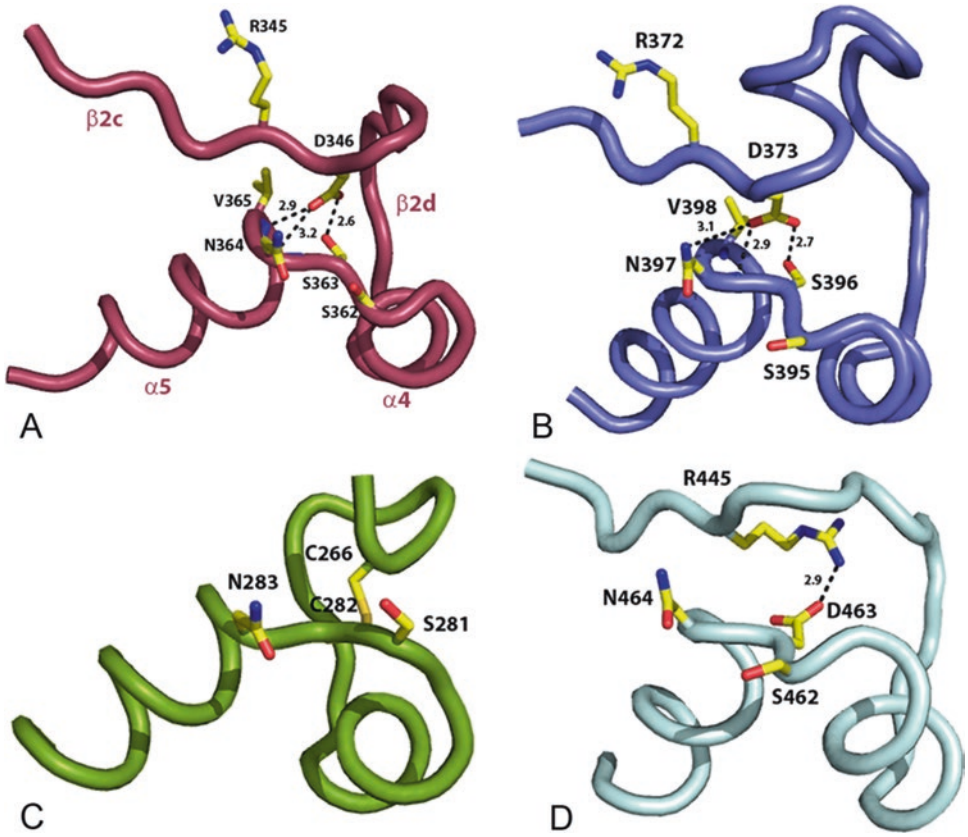


Fig. 13.14 Conservation of the interaction between the X residue of the second conserved motif SXN with a residue in the preceding loop [250]. (**a**) PBP2 from *N. gonorrhoeae* (PDB # 3EQU), showing the interaction between Asp346 in the loop and Ser363 (the X residue). The SXN motif is shown. (**b**) PBP2x from *S. pneumoniae* (PDB # 1QME), showing the interaction between Asp373 and Ser396. (**c**) *M. tuberculosis* PBPA (PDB # 3LO7), showing a disulfide bond between Cys266 and Cys282. (**d**) PBP2a from *S. aureus* (PDB # 1VQQ), showing the interaction between Arg445 and Asp463. Hydrogen bonds are depicted by the *dotted lines*

The A501V substitution in gonococcal sequences is another example of a point mutation that arose in addition to mosaicism. Indeed, the A501V substitution is absent from related species that contributed sequence fragments to resistant *N. gonorrhoeae* [251]. The A501V mutation was found to be somewhat correlated with strains expressing resistance to ceftriaxone, a third generation cephalosporin [240]. This was confirmed experimentally, as introduction of the A501V mutation in a mosaic PBP2 normally devoid of this substitution increased the resistance to ceftriaxone and cefixime while decreasing the resistance to penicillin [252]. The effect was mirrored in vitro on the acylation efficiency k_2/K_D with cefixime and penicillin, in that introduction of the A501V substitution increased the reactivity with penicillin and decreased that with cefixime. Odd results were reported with ceftriaxone, the mutation increased the reactivity of the enzyme, in contradiction with the physiological effect [252]. Position 501 is close to the active site Ser310 at the beginning of the loop connecting strands β3 and β4 which is disordered in the crystal structure of PBP2 [249]. An A501P substitution is found in the mosaic sequence XXXIX from a strain that showed high resistance to third generation cephalosporins [253].

Due to the process of homologous recombination that swaps large gene fragments, it is usually difficult to pinpoint the substitutions that contribute to the diminished reactivity of altered PBPs for β-lactams. In a study of PBP2, recombination of partial sequences of penA from a clinical strain particularly resistant to cefixime, allowed to home in on a subset of substitutions that contribute to resistance [239]. Further narrowing by site-directed mutagenesis pointed out to I312M, V316T, and G545S as contributing most to the reduction of reactivity towards most cephems. The I312M substitution takes place within the SAIK first catalytic motif, and is therefore analogous to the M339F substitution characterized in *S. pneumoniae* PBP2x [154]. The V316T substitution is in the middle of helix α 2 which starts with the active site S311. V316 is one-turn downstream of K314 from the SAIK catalytic motif, and their side chains protrude on the same side of α2. It is therefore likely that the V316T substitution impacts on the spatial arrangement of the active site. G545 sits at the beginning of α11 facing strand β3 that lines the active site. The introduction of a serine side chain at position 545 likely modifies the conformation of β3 and the active site. These effects are certainly subtle since they appear to affect differently the resistance to various cephems [239, 252]. These three mutations display epistasis with other substitutions present in the mosaic PBP2 from which they were identified, in that their introduction in a wild-type strain confers only modest resistance, whereas their

reversion in the originating mosaic PBP2 abolishes resistance [252].

Other substitutions located in the loop connecting strands β3 and β4, F504L, A510V, and N512Y, were found to contribute to β-lactam resistance, like the A501V point mutation mentioned above. However, reversion of N512Y greatly diminished resistance to ceftriaxone and cefixime, while not affecting resistance to penicillin. Instead, reversion of A510V or F504L had little effect on resistance to cephems but diminished the resistance to penicillin [252]. The physiological results of the mutations were broadly in agreement with the measured kinetics of the reaction between recombinant enzymes and β-lactams. PBP2 is 20 times more reactive with cephalosporins than with penicillin. The acylation efficiency k_2/K_D for both penicillin and cephalosporins was decreased 150-fold in the mosaic PBP2 investigated [252].

Other substitutions in helix α11 that is lining strand β4 have been proposed to contribute to cephalosporin resistance: G542S and P551S/L [254]. These positions could potentially also affect the nearby β3–β4 loop. These findings emphasize the importance of the β3–β4 loop for the reaction with β-lactams, as noted before with pneumococcal PBP2x [189]. However, the differential effects of individual mutations with different β-lactams indicate that a simple flexibility explanation is unsatisfactory, and molecular details are more complicated.

In susceptible strains, the meningococcal PBP2 sequence is 99 % identical to that from *N. gonorrhoeae*. It is therefore unsurprising that identical substitutions and mechanisms for reducing the acylation efficiency are observed in meningococci, possibly arising from similar recombination events [231, 232].

Thus, it appears that *penA* alleles that confer penicillin resistance have arisen both from the recruitment of sequence blocks from naturally resistant species, such as *N. flavescens*, and new mutations such as a codon insertion or substitution. When, how often, and in which species these recombination and mutation events have occurred are difficult questions. As commensal *Neisseria* species readily exchange genetic material, the *penA* alleles conferring resistance may be considered as forming a common gene pool, which is shared by several species [255, 256].

The cell wall of meningococcal strains with altered *penA* alleles has a greater amount of unprocessed pentapeptides, suggesting that the transpeptidase and/or carboxypeptidase activity of low-affinity PBP2 is modified [257].

Early studies hinted at the possibility that PBP1, the class A PBP, also had decreased reactivity for penicillin in gonococci [227], but subsequent studies failed to uncover mosaicity in the *ponA* gene encoding PBP1. Recently, an allele of *ponA* encoding PBP1 with the single substitution L421P was found to contribute to the high resistance of some *N. gonorrhoeae* strains [258]. This substitution is 40 residues N-terminal to the catalytic S461. The L421P substitution was shown in vitro to diminish about fourfold the acylation efficiency of PBP1 by various β-lactams [258].

Note that three non-*pbp* loci have been found to contribute to β-lactam resistance in *Neisseria* species. The *mtr* locus encodes an efflux pump [259], while *penB* codes for a porin [260]. The nature of the third locus *penC*, which is required to allow phenotypic expression of the *ponA* mutation, remains undetermined [258].

7.2 *Haemophilus influenza*

Most resistant clinical isolates of *Haemophilus influenza* evade the action of β-lactams by producing a β-lactamase [261]. However, numerous β-lactamase-negative ampicillin-resistant (BLNAR) strains have been isolated, particularly in Japan [262]. First documented in 1980 [261], BLNAR strains were found to express PBPs with a reduced reactivity towards penicillin [263]. Early studies that monitored the PBPs by reaction with radiolabeled penicillin found modifications in PBP2, PBP3, PBP4, PBP5, and PBP6, depending on the resistant strain [264, 265]. Further scrutiny and gene sequencing confirmed only the role of modifications in PBP3, the division of a specific class B PBP [266–268]. Truncation of PBP4, a low molecular weight PBP, was found in some BLNAR strains, but this anomaly was not correlated with resistance [269]. Another study failed to find significant substitutions in the high molecular weight PBPs: PBP1a, PBP1b, and PBP2 [270].

Sequencing of the gene fragment encoding the transpeptidase domain of PBP3 revealed in excess of 30 mutation patterns, with a number of mutations per sequence ranging from 1 to 9, affecting 23 different positions [269–275]. These PBP3 sequences show mostly an accumulation of point mutations, but gene mosaicism has been detected, resulting from horizontal gene transfer between *H. influenzae* and *Haemophilus haemolyticus* [276, 277]. Also, horizontal transfer of genes encoding variants of PBP3 has been observed in the laboratory in co-cultures of BLNAR and susceptible strains [276]. Various classification schemes have been proposed [269, 272, 273, 278]. Seven groups are now recognized (I, IIa,b,c,d, III-like and M (miscellanous)). Some sequences are characterized by the presence of an R517H substitution (group I and III), while others have the N526K mutation (groups II). Both substitutions are relatively close to the third KTG514 catalytic motif. Position 517 with respect to the KTG motif is analogous to the position 552, which is also mutated in a group of PBP2x sequences from *S. Pneumoniae* [157]. Sequences that contain the N526K substitution can also possess the three additional

mutations M377I, S385T, and L389F surrounding the second SSN381 catalytic motif (group III). Site-directed mutagenesis and transformation experiments have shown that S385T and L389F increase the resistance conferred by N526K [278], confirming statistical evidence from clinical isolates [274]. M377I does not increase the resistance conferred by N526K, but may be a neutral mutation linked to the S385T substitution [278]. Modeling of the structure of *H. influenza* PBP3 on that of *S. pneumoniae* PBP2x showed that residues 517, 526, 377, 385, and 389 are probably lining the active site cavity [269]. It has been noted that the PBP3 sequence of group III is associated with a high resistance to cefotaxime and cefixime, whereas group I and group II sequences confer only weak resistance to these cephalosporins [272, 273]. Two substitutions, V511A preceding the KTG motif, sometimes found in group IIb sequences, and V329A within the STVK catalytic motif, were shown to be responsible to elevated resistance to amoxicillin [279]. BLNAR strains with a very high heterogenous resistance to imipenem have been isolated [280]. The presence of a mutated PBP3 from group IIb is required, but fails to account entirely for this unusual resistance.

Interestingly, PBP3 mutations that confer resistance do not appear to impart a fitness cost. In an epithelial cell invasion model, the most invasive strains tested were BLNAR with a PBP3 carrying substitutions in position 377, 385, 389, and 526 [281]. In this experiment, however, strains were not isogenic and a possible deleterious effect of the PBP3 variant may have been offset by other genes.

The affinity for penicillin of a few *H. influenza* PBP3 variants has been measured in vitro [270]. PBP3 of group II, including one variant that has only the N526K mutation, had lower affinity than a PBP3 of group I, in agreement with the resistance level of the originating strains. Surprisingly a PBP3 with only the R517H substitution, the mutation defining group I sequences, had the same high affinity as a wild-type PBP3. This substitution in isolation therefore cannot confer resistance.

BLNAR strains with very high level of resistance combine mechanisms that involve alteration of PBP3 and an efflux pump (AcrAB) [270]. In the laboratory, introduction of a β-lactamase encoding plasmid in BLNAR strain increases the level of resistance [282]. In the clinic, it has now been evidenced that a low-affinity PBP3 can also be found in strains expressing a β-lactamase (BLPAR) [271]. Both mechanisms can cooperate to increase the resistance or to resist to combinations of β-lactams and β-lactamase inhibitors such as the widely used amoxicillin/clavulanate formulations (BLPACR) [272, 283–285]. The same mechanisms were found in various clinical isolates of the related species *Haemophilus parainfluenza* [286].

8 Other Pathogens

Modified PBPs as a means to resist β-lactams has been documented in a few other pathogens, including species where the most frequently encountered mode of resistance is the production of a β-lactamase. Some examples will be briefly presented below.

The genome of *Helicobacter pylori* encodes three recognizable PBPs. These are the homologues of the class B PBP2 and PBP3, and of the class A PBP1a from *E. coli*. Using a fluorescein-labeled penicillin, a fourth low molecular weight penicillin-binding protein was identified [287]. Its sequence shows no homology with proteins of the ASPRE family and the catalytic motifs cannot be recognized in their usual positions. The status of this protein with respect to the subject of this review is therefore uncertain.

Clinical amoxicillin-resistant *H. pylori* strains have been isolated that lose their resistance following storage as frozen samples [288]. This type of unstable resistance may be related to the transient loss of expression of the fourth mysterious penicillin-binding protein [289].

The isolation of stable amoxicillin-resistant strains was also reported [290–293]. In one strain, the resistance was shown to result entirely from the single point mutation S414R in PBP1a, although another substitution was also present [290]. Two other stable resistant strains were found to have the three substitutions T556S, N562Y, and T593A as well as the insertion of a Glu after residue 464 [292]. One strain had ten substitutions, all of them in the second half of the transpeptidase domain, including the T556S and N562Y mutations [294]. It may be noteworthy that the T556S is within the third catalytic motif KTG. The T556S substitution was also found in a clinical resistant strain combined with a C-terminal truncation after residue 636. Both the KTG to KSG substitution and the truncation were shown to contribute to the resistance [295]. In a Korean study, the PBP1 sequence from nine different isolates with varying levels of amoxicillin resistance harbored the same six substitutions: V16I, V45I, S414R, N562Y, T593A, 5595S, and A599T. When the first two and last four substitutions were introduced separately in a recipient susceptible strain, only the last four mutations produced a decrease of the susceptibility to amoxicillin [296]. A careful study of a PBP1a variant with 13 substitutions found that three of them could account for all the amoxicillin resistance conferred: S543R, F473L, and V469M. The last of these was proposed to have a compensatory role, as the double mutant S543R, F473L could never be obtained [297].

In vitro selection on amoxicillin also yielded strains with modified PBP1a [298, 299]. The PBP1a of one such strain had four substitutions, including the S414R mutation [300],

another strain had the single substitution T438M conferring an eightfold increase of the MIC for amoxicillin [298].

Altogether, regarding PBP1a in *H. pylori*, six substitutions have been shown unambiguously to contribute to resistance to amoxicillin, either alone or in various combinations: S414R, T438M, F473L, S543R, T556S, and N562Y, as reviewed in [297]. The possible mechanisms by which some of these mutations decrease the reactivity with β-lactam have been explored by homology modeling the structure of PBP1a from *H. pylori* on that of PBP1a from *S. pneumoniae* [297]. Whereas T438M, T556S, and N562Y may directly affect the active site binding pocket, S414R and S543R change the electrostatics at the entry of the binding cleft and may affect access to the active site [297].

Although the role of variants of the class A PBP1a in β-lactam resistance is firmly established, mutations in PBP2 and PBP3 have also been reported in two clinical isolates [301]. PBP2 and PBP3 are the septal and peripheral class B PBPs, respectively. Subsequent work demonstrated that the variant PBP2 with 4 substitutions (A296V, S494H, A541M, and E572G) could alone confer resistance to cephalosporins, but had little effect on the resistance to amoxicillin or penicillin. Both PBP2 and PBP3 variants, the latter with the single A499V mutation, increased the resistance to β-lactams provided by a PBP1a variants with six mutations including the aforementioned S414R and N562Y [301].

To our knowledge, no clinical isolates of *Escherichia coli* were found to resist through the expression of modified PBPs. However, as a laboratory workhorse, *E. coli* was used to demonstrate that β-lactam pressure can select for altered PBPs [302]. Several point mutations in PBP3 were found to confer resistance to cephalexin and other cephalosporins. Note that *E. coli* PBP3 is the class B PBP dedicated to division. Interestingly, the substitution T308A, next to the active site S307, is analogous to the PBP2x T338A and PBP1a T471A that confer resistance to *S. pneumoniae* [303, 304]. Another mutation was found in the second catalytic motif, changing SSN361 into SSS361 [303].

A few reports must be added to complete this overview of pathogens with modified PBPs. PBP alteration has also been found in imipenem-resistant clinical isolates of *Proteus mirabilis* [305] and *Pseudomonas aeruginosa* [306]. A cefsulodin-resistant clinical isolate of *P. aeruginosa* also had one PBP with reduced affinity, although not the same as the imipenem-resistant isolate [307]. Overexpression of PBP3, in addition to decreased outer-membrane permeability, was found in a highly resistant strain of *Salmonella muenchen* [308]. The various levels of resistance of several strains of *Acinetobacter calcoaceticus* could be correlated with the production of PBPs with altered expression or affinity for β-lactams [309]. In the laboratory, imipenem could select a resistant clone of *Acinetobacter baumanii* with an altered PBP [310]. Alterations in the profile of PBPs revealed by the fluorescent penicillin analogue Bocillin were found in clinical isolates of *A. baumanii* [311]. However, in a more detailed study, no correlation was found between various instances of polymorphism in PBP coding genes and β-lactam resistance [312].

Alterations of PBP3 or PBP2 were selected in laboratory mutants of *Listeria monocytogenes* [313, 314]. Altered PBPs were also found in laboratory-resistant mutants of the *Bacteroides fragilis* group [315] and of *Rhodococcus equi* [316].

Pathogens have been submitted to severe antibiotic pressure over the past five decades, leading to the emergence of resistant strains. In a natural setting as well, β-lactam producing bacteria need to be protected against the drugs of their own making. Two examples have been documented, which involve low-affinity PBPs. Expression of a particular PBP is responsible in part for the resistance of β-lactam-producing *Streptomyces clavuligenus* [317]. None of the eight PBPs of cephamycin C-producing *Nocardia lactamdurans* bind the β-lactam secreted by this bacteria, although it also express a β-lactamase [318].

9 Are the PBPs Sustainable Targets?

The PBPs involved in the β-lactam resistance of the major pathogens are summarized in Table 13.2. The use of β-lactams to treat staphylococcal, enterococcal, and pneumococcal infections is already largely compromised. The isolation of strains with modified PBPs from species that usually resist by producing β-lactamases is worrying. The long-term efficacy of β-lactams may thus be compromised even in the advent of efficient β-lactamase inhibitors. It is therefore reasonable to ask whether PBPs are still valid targets for future antimicrobial therapies.

Half a century of β-lactam therapy has largely validated the targeting of PBPs. The uniquely eubacterial synthesis of peptidoglycan is a good predictor of the near absence of negative secondary effects in vertebrates. These two reasons justify the continued effort to target the PBPs. In which direction should the research effort be headed?

The main lesson from detailed kinetic studies of the reaction between PBPs and β-lactams is that these antibiotics are a poor fit to the enzyme active site. The high dissociation constant of the non-covalent complex guarantees the broad specificity of the β-lactams, but also hints that attempts to improve their affinities may be misguided. Moreover, crystal structures of PBPs complexed covalently to various antibiotics can only suggest what might be the interactions taking place in the preacylation complexes. The structure of a preacylation complex would help to understand both the non-covalent affinity (K_d) and the acylation rate (k_2), the latter being most affected in altered PBPs.

Instead of focusing in the reaction between PBPs and β-lactams, research should be directed towards what may be

Table 13.2 High molecular weight PBPs of organisms that resist β-lactams by expressing low affinity PBPs

	Class A (bifunctional)			Class B (monofunctional)		
Staphylococcus aureus	PBP2			PBP1	PBP3	PBP2a ↗ *mecA*
Enterococci	PBP1a *ponA*	PBP1b *pbpZ*	PBP2a *pbpF*	PBPC(B) *pbpB*	PBP2b *pbpA*	PBP5 ↗
Streptococcus pneumoniae	PBP1a	PBP1b	PBP2a	PBP2x	PBP2b	
Neisseria	PBP1 *ponA*			PBP2 *penA*		
Haemophilus influenza	PBP1a	PBP1b		PBP3 *ftsI*	PBP2	
Helicobacter pylori	PBP1a			PBP3	PBP2	

Low affinity PBPs are boxed. Hatched borders indicate an intrinsic low affinity. An arrow indicate overexpression. No shading indicates point mutations, light shading indicates mosaicity, and dark shading indicates acquisition of exogenous origin. Alternative gene names are given below their respective product.

PBPs' Achile's heel: their physiological reaction of transpeptidation. Indeed, the remarkable feature of the low-affinity PBPs is their retained capacity to catalyze peptidoglycan cross-linking, even though the acylation chemistry is expected to be similar with β-lactams and D-Ala-D-Ala-containing substrates. Understanding how the natural PBP substrates maintain the reactivity of the catalytic serine even in PBPs from resistant bacteria should help the design of novel compounds. Such new drugs could react with all PBPs, regardless of their reactivity with β-lactams [319]. Alternatively new molecules might serve as adjuvant to restore or maintain the reactivity of all PBPs towards traditional β-lactams.

References

1. Tipper DJ, Strominger JL. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci U S A. 1965;54(4):1133–41.
2. Wise Jr EM, Park JT. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc Natl Acad Sci U S A. 1965;54(1):75–81.
3. Blumberg PM, Strominger JL. Isolation by covalent affinity chromatography of the penicillin-binding components from membranes of Bacillus subtilis. Proc Natl Acad Sci U S A. 1972;69(12):3751–5.
4. Joris B, Ledent P, Dideberg O, Fonze E, Lamotte-Brasseur J, Kelly JA, et al. Comparison of the sequences of class A beta-lactamases and of the secondary structure elements of penicillin-recognizing proteins. Antimicrob Agents Chemother. 1991;35(11):2294–301.
5. Pares S, Mouz N, Petillot Y, Hakenbeck R, Dideberg O. X-ray structure of Streptococcus pneumoniae PBP2x, a primary penicillin target enzyme. Nat Struct Biol. 1996;3(3):284–9.
6. Lobkovsky E, Moews PC, Liu H, Zhao H, Frere JM, Knox JR. Evolution of an enzyme activity: crystallographic structure at 2-A resolution of cephalosporinase from the ampC gene of Enterobacter cloacae P99 and comparison with a class A penicillinase. Proc Natl Acad Sci U S A. 1993;90(23):11257–61.
7. Oliva M, Dideberg O, Field MJ. Understanding the acylation mechanisms of active-site serine penicillin-recognizing proteins: a molecular dynamics simulation study. Proteins. 2003;53(1):88–100.
8. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis.

FEMS Microbiol Rev. 2008;32(2):234–58. doi:10.1111/j.1574-6976.2008.00105.x.
9. Bertsche U, Breukink E, Kast T, Vollmer W. In vitro murein peptidoglycan synthesis by dimers of the bifunctional transglycosylase-transpeptidase PBP1B from Escherichia coli. J Biol Chem. 2005;280(45):38096–101.
10. Born P, Breukink E, Vollmer W. In vitro synthesis of cross-linked murein and its attachment to sacculi by PBP1A from Escherichia coli. J Biol Chem. 2006;281(37):26985–93.
11. Schiffer G, Holtje JV. Cloning and characterization of PBP 1C, a third member of the multimodular class A penicillin-binding proteins of Escherichia coli. J Biol Chem. 1999;274(45):32031–9.
12. Schwartz B, Markwalder JA, Wang Y. Lipid II: total synthesis of the bacterial cell wall precursor and utilization as a substrate for glycosyltransfer and transpeptidation by penicillin binding protein (PBP) 1b of Escherichia coli. J Am Chem Soc. 2001;123(47):11638–43.
13. Terrak M, Ghosh TK, van Heijenoort J, Van Beeumen J, Lampilas M, Aszodi J, et al. The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of Escherichia coli. Mol Microbiol. 1999;34(2):350–64.
14. Di Guilmi AM, Dessen A, Dideberg O, Vernet T. The glycosyltransferase domain of penicillin-binding protein 2a from Streptococcus pneumoniae catalyzes the polymerization of murein glycan chains. J Bacteriol. 2003;185(15):4418–23.
15. Zapun A, Philippe J, Abrahams KA, Signor L, Roper DI, Breukink E, et al. In vitro reconstitution of peptidoglycan assembly from the Gram-positive pathogen Streptococcus pneumoniae. ACS Chem Biol. 2013;8(12):2688–96. doi:10.1021/cb400575t.
16. Kohlrausch U, Holtje JV. Analysis of murein and murein precursors during antibiotic-induced lysis of Escherichia coli. J Bacteriol. 1991;173(11):3425–31.
17. Banzhaf M, van den Berg van Saparoea B, Terrak M, Fraipont C, Egan A, Philippe J, et al. Cooperativity of peptidoglycan synthases active in bacterial cell elongation. Mol Microbiol. 2012;85(1):179–94. doi:10.1111/j.1365-2958.2012.08103.x.
18. Piette A, Fraipont C, Den Blaauwen T, Aarsman ME, Pastoret S, Nguyen-Disteche M. Structural determinants required to target penicillin-binding protein 3 to the septum of Escherichia coli. J Bacteriol. 2004;186(18):6110–7.
19. Harris F, Brandenburg K, Seydel U, Phoenix D. Investigations into the mechanisms used by the C-terminal anchors of Escherichia coli penicillin-binding proteins 4, 5, 6 and 6b for membrane interaction. Eur J Biochem. 2002;269(23):5821–9.
20. Amanuma H, Strominger JL. Purification and properties of penicillin-binding proteins 5 and 6 from Escherichia coli membranes. J Biol Chem. 1980;255(23):11173–80.
21. Baquero MR, Bouzon M, Quintela JC, Ayala JA, Moreno F. dacD, an Escherichia coli gene encoding a novel penicillin-binding protein (PBP6b) with DD-carboxypeptidase activity. J Bacteriol. 1996;178(24):7106–11.
22. Korat B, Mottl H, Keck W. Penicillin-binding protein 4 of Escherichia coli: molecular cloning of the dacB gene, controlled overexpression, and alterations in murein composition. Mol Microbiol. 1991;5(3):675–84.
23. Romeis T, Holtje JV. Penicillin-binding protein 7/8 of Escherichia coli is a DD-endopeptidase. Eur J Biochem. 1994;224(2):597–604.
24. Botta GA, Park JT. Evidence for involvement of penicillin-binding protein 3 in murein synthesis during septation but not during cell elongation. J Bacteriol. 1981;145(1):333–40.
25. de Pedro MA, Donachie WD, Holtje JV, Schwarz H. Constitutive septal murein synthesis in Escherichia coli with impaired activity of the morphogenetic proteins RodA and penicillin-binding protein 2. J Bacteriol. 2001;183(14):4115–26.
26. Den Blaauwen T, Aarsman ME, Vischer NO, Nanninga N. Penicillin-binding protein PBP2 of Escherichia coli localizes preferentially in the lateral wall and at mid-cell in comparison with the old cell pole. Mol Microbiol. 2003;47(2):539–47.
27. Wientjes FB, Nanninga N. Rate and topography of peptidoglycan synthesis during cell division in Escherichia coli: concept of a leading edge. J Bacteriol. 1989;171(6):3412–9.
28. Bertsche U, Kast T, Wolf B, Fraipont C, Aarsman ME, Kannenberg K, et al. Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli. Mol Microbiol. 2006;61(3):675–90. doi:10.1111/j.1365-2958.2006.05280.x.
29. Higgins ML, Shockman GD. Study of cycle of cell wall assembly in Streptococcus faecalis by three-dimensional reconstructions of thin sections of cells. J Bacteriol. 1976;127(3):1346–58.
30. Lleo MM, Canepari P, Satta G. Bacterial cell shape regulation: testing of additional predictions unique to the two-competing-sites model for peptidoglycan assembly and isolation of conditional rod-shaped mutants from some wild-type cocci. J Bacteriol. 1990;172(7):3758–71.
31. Tsui HC, Boersma MJ, Vella SA, Kocaoglu O, Kuru E, Peceny JK, et al. Pbp2x localizes separately from Pbp2b and other peptidoglycan synthesis proteins during later stages of cell division of Streptococcus pneumoniae D39. Mol Microbiol. 2014;94(1):21–40. doi:10.1111/mmi.12745.
32. Pinho MG, Errington J. Dispersed mode of Staphylococcus aureus cell wall synthesis in the absence of the division machinery. Mol Microbiol. 2003;50(3):871–81.
33. Massidda O, Anderluzzi D, Friedli L, Feger G. Unconventional organization of the division and cell wall gene cluster of Streptococcus pneumoniae. Microbiology. 1998;144(Pt 11):3069–78.
34. de Pedro MA, Holtje JV, Schwarz H. Fast lysis of Escherichia coli filament cells requires differentiation of potential division sites. Microbiology. 2002;148(Pt 1):79–86.
35. Giesbrecht P, Kersten T, Maidhof H, Wecke J. Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. Microbiol Mol Biol Rev. 1998;62(4):1371–414.
36. Pucci MJ, Hinks ET, Dicker DT, Higgins ML, Daneo-Moore L. Inhibition of beta-lactam antibiotics at two different times in the cell cycle of Streptococcus faecium ATCC 9790. J Bacteriol. 1986;165(3):682–8.
37. Jevons MP, Coe AW, Parker MT. Methicillin resistance in staphylococci. Lancet. 1963;1:904–7.
38. Deurenberg RH, Stobberingh EE. The evolution of Staphylococcus aureus. Infect Genet Evol. 2008;8(6):747–63. doi:10.1016/j.meegid.2008.07.007.
39. Kos VN, Desjardins CA, Griggs A, Cerqueira G, Van Tonder A, Holden MT, et al. Comparative genomics of vancomycin-resistant Staphylococcus aureus strains and their positions within the clade most commonly associated with Methicillin-resistant S. aureus hospital-acquired infection in the United States. MBio. 2012;3(3):e00112–12. doi:10.1128/mBio.00112-12.
40. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, et al. Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus. Science. 2003;302(5650):1569–71.
41. Saravolatz LD, Pawlak J, Saravolatz SN, Johnson LB. In vitro activity of retapamulin against Staphylococcus aureus resistant to various antimicrobial agents. Antimicrob Agents Chemother. 2013;57(9):4547–50. doi:10.1128/AAC.00282-13.
42. Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in Staphylococcus aureus. J Bacteriol. 1984;158(2):513–6.
43. Utsui Y, Yokota T. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 1985;28(3):397–403.

44. Parvez MA, Shibata H, Nakano T, Niimi S, Fujii N, Arakaki N, et al. No relationship exists between PBP 2a amounts expressed in different MRSA strains obtained clinically and their beta-lactam MIC values. J Med Invest. 2008;55(3–4):246–53.
45. Garcia-Castellanos R, Mallorqui-Fernandez G, Marrero A, Potempa J, Coll M, Gomis-Ruth FX. On the transcriptional regulation of methicillin resistance: MecI repressor in complex with its operator. J Biol Chem. 2004;279(17):17888–96.
46. Amoroso A, Boudet J, Berzigotti S, Duval V, Teller N, Mengin-Lecreulx D, et al. A peptidoglycan fragment triggers beta-lactam resistance in Bacillus licheniformis. PLoS Pathog. 2012; 8(3):e1002571. doi:10.1371/journal.ppat.1002571.
47. Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, et al. Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2001;45(5):1323–36.
48. Ender M, McCallum N, Adhikari R, Berger-Bachi B. Fitness cost of SCCmec and methicillin resistance levels in Staphylococcus aureus. Antimicrob Agents Chemother. 2004;48(6):2295–7.
49. Hiramatsu K, Cui L, Kuroda M, Ito T. The emergence and evolution of methicillin-resistant Staphylococcus aureus. Trends Microbiol. 2001;9(10):486–93.
50. Dordel J, Kim C, Chung M, Pardos de la Gandara M, Holden MT, Parkhill J, et al. Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus. MBio. 2014;5(2):e01000. doi:10.1128/mBio.01000-13.
51. Pinho MG, de Lencastre H, Tomasz A. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc Natl Acad Sci U S A. 2001; 98(19):10886–91.
52. Pinho MG, Filipe SR, de Lencastre H, Tomasz A. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphylococcus aureus. J Bacteriol. 2001;183(22): 6525–31.
53. Navratna V, Nadig S, Sood V, Prasad K, Arakere G, Gopal B. Molecular basis for the role of Staphylococcus aureus penicillin binding protein 4 in antimicrobial resistance. J Bacteriol. 2010;192(1):134–44. doi:10.1128/JB.00822-09.
54. Leski TA, Tomasz A. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J Bacteriol. 2005;187(5):1815–24.
55. Memmi G, Filipe SR, Pinho MG, Fu Z, Cheung A. Staphylococcus aureus PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob Agents Chemother. 2008;52(11):3955–66. doi:10.1128/AAC.00049-08.
56. Farha MA, Leung A, Sewell EW, D'Elia MA, Allison SE, Ejim L, et al. Inhibition of WTA synthesis blocks the cooperative action of PBPs and sensitizes MRSA to beta-lactams. ACS Chem Biol. 2013;8(1):226–33. doi:10.1021/cb300413m.
57. Atilano ML, Pereira PM, Yates J, Reed P, Veiga H, Pinho MG, et al. Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in Staphylococcus aureus. Proc Natl Acad Sci U S A. 2010;107(44):18991–6. doi:10.1073/pnas.1004304107.
58. Brown S, Xia G, Luhachack LG, Campbell J, Meredith TC, Chen C, et al. Methicillin resistance in Staphylococcus aureus requires glycosylated wall teichoic acids. Proc Natl Acad Sci U S A. 2012;109(46):18909–14. doi:10.1073/pnas.1209126109.
59. De Lencastre H, Wu SW, Pinho MG, Ludovice AM, Filipe S, Gardete S, et al. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in Staphylococcus aureus strain COL that impact on the expression of resistance to methicillin. Microb Drug Resist. 1999;5(3):163–75.
60. Henze U, Sidow T, Wecke J, Labischinski H, Berger-Bachi B. Influence of femB on methicillin resistance and peptidoglycan metabolism in Staphylococcus aureus. J Bacteriol. 1993;175(6): 1612–20.
61. Stranden AM, Ehlert K, Labischinski H, Berger-Bachi B. Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a femAB null mutant of methicillin-resistant Staphylococcus aureus. J Bacteriol. 1997;179(1):9–16.
62. Arbeloa A, Hugonnet JE, Sentilhes AC, Josseaume N, Dubost L, Monsempes C, et al. Synthesis of mosaic peptidoglycan cross-bridges by hybrid peptidoglycan assembly pathways in gram-positive bacteria. J Biol Chem. 2004;279(40):41546–56.
63. Giannouli S, Labrou M, Kyritsis A, Ikonomidis A, Pournaras S, Stathopoulos C, et al. Detection of mutations in the FemXAB protein family in oxacillin-susceptible mecA-positive Staphylococcus aureus clinical isolates. J Antimicrob Chemother. 2010;65(4):626–33. doi:10.1093/jac/dkq039.
64. Komatsuzawa H, Ohta K, Labischinski H, Sugai M, Suginaka H. Characterization of fmtA, a gene that modulates the expression of methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother. 1999;43(9):2121–5.
65. Fan X, Liu Y, Smith D, Konermann L, Siu KW, Golemi-Kotra D. Diversity of penicillin-binding proteins. Resistance factor FmtA of Staphylococcus aureus. J Biol Chem. 2007;282(48): 35143–52. doi:10.1074/jbc.M706296200.
66. Qamar A, Golemi-Kotra D. Dual roles of FmtA in Staphylococcus aureus cell wall biosynthesis and autolysis. Antimicrob Agents Chemother. 2012;56(7):3797–805. doi:10.1128/AAC.00187-12.
67. Antignac A, Tomasz A. Reconstruction of the phenotypes of methicillin-resistant Staphylococcus aureus by replacement of the staphylococcal cassette chromosome mec with a plasmid-borne copy of Staphylococcus sciuri pbpD gene. Antimicrob Agents Chemother. 2009;53(2):435–41. doi:10.1128/AAC.01099-08.
68. Couto I, Wu SW, Tomasz A, de Lencastre H. Development of methicillin resistance in clinical isolates of Staphylococcus sciuri by transcriptional activation of the mecA homologue native to s. J Bacteriol. 2003;185(2):645–53.
69. Wu S, de Lencastre H, Tomasz A. Genetic organization of the mecA region in methicillin-susceptible and methicillin-resistant strains of Staphylococcus sciuri. J Bacteriol. 1998;180(2): 236–42.
70. Tsubakishita S, Kuwahara-Arai K, Sasaki T, Hiramatsu K. Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. Antimicrob Agents Chemother. 2010;54(10):4352–9. doi:10.1128/AAC.00356-10.
71. Pierre J, Williamson R, Bornet M, Gutmann L. Presence of an additional penicillin-binding protein in methicillin-resistant Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, and Staphylococcus simulans with a low affinity for methicillin, cephalothin, and cefamandole. Antimicrob Agents Chemother. 1990;34(9):1691–4.
72. Harrison EM, Paterson GK, Holden MT, Larsen J, Stegger M, Larsen AR, et al. Whole genome sequencing identifies zoonotic transmission of MRSA isolates with the novel mecA homologue mecC. EMBO Mol Med. 2013;5(4):509–15. doi:10.1002/emmm.201202413.
73. Shore AC, Deasy EC, Slickers P, Brennan G, O'Connell B, Monecke S, et al. Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent mecA, mecI, mecR1, blaZ, and ccr genes in human clinical isolates of clonal complex 130 methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2011;55(8):3765–73. doi:10.1128/AAC.00187-11.
74. Kim C, Milheirico C, Gardete S, Holmes MA, Holden MT, de Lencastre H, et al. Properties of a novel PBP2A protein homolog from Staphylococcus aureus strain LGA251 and its contribution

to the beta-lactam-resistant phenotype. J Biol Chem. 2012; 287(44):36854–63. doi:10.1074/jbc.M112.395962.
75. Chambers HF, Sachdeva MJ, Hackbarth CJ. Kinetics of penicillin binding to penicillin-binding proteins of Staphylococcus aureus. Biochem J. 1994;301(Pt 1):139–44.
76. Graves-Woodward K, Pratt RF. Reaction of soluble penicillin-binding protein 2a of methicillin-resistant Staphylococcus aureus with beta-lactams and acyclic substrates: kinetics in homogeneous solution. Biochem J. 1998;332(Pt 3):755–61.
77. Lu WP, Sun Y, Bauer MD, Paule S, Koenigs PM, Kraft WG. Penicillin-binding protein 2a from methicillin-resistant Staphylococcus aureus: kinetic characterization of its interactions with beta-lactams using electrospray mass spectrometry. Biochemistry. 1999;38(20):6537–46.
78. Lu WP, Kincaid E, Sun Y, Bauer MD. Kinetics of beta-lactam interactions with penicillin-susceptible and -resistant penicillin-binding protein 2x proteins from Streptococcus pneumoniae. Involvement of acylation and deacylation in beta-lactam resistance. J Biol Chem. 2001;276(34):31494–501.
79. Mouz N, Gordon E, Di Guilmi AM, Petit I, Petillot Y, Dupont Y, et al. Identification of a structural determinant for resistance to beta-lactam antibiotics in Gram-positive bacteria. Proc Natl Acad Sci U S A. 1998;95(23):13403–6.
80. Fuda C, Suvorov M, Vakulenko SB, Mobashery S. The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant Staphylococcus aureus. J Biol Chem. 2004;279(39):40802–6.
81. Lim D, Strynadka NC. Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant Staphylococcus aureus. Nat Struct Biol. 2002;9(11):870–6.
82. Beadle BM, Nicholas RA, Shoichet BK. Interaction energies between beta-lactam antibiotics and E. coli penicillin-binding protein 5 by reversible thermal denaturation. Protein Sci. 2001; 10(6):1254–9.
83. Fuda C, Hesek D, Lee M, Heilmayer W, Novak R, Vakulenko SB, et al. Mechanistic basis for the action of new cephalosporin antibiotics effective against methicillin- and vancomycin-resistant Staphylococcus aureus. J Biol Chem. 2006;281(15):10035–41.
84. Fuda C, Hesek D, Lee M, Morio K, Nowak T, Mobashery S. Activation for catalysis of penicillin-binding protein 2a from methicillin-resistant Staphylococcus aureus by bacterial cell wall. J Am Chem Soc. 2005;127(7):2056–7.
85. Kosowska-Shick K, McGhee PL, Appelbaum PC. Affinity of ceftaroline and other beta-lactams for penicillin-binding proteins from Staphylococcus aureus and Streptococcus pneumoniae. Antimicrob Agents Chemother. 2010;54(5):1670–7. doi:10.1128/AAC.00019-10.
86. Otero LH, Rojas-Altuve A, Llarrull LI, Carrasco-Lopez C, Kumarasiri M, Lastochkin E, et al. How allosteric control of Staphylococcus aureus penicillin binding protein 2a enables methicillin resistance and physiological function. Proc Natl Acad Sci U S A. 2013;110(42):16808–13. doi:10.1073/pnas.1300118110.
87. Fishovitz J, Rojas-Altuve A, Otero LH, Dawley M, Carrasco-Lopez C, Chang M, et al. Disruption of allosteric response as an unprecedented mechanism of resistance to antibiotics. J Am Chem Soc. 2014;136(28):9814–7. doi:10.1021/ja5030657.
88. Mendes RE, Tsakris A, Sader HS, Jones RN, Biek D, McGhee P, et al. Characterization of methicillin-resistant Staphylococcus aureus displaying increased MICs of ceftaroline. J Antimicrob Chemother. 2012;67(6):1321–4. doi:10.1093/jac/dks069.
89. Ba X, Harrison EM, Edwards GF, Holden MT, Larsen AR, Petersen A, et al. Novel mutations in penicillin-binding protein genes in clinical Staphylococcus aureus isolates that are methicillin resistant on susceptibility testing, but lack the mec gene. J Antimicrob Chemother. 2014;69(3):594–7. doi:10.1093/jac/dkt418.
90. Banerjee R, Gretes M, Harlem C, Basuino L, Chambers HF. A mecA-negative strain of methicillin-resistant Staphylococcus aureus with high-level beta-lactam resistance contains mutations in three genes. Antimicrob Agents Chemother. 2010;54(11):4900–2. doi:10.1128/AAC.00594-10.
91. Henze UU, Berger-Bachi B. Penicillin-binding protein 4 overproduction increases beta-lactam resistance in Staphylococcus aureus. Antimicrob Agents Chemother. 1996;40(9):2121–5.
92. Tomasz A, Drugeon HB, de Lencastre HM, Jabes D, McDougall L, Bille J. New mechanism for methicillin resistance in Staphylococcus aureus: clinical isolates that lack the PBP 2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob Agents Chemother. 1989;33(11):1869–74.
93. Hackbarth CJ, Kocagoz T, Kocagoz S, Chambers HF. Point mutations in Staphylococcus aureus PBP 2 gene affect penicillin-binding kinetics and are associated with resistance. Antimicrob Agents Chemother. 1995;39(1):103–6.
94. Contreras-Martel C, Amoroso A, Woon EC, Zervosen A, Inglis S, Martins A, et al. Structure-guided design of cell wall biosynthesis inhibitors that overcome beta-lactam resistance in Staphylococcus aureus (MRSA). ACS Chem Biol. 2011;6(9):943–51. doi:10.1021/cb2001846.
95. Turk S, Verlaine O, Gerards T, Zivec M, Humljan J, Sosic I, et al. New noncovalent inhibitors of penicillin-binding proteins from penicillin-resistant bacteria. PLoS One. 2011;6(5):e19418. doi:10.1371/journal.pone.0019418.
96. Bernal P, Lemaire S, Pinho MG, Mobashery S, Hinds J, Taylor PW. Insertion of epicatechin gallate into the cytoplasmic membrane of methicillin-resistant Staphylococcus aureus disrupts penicillin-binding protein (PBP) 2a-mediated beta-lactam resistance by delocalizing PBP2. J Biol Chem. 2010;285(31):24055–65. doi:10.1074/jbc.M110.114793.
97. Yao X, Lu CD. A PBP 2 mutant devoid of the transpeptidase domain abolishes spermine-beta-lactam synergy in Staphylococcus aureus Mu50. Antimicrob Agents Chemother. 2012;56(1):83–91. doi:10.1128/AAC.05415-11.
98. Perez JL, Riera L, Valls F, Berrocal CI, Berrocal L. A comparison of the in-vitro activity of seventeen antibiotics against Streptococcus faecalis. J Antimicrob Chemother. 1987;20(3):357–62.
99. Moellering Jr RC, Korzeniowski OM, Sande MA, Wennersten CB. Species-specific resistance to antimocrobial synergism in Streptococcus faecium and Streptococcus faecalis. J Infect Dis. 1979;140(2):203–8.
100. Arbeloa A, Segal H, Hugonnet JE, Josseaume N, Dubost L, Brouard JP, et al. Role of class A penicillin-binding proteins in PBP5-mediated beta-lactam resistance in Enterococcus faecalis. J Bacteriol. 2004;186(5):1221–8.
101. Fontana R, Cerini R, Longoni P, Grossato A, Canepari P. Identification of a streptococcal penicillin-binding protein that reacts very slowly with penicillin. J Bacteriol. 1983;155(3): 1343–50.
102. Fontana R, Grossato A, Rossi L, Cheng YR, Satta G. Transition from resistance to hypersusceptibility to beta-lactam antibiotics associated with loss of a low-affinity penicillin-binding protein in a Streptococcus faecium mutant highly resistant to penicillin. Antimicrob Agents Chemother. 1985;28(5):678–83.
103. Lleo MM, Canepari P, Cornaglia G, Fontana R, Satta G. Bacteriostatic and bactericidal activities of beta-lactams against Streptococcus (Enterococcus) faecium are associated with saturation of different penicillin-binding proteins. Antimicrob Agents Chemother. 1987;31(10):1618–26.
104. Sifaoui F, Arthur M, Rice L, Gutmann L. Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in Enterococcus faecium. Antimicrob Agents Chemother. 2001;45(9):2594–7.

105. Williamson R, le Bouguenec C, Gutmann L, Horaud T. One or two low affinity penicillin-binding proteins may be responsible for the range of susceptibility of Enterococcus faecium to benzylpenicillin. J Gen Microbiol. 1985;131(Pt 8):1933–40.
106. Duez C, Zorzi W, Sapunaric F, Amoroso A, Thamm I, Coyette J. The penicillin resistance of Enterococcus faecalis JH2-2r results from an overproduction of the low-affinity penicillin-binding protein PBP4 and does not involve a psr-like gene. Microbiology. 2001;147(Pt 9):2561–9.
107. Fontana R, Aldegheri M, Ligozzi M, Lopez H, Sucari A, Satta G. Overproduction of a low-affinity penicillin-binding protein and high-level ampicillin resistance in Enterococcus faecium. Antimicrob Agents Chemother. 1994;38(9):1980–3.
108. Hsieh SE, Hsu LL, Hsu WH, Chen CY, Chen HJ, Liao CT. Importance of amino acid alterations and expression of penicillin-binding protein 5 to ampicillin resistance of Enterococcus faecium in Taiwan. Int J Antimicrob Agents. 2006;28(6):514–9. doi:10.1016/j.ijantimicag.2006.07.027.
109. Klare I, Rodloff AC, Wagner J, Witte W, Hakenbeck R. Overproduction of a penicillin-binding protein is not the only mechanism of penicillin resistance in Enterococcus faecium. Antimicrob Agents Chemother. 1992;36(4):783–7.
110. Rybkine T, Mainardi JL, Sougakoff W, Collatz E, Gutmann L. Penicillin-binding protein 5 sequence alterations in clinical isolates of Enterococcus faecium with different levels of beta-lactam resistance. J Infect Dis. 1998;178(1):159–63.
111. Zorzi W, Zhou XY, Dardenne O, Lamotte J, Raze D, Pierre J, et al. Structure of the low-affinity penicillin-binding protein 5 PBP5fm in wild-type and highly penicillin-resistant strains of Enterococcus faecium. J Bacteriol. 1996;178(16):4948–57.
112. Rice LB, Carias LL, Rudin S, Lakticova V, Wood A, Hutton-Thomas R. Enterococcus faecium low-affinity pbp5 is a transferable determinant. Antimicrob Agents Chemother. 2005;49(12):5007–12. doi:10.1128/AAC.49.12.5007-5012.2005.
113. Piras G, el Kharroubi A, van Beeumen J, Coeme E, Coyette J, Ghuysen JM. Characterization of an Enterococcus hirae penicillin-binding protein 3 with low penicillin affinity. J Bacteriol. 1990;172(12):6856–62.
114. Piras G, Raze D, el Kharroubi A, Hastir D, Englebert S, Coyette J, et al. Cloning and sequencing of the low-affinity penicillin-binding protein 3r-encoding gene of Enterococcus hirae S185: modular design and structural organization of the protein. J Bacteriol. 1993;175(10):2844–52.
115. Ligozzi M, Pittaluga F, Fontana R. Modification of penicillin-binding protein 5 associated with high-level ampicillin resistance in Enterococcus faecium. Antimicrob Agents Chemother. 1996;40(2):354–7.
116. Galloway-Pena JR, Rice LB, Murray BE. Analysis of PBP5 of early U.S. isolates of Enterococcus faecium: sequence variation alone does not explain increasing ampicillin resistance over time. Antimicrob Agents Chemother. 2011;55(7):3272–7. doi:10.1128/AAC.00099-11.
117. Jureen R, Mohn SC, Harthug S, Haarr L, Langeland N. Role of penicillin-binding protein 5 C-terminal amino acid substitutions in conferring ampicillin resistance in Norwegian clinical strains of Enterococcus faecium. APMIS. 2004;112(4–5):291–8.
118. Rice LB, Bellais S, Carias LL, Hutton-Thomas R, Bonomo RA, Caspers P, et al. Impact of specific pbp5 mutations on expression of beta-lactam resistance in Enterococcus faecium. Antimicrob Agents Chemother. 2004;48(8):3028–32.
119. Sauvage E, Kerff F, Fonze E, Herman R, Schoot B, Marquette JP, et al. The 2.4-A crystal structure of the penicillin-resistant penicillin-binding protein PBP5fm from Enterococcus faecium in complex with benzylpenicillin. Cell Mol Life Sci. 2002;59(7):1223–32.
120. Hujer AM, Kania M, Gerken T, Anderson VE, Buynak JD, Ge X, et al. Structure-activity relationships of different beta-lactam antibiotics against a soluble form of Enterococcus faecium PBP5, a type II bacterial transpeptidase. Antimicrob Agents Chemother. 2005;49(2):612–8.
121. Rice LB, Carias LL, Rudin S, Hutton R, Marshall S, Hassan M, et al. Role of class A penicillin-binding proteins in the expression of beta-lactam resistance in Enterococcus faecium. J Bacteriol. 2009;191(11):3649–56. doi:10.1128/JB.01834-08.
122. Ligozzi M, Pittaluga F, Fontana R. Identification of a genetic element (psr) which negatively controls expression of Enterococcus hirae penicillin-binding protein 5. J Bacteriol. 1993;175(7):2046–51.
123. Sapunaric F, Franssen C, Stefanic P, Amoroso A, Dardenne O, Coyette J. Redefining the role of psr in beta-lactam resistance and cell autolysis of Enterococcus hirae. J Bacteriol. 2003;185(20):5925–35.
124. Rice LB, Carias LL, Hutton-Thomas R, Sifaoui F, Gutmann L, Rudin SD. Penicillin-binding protein 5 and expression of ampicillin resistance in Enterococcus faecium. Antimicrob Agents Chemother. 2001;45(5):1480–6.
125. Ono S, Muratani T, Matsumoto T. Mechanisms of resistance to imipenem and ampicillin in Enterococcus faecalis. Antimicrob Agents Chemother. 2005;49(7):2954–8.
126. Murray BE. Beta-lactamase-producing enterococci. Antimicrob Agents Chemother. 1992;36(11):2355–9.
127. Mainardi JL, Fourgeaud M, Hugonnet JE, Dubost L, Brouard JP, Ouazzani J, et al. A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. J Biol Chem. 2005;280(46):38146–52.
128. Mainardi JL, Legrand R, Arthur M, Schoot B, van Heijenoort J, Gutmann L. Novel mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in Enterococcus faecium. J Biol Chem. 2000;275(22):16490–6.
129. Mainardi JL, Morel V, Fourgeaud M, Cremniter J, Blanot D, Legrand R, et al. Balance between two transpeptidation mechanisms determines the expression of beta-lactam resistance in Enterococcus faecium. J Biol Chem. 2002;277(39):35801–7.
130. Sacco E, Hugonnet JE, Josseaume N, Cremniter J, Dubost L, Marie A, et al. Activation of the L, D-transpeptidation peptidoglycan cross-linking pathway by a metallo-D,D-carboxypeptidase in Enterococcus faecium. Mol Microbiol. 2010;75(4):874–85. doi:10.1111/j.1365-2958.2009.07014.x.
131. Hakenbeck R, Tarpay M, Tomasz A. Multiple changes of penicillin-binding proteins in penicillin-resistant clinical isolates of Streptococcus pneumoniae. Antimicrob Agents Chemother. 1980;17(3):364–71.
132. Zighelboim S, Tomasz A. Penicillin-binding proteins of multiply antibiotic-resistant South African strains of Streptococcus pneumoniae. Antimicrob Agents Chemother. 1980;17(3):434–42.
133. Laible G, Spratt BG, Hakenbeck R. Interspecies recombinational events during the evolution of altered PBP 2x genes in penicillin-resistant clinical isolates of Streptococcus pneumoniae. Mol Microbiol. 1991;5(8):1993–2002.
134. Dowson CG, Hutchison A, Brannigan JA, George RC, Hansman D, Linares J, et al. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of Streptococcus pneumoniae. Proc Natl Acad Sci U S A. 1989;86(22):8842–6.
135. Martin C, Sibold C, Hakenbeck R. Relatedness of penicillin-binding protein 1a genes from different clones of penicillin-resistant Streptococcus pneumoniae isolated in South Africa and Spain. Embo J. 1992;11(11):3831–6.
136. Claverys JP, Prudhomme M, Mortier-Barriere I, Martin B. Adaptation to the environment: Streptococcus pneumoniae, a paradigm for recombination-mediated genetic plasticity? Mol Microbiol. 2000;35(2):251–9.
137. Smith AM, Klugman KP. Alterations in penicillin-binding protein 2B from penicillin-resistant wild-type strains of Streptococcus pneumoniae. Antimicrob Agents Chemother. 1995;39(4):859–67.

138. Sibold C, Henrichsen J, Konig A, Martin C, Chalkley L, Hakenbeck R. Mosaic pbpX genes of major clones of penicillin-resistant Streptococcus pneumoniae have evolved from pbpX genes of a penicillin-sensitive Streptococcus oralis. Mol Microbiol. 1994;12(6):1013–23.
139. Chi F, Nolte O, Bergmann C, Ip M, Hakenbeck R. Crossing the barrier: Evolution and spread of a major class of mosaic pbp2x in Streptococcus pneumoniae, S. mitis and S. oralis. Int J Med Microbiol. 2007;297(7–8):503–12.
140. Dowson CG, Hutchison A, Woodford N, Johnson AP, George RC, Spratt BG. Penicillin-resistant viridans streptococci have obtained altered penicillin-binding protein genes from penicillin-resistant strains of Streptococcus pneumoniae. Proc Natl Acad Sci U S A. 1990;87(15):5858–62.
141. Reichmann P, Konig A, Linares J, Alcaide F, Tenover FC, McDougal L, et al. A global gene pool for high-level cephalosporin resistance in commensal Streptococcus species and Streptococcus pneumoniae. J Infect Dis. 1997;176(4):1001–12.
142. Coffey TJ, Dowson CG, Daniels M, Zhou J, Martin C, Spratt BG, et al. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of Streptococcus pneumoniae. Mol Microbiol. 1991;5(9):2255–60.
143. Zhou J, Enright MC, Spratt BG. Identification of the major Spanish clones of penicillin-resistant pneumococci via the Internet using multilocus sequence typing. J Clin Microbiol. 2000;38(3):977–86.
144. McGee L, McDougal L, Zhou J, Spratt BG, Tenover FC, George R, et al. Nomenclature of major antimicrobial-resistant clones of Streptococcus pneumoniae defined by the pneumococcal molecular epidemiology network. J Clin Microbiol. 2001;39(7):2565–71.
145. Stanhope MJ, Walsh SL, Becker JA, Miller LA, Lefebure T, Lang P, et al. The relative frequency of intraspecific lateral gene transfer of penicillin binding proteins 1a, 2b, and 2x, in amoxicillin resistant Streptococcus pneumoniae. Infect Genet Evol. 2007;7(4):520–34. doi:10.1016/j.meegid.2007.03.004.
146. Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of Streptococcus pneumoniae are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob Agents Chemother. 1996;40(4):829–34.
147. Krauss J, van der Linden M, Grebe T, Hakenbeck R. Penicillin-binding proteins 2x and 2b as primary PBP targets in Streptococcus pneumoniae. Microb Drug Resist. 1996;2(2):183–6.
148. Sifaoui F, Kitzis MD, Gutmann L. In vitro selection of one-step mutants of Streptococcus pneumoniae resistant to different oral beta-lactam antibiotics is associated with alterations of PBP2x. Antimicrob Agents Chemother. 1996;40(1):152–6.
149. Coffey TJ, Daniels M, McDougal LK, Dowson CG, Tenover FC, Spratt BG. Genetic analysis of clinical isolates of Streptococcus pneumoniae with high-level resistance to expanded-spectrum cephalosporins. Antimicrob Agents Chemother. 1995;39(6):1306–13.
150. Asahi Y, Takeuchi Y, Ubukata K. Diversity of substitutions within or adjacent to conserved amino acid motifs of penicillin-binding protein 2X in cephalosporin-resistant Streptococcus pneumoniae isolates. Antimicrob Agents Chemother. 1999;43(5):1252–5.
151. Laible G, Hakenbeck R. Penicillin-binding proteins in beta-lactam-resistant laboratory mutants of Streptococcus pneumoniae. Mol Microbiol. 1987;1(3):355–63.
152. Hakenbeck R, Tornette S, Adkinson NF. Interaction of non-lytic beta-lactams with penicillin-binding proteins in Streptococcus pneumoniae. J Gen Microbiol. 1987;133(Pt 3):755–60.
153. Maurer P, Koch B, Zerfass I, Krauss J, van der Linden M, Frere JM, et al. Penicillin-binding protein 2x of Streptococcus pneumoniae: three new mutational pathways for remodelling an essential enzyme into a resistance determinant. J Mol Biol. 2008;376(5):1403–16. doi:10.1016/j.jmb.2007.12.058.
154. Chesnel L, Pernot L, Lemaire D, Champelovier D, Croize J, Dideberg O, et al. The structural modifications induced by the M339F substitution in PBP2x from Streptococcus pneumoniae further decreases the susceptibility to beta-lactams of resistant strains. J Biol Chem. 2003;278(45):44448–56.
155. Dowson CG, Hutchison A, Spratt BG. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of Streptococcus pneumoniae. Mol Microbiol. 1989;3(1):95–102.
156. du Plessis M, Bingen E, Klugman KP. Analysis of penicillin-binding protein genes of clinical isolates of Streptococcus pneumoniae with reduced susceptibility to amoxicillin. Antimicrob Agents Chemother. 2002;46(8):2349–57.
157. Pernot L, Chesnel L, Le Gouellec A, Croize J, Vernet T, Dideberg O, et al. A PBP2x from a clinical isolate of Streptococcus pneumoniae exhibits an alternative mechanism for reduction of susceptibility to beta-lactam antibiotics. J Biol Chem. 2004;279(16):16463–70.
158. Reichmann P, Konig A, Marton A, Hakenbeck R. Penicillin-binding proteins as resistance determinants in clinical isolates of Streptococcus pneumoniae. Microb Drug Resist. 1996;2(2):177–81.
159. Smith AM, Klugman KP. Alterations in PBP 1A essential-for high-level penicillin resistance in Streptococcus pneumoniae. Antimicrob Agents Chemother. 1998;42(6):1329–33.
160. Pagliero E, Chesnel L, Hopkins J, Croize J, Dideberg O, Vernet T, et al. Biochemical characterization of Streptococcus pneumoniae penicillin-binding protein 2b and its implication in beta-lactam resistance. Antimicrob Agents Chemother. 2004;48(5):1848–55.
161. Barcus VA, Ghanekar K, Yeo M, Coffey TJ, Dowson CG. Genetics of high level penicillin resistance in clinical isolates of Streptococcus pneumoniae. FEMS Microbiol Lett. 1995;126(3):299–303.
162. Munoz R, Dowson CG, Daniels M, Coffey TJ, Martin C, Hakenbeck R, et al. Genetics of resistance to third-generation cephalosporins in clinical isolates of Streptococcus pneumoniae. Mol Microbiol. 1992;6(17):2461–5.
163. Sanbongi Y, Ida T, Ishikawa M, Osaki Y, Kataoka H, Suzuki T, et al. Complete sequences of six penicillin-binding protein genes from 40 Streptococcus pneumoniae clinical isolates collected in Japan. Antimicrob Agents Chemother. 2004;48(6):2244–50.
164. Ubukata K, Chiba N, Hasegawa K, Kobayashi R, Iwata S, Sunakawa K. Antibiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of Streptococcus pneumoniae strains responsible for meningitis in Japan, 1999 to 2002. Antimicrob Agents Chemother. 2004;48(5):1488–94.
165. Ferroni A, Berche P. Alterations to penicillin-binding proteins 1A, 2B and 2X amongst penicillin-resistant clinical isolates of Streptococcus pneumoniae serotype 23F from the nasopharyngeal flora of children. J Med Microbiol. 2001;50(9):828–32.
166. Izdebski R, Rutschmann J, Fiett J, Sadowy E, Gniadkowski M, Hryniewicz W, et al. Highly variable penicillin resistance determinants PBP 2x, PBP 2b, and PBP 1a in isolates of two Streptococcus pneumoniae clonal groups, Poland 23F-16 and Poland 6B-20. Antimicrob Agents Chemother. 2008;52(3):1021–7. doi:10.1128/AAC.01082-07.
167. Nagai K, Davies TA, Jacobs MR, Appelbaum PC. Effects of amino acid alterations in penicillin-binding proteins (PBPs) 1a, 2b, and 2x on PBP affinities of penicillin, ampicillin, amoxicillin, cefditoren, cefuroxime, cefprozil, and cefaclor in 18 clinical isolates of penicillin-susceptible, -intermediate, and -resistant pneumococci. Antimicrob Agents Chemother. 2002;46(5):1273–80.
168. Nichol KA, Zhanel GG, Hoban DJ. Penicillin-binding protein 1A, 2B, and 2X alterations in Canadian isolates of penicillin-resistant Streptococcus pneumoniae. Antimicrob Agents Chemother. 2002;46(10):3261–4.

169. Overweg K, Bogaert D, Sluijter M, de Groot R, Hermans PW. Molecular characteristics of penicillin-binding protein genes of penicillin-nonsusceptible Streptococcus pneumoniae isolated in the Netherlands. Microb Drug Resist. 2001;7(4):323–34.
170. Granger D, Boily-Larouche G, Turgeon P, Weiss K, Roger M. Molecular characteristics of pbp1a and pbp2b in clinical Streptococcus pneumoniae isolates in Quebec, Canada. J Antimicrob Chemother. 2006;57(1):61–70. doi:10.1093/jac/dki401.
171. McDougal LK, Rasheed JK, Biddle JW, Tenover FC. Identification of multiple clones of extended-spectrum cephalosporin-resistant Streptococcus pneumoniae isolates in the United States. Antimicrob Agents Chemother. 1995;39(10):2282–8.
172. Smith AM, Klugman KP. Alterations in MurM, a cell wall muropeptide branching enzyme, increase high-level penicillin and cephalosporin resistance in Streptococcus pneumoniae. Antimicrob Agents Chemother. 2001;45(8):2393–6.
173. Cafini F, Aguilar L, Sevillano D, Gimenez MJ, Alou L, Fenoll A, et al. Decrease in bacterial load versus resistance selection of pneumococcal subpopulations by beta-lactam physiological concentrations over time: an in vitro pharmacodynamic simulation. Microb Drug Resist. 2008;14(1):13–21. doi:10.1089/mdr.2008.0783.
174. Coffey TJ, Daniels M, Enright MC, Spratt BG. Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of Streptococcus pneumoniae arose by large recombinational replacements of the cpsA-pbp1a region. Microbiology. 1999;145 (Pt 8):2023–31.
175. Enright MC, Spratt BG. Extensive variation in the ddl gene of penicillin-resistant Streptococcus pneumoniae results from a hitchhiking effect driven by the penicillin-binding protein 2b gene. Mol Biol Evol. 1999;16(12):1687–95.
176. Stanhope MJ, Lefebure T, Walsh SL, Becker JA, Lang P, Pavinski Bitar PD, et al. Positive selection in penicillin-binding proteins 1a, 2b, and 2x from Streptococcus pneumoniae and its correlation with amoxicillin resistance development. Infect Genet Evol. 2008;8(3):331–9. doi:10.1016/j.meegid.2008.02.001.
177. Chesnel L, Zapun A, Mouz N, Dideberg O, Vernet T. Increase of the deacylation rate of PBP2x from Streptococcus pneumoniae by single point mutations mimicking the class A beta-lactamases. Eur J Biochem. 2002;269(6):1678–83.
178. Di Guilmi AM, Mouz N, Petillot Y, Forest E, Dideberg O, Vernet T. Deacylation kinetics analysis of Streptococcus pneumoniae penicillin-binding protein 2x mutants resistant to beta-lactam antibiotics using electrospray ionization- mass spectrometry. Anal Biochem. 2000;284(2):240–6.
179. Jamin M, Damblon C, Millier S, Hakenbeck R, Frere JM. Penicillin-binding protein 2x of Streptococcus pneumoniae: enzymic activities and interactions with beta-lactams. Biochem J. 1993;292(Pt 3):735–41.
180. Thomas B, Wang Y, Stein RL. Kinetic and mechanistic studies of penicillin-binding protein 2x from Streptococcus pneumoniae. Biochemistry. 2001;40(51):15811–23.
181. Gordon E, Mouz N, Duee E, Dideberg O. The crystal structure of the penicillin-binding protein 2x from Streptococcus pneumoniae and its acyl-enzyme form: implication in drug resistance. J Mol Biol. 2000;299(2):477–85.
182. Dessen A, Mouz N, Gordon E, Hopkins J, Dideberg O. Crystal structure of PBP2x from a highly penicillin-resistant Streptococcus pneumoniae clinical isolate: a mosaic framework containing 83 mutations. J Biol Chem. 2001;276(48):45106–12.
183. Morlot C, Bayle L, Jacq M, Fleurie A, Tourcier G, Galisson F, et al. Interaction of Penicillin-Binding Protein 2x and Ser/Thr protein kinase StkP, two key players in Streptococcus pneumoniae R6 morphogenesis. Mol Microbiol. 2013. doi:10.1111/mmi.12348.
184. Peters K, Schweizer I, Beilharz K, Stahlmann C, Veening JW, Hakenbeck R, et al. Streptococcus pneumoniae PBP2x mid-cell localization requires the C-terminal PASTA domains and is essential for cell shape maintenance. Mol Microbiol. 2014;92(4):733–55. doi:10.1111/mmi.12588.
185. Granger D, Boily-Larouche G, Turgeon P, Weiss K, Roger M. Genetic analysis of pbp2x in clinical Streptococcus pneumoniae isolates in Quebec, Canada. J Antimicrob Chemother. 2005;55(6):832–9. doi:10.1093/jac/dki118.
186. Mouz N, Di Guilmi AM, Gordon E, Hakenbeck R, Dideberg O, Vernet T. Mutations in the active site of penicillin-binding protein PBP2x from Streptococcus pneumoniae. Role in the specificity for beta-lactam antibiotics. J Biol Chem. 1999;274(27): 19175–80.
187. Davies TA, Flamm RK, Lynch AS. Activity of ceftobiprole against Streptococcus pneumoniae isolates exhibiting high-level resistance to ceftriaxone. Int J Antimicrob Agents. 2012;39(6):534–8. doi:10.1016/j.ijantimicag.2012.02.016.
188. Soriano F, Cafini F, Aguilar L, Tarrago D, Alou L, Gimenez MJ, et al. Breakthrough in penicillin resistance? Streptococcus pneumoniae isolates with penicillin/cefotaxime MICs of 16 mg/L and their genotypic and geographical relatedness. J Antimicrob Chemother. 2008;62(6):1234–40. doi:10.1093/jac/dkn392.
189. Carapito R, Chesnel L, Vernet T, Zapun A. Pneumococcal beta-lactam resistance due to a conformational change in penicillin-binding protein 2x. J Biol Chem. 2006;281(3):1771–7.
190. Smith AM, Klugman KP. Amino acid mutations essential to production of an altered PBP 2X conferring high-level beta-lactam resistance in a clinical isolate of Streptococcus pneumoniae. Antimicrob Agents Chemother. 2005;49(11):4622–7.
191. Contreras-Martel C, Dahout-Gonzalez C, Martins Ados S, Kotnik M, Dessen A. PBP active site flexibility as the key mechanism for beta-lactam resistance in pneumococci. J Mol Biol. 2009; 387(4):899–909. doi:10.1016/j.jmb.2009.02.024.
192. Yamane A, Nakano H, Asahi Y, Ubukata K, Konno M. Directly repeated insertion of 9-nucleotide sequence detected in penicillin-binding protein 2B gene of penicillin-resistant Streptococcus pneumoniae. Antimicrob Agents Chemother. 1996; 40(5):1257–9.
193. Cafini F, del Campo R, Alou L, Sevillano D, Morosini MI, Baquero F, et al. Alterations of the penicillin-binding proteins and murM alleles of clinical Streptococcus pneumoniae isolates with high-level resistance to amoxicillin in Spain. J Antimicrob Chemother. 2006;57(2):224–9. doi:10.1093/jac/dki442.
194. Kosowska K, Jacobs MR, Bajaksouzian S, Koeth L, Appelbaum PC. Alterations of penicillin-binding proteins 1A, 2X, and 2B in Streptococcus pneumoniae isolates for which amoxicillin MICs are higher than penicillin MICs. Antimicrob Agents Chemother. 2004;48(10):4020–2.
195. Song JH, Yang JW, Jin JH, Kim SW, Kim CK, Lee H, et al. Molecular characterization of multidrug-resistant Streptococcus pneumoniae isolates in Korea. The Asian Network for Surveillance of Resistant Pathogens (ANSORP) Study Group. J Clin Microbiol. 2000;38(4):1641–4.
196. Kell CM, Jordens JZ, Daniels M, Coffey TJ, Bates J, Paul J, et al. Molecular epidemiology of penicillin-resistant pneumococci isolated in Nairobi, Kenya. Infect Immun. 1993;61(10):4382–91.
197. Contreras-Martel C, Job V, Di Guilmi AM, Vernet T, Dideberg O, Dessen A. Crystal structure of penicillin-binding protein 1a (PBP1a) reveals a mutational hotspot implicated in beta-lactam resistance in Streptococcus pneumoniae. J Mol Biol. 2006; 355(4):684–96.
198. Job V, Carapito R, Vernet T, Dessen A, Zapun A. Common alterations in PBP1a from resistant Streptococcus pneumoniae decrease its reactivity toward beta-lactams: structural insights. J Biol Chem. 2008;283(8):4886–94. doi:10.1074/jbc.M706181200.
199. Di Guilmi AM, Mouz N, Andrieu JP, Hoskins J, Jaskunas SR, Gagnon J, et al. Identification, purification, and characterization of transpeptidase and glycosyltransferase domains of Streptococcus

pneumoniae penicillin-binding protein 1a. J Bacteriol. 1998; 180(21):5652–9.
200. Asahi Y, Ubukata K. Association of a thr-371 substitution in a conserved amino acid motif of penicillin-binding protein 1A with penicillin resistance of Streptococcus pneumoniae. Antimicrob Agents Chemother. 1998;42(9):2267–73.
201. Smith AM, Klugman KP. Site-specific mutagenesis analysis of PBP 1A from a penicillin-cephalosporin-resistant pneumococcal isolate. Antimicrob Agents Chemother. 2003;47(1):387–9.
202. Hakenbeck R, Konig A, Kern I, van der Linden M, Keck W, Billot-Klein D, et al. Acquisition of five high-Mr penicillin-binding protein variants during transfer of high-level beta-lactam resistance from Streptococcus mitis to Streptococcus pneumoniae. J Bacteriol. 1998;180(7):1831–40.
203. Du Plessis M, Smith AM, Klugman KP. Analysis of penicillin-binding protein lb and 2a genes from Streptococcus pneumoniae. Microb Drug Resist. 2000;6(2):127–31.
204. Chesnel L, Carapito R, Croize J, Dideberg O, Vernet T, Zapun A. Identical penicillin-binding domains in penicillin-binding proteins of Streptococcus pneumoniae clinical isolates with different levels of beta-lactam resistance. Antimicrob Agents Chemother. 2005;49(7):2895–902.
205. Smith AM, Feldman C, Massidda O, McCarthy K, Ndiweni D, Klugman KP. Altered PBP 2A and its role in the development of penicillin, cefotaxime, and ceftriaxone resistance in a clinical isolate of Streptococcus pneumoniae. Antimicrob Agents Chemother. 2005;49(5):2002–7.
206. Hoskins J, Matsushima P, Mullen DL, Tang J, Zhao G, Meier TI, et al. Gene disruption studies of penicillin-binding proteins 1a, 1b, and 2a in Streptococcus pneumoniae. J Bacteriol. 1999; 181(20):6552–5.
207. Schuster C, Dobrinski B, Hakenbeck R. Unusual septum formation in Streptococcus pneumoniae mutants with an alteration in the D, D-carboxypeptidase penicillin-binding protein 3. J Bacteriol. 1990;172(11):6499–505.
208. Paik J, Kern I, Lurz R, Hakenbeck R. Mutational analysis of the Streptococcus pneumoniae bimodular class A penicillin-binding proteins. J Bacteriol. 1999;181(12):3852–6.
209. Zhao G, Meier TI, Hoskins J, McAllister KA. Identification and characterization of the penicillin-binding protein 2a of Streptococcus pneumoniae and its possible role in resistance to beta-lactam antibiotics. Antimicrob Agents Chemother. 2000;44 (6):1745–8.
210. Kempf M, Baraduc R, Bonnabau H, Brun M, Chabanon G, Chardon H, et al. Epidemiology and antimicrobial resistance of Streptococcus pneumoniae in France in 2007: data from the pneumococcus surveillance network. Microb Drug Resist. 2011;17(1):31–6. doi:10.1089/mdr.2010.0031.
211. Trzcinski K, Thompson CM, Gilbey AM, Dowson CG, Lipsitch M. Incremental increase in fitness cost with increased beta -lactam resistance in pneumococci evaluated by competition in an infant rat nasal colonization model. J Infect Dis. 2006;193(9):1296–303. doi:10.1086/501367.
212. Albarracin Orio AG, Pinas GE, Cortes PR, Cian MB, Echenique J. Compensatory evolution of pbp mutations restores the fitness cost imposed by beta-lactam resistance in Streptococcus pneumoniae. PLoS Pathog. 2011;7(2):e1002000. doi:10.1371/journal.ppat.1002000.
213. Maurer P, Todorova K, Sauerbier J, Hakenbeck R. Mutations in Streptococcus pneumoniae penicillin-binding protein 2x: importance of the C-terminal penicillin-binding protein and serine/threonine kinase-associated domains for beta-lactam binding. Microb Drug Resist. 2012;18(3):314–21. doi:10.1089/mdr.2012.0022.
214. Yeats C, Finn RD, Bateman A. The PASTA domain: a beta-lactam-binding domain. Trends Biochem Sci. 2002;27(9):438.
215. Garcia-Bustos J, Tomasz A. A biological price of antibiotic resistance: major changes in the peptidoglycan structure of penicillin-resistant pneumococci. Proc Natl Acad Sci U S A. 1990; 87(14):5415–9.
216. Severin A, Figueiredo AM, Tomasz A. Separation of abnormal cell wall composition from penicillin resistance through genetic transformation of Streptococcus pneumoniae. J Bacteriol. 1996;178(7):1788–92.
217. Filipe SR, Tomasz A. Inhibition of the expression of penicillin resistance in Streptococcus pneumoniae by inactivation of cell wall muropeptide branching genes. Proc Natl Acad Sci U S A. 2000;97(9):4891–6.
218. Weber B, Ehlert K, Diehl A, Reichmann P, Labischinski H, Hakenbeck R. The fib locus in Streptococcus pneumoniae is required for peptidoglycan crosslinking and PBP-mediated beta-lactam resistance. FEMS Microbiol Lett. 2000;188(1):81–5.
219. Mascher T, Heintz M, Zahner D, Merai M, Hakenbeck R. The CiaRH system of Streptococcus pneumoniae prevents lysis during stress induced by treatment with cell wall inhibitors and by mutations in pbp2x involved in beta-lactam resistance. J Bacteriol. 2006;188(5):1959–68.
220. Davies TA, Shang W, Bush K. Activities of ceftobiprole and other beta-lactams against Streptococcus pneumoniae clinical isolates from the United States with defined substitutions in penicillin-binding proteins PBP 1a, PBP 2b, and PBP 2x. Antimicrob Agents Chemother. 2006;50(7):2530–2.
221. McGee L, Biek D, Ge Y, Klugman M, du Plessis M, Smith AM, et al. In vitro evaluation of the antimicrobial activity of ceftaroline against cephalosporin-resistant isolates of Streptococcus pneumoniae. Antimicrob Agents Chemother. 2009;53(2):552–6. doi:10.1128/AAC.01324-08.
222. Saravolatz LD, Stein GE, Johnson LB. Ceftaroline: a novel cephalosporin with activity against methicillin-resistant Staphylococcus aureus. Clin Infect Dis. 2011;52(9):1156–63. doi:10.1093/cid/cir147.
223. Zervosen A, Zapun A, Frere JM. Inhibition of Streptococcus pneumoniae penicillin-binding protein 2x and Actinomadura R39 DD-peptidase activities by ceftaroline. Antimicrob Agents Chemother. 2013;57(1):661–3. doi:10.1128/AAC.01593-12.
224. Macheboeuf P, Fischer DS, Brown Jr T, Zervosen A, Luxen A, Joris B, et al. Structural and mechanistic basis of penicillin-binding protein inhibition by lactivicins. Nat Chem Biol. 2007;3(9):565–9. doi:10.1038/nchembio.2007.21.
225. Hermida M, Roy C, Baro MT, Reig R, Tirado M. Characterization of penicillinase-producing strains of Neisseria gonorrhoeae. Eur J Clin Microbiol Infect Dis. 1993;12(1):45–8.
226. Backman A, Orvelid P, Vazquez JA, Skold O, Olcen P. Complete sequence of a beta-lactamase-encoding plasmid in Neisseria meningitidis. Antimicrob Agents Chemother. 2000;44(1):210–2.
227. Dougherty TJ, Koller AE, Tomasz A. Penicillin-binding proteins of penicillin-susceptible and intrinsically resistant Neisseria gonorrhoeae. Antimicrob Agents Chemother. 1980;18(5):730–7.
228. Mendelman PM, Campos J, Chaffin DO, Serfass DA, Smith AL, Saez-Nieto JA. Relative penicillin G resistance in Neisseria meningitidis and reduced affinity of penicillin-binding protein 3. Antimicrob Agents Chemother. 1988;32(5):706–9.
229. Spratt BG. Hybrid penicillin-binding proteins in penicillin-resistant strains of Neisseria gonorrhoeae. Nature. 1988; 332(6160):173–6.
230. Spratt BG, Zhang QY, Jones DM, Hutchison A, Brannigan JA, Dowson CG. Recruitment of a penicillin-binding protein gene from Neisseria flavescens during the emergence of penicillin resistance in Neisseria meningitidis. Proc Natl Acad Sci U S A. 1989;86(22):8988–92.
231. Taha MK, Vazquez JA, Hong E, Bennett DE, Bertrand S, Bukovski S, et al. Target gene sequencing to characterize the penicillin G

susceptibility of Neisseria meningitidis. Antimicrob Agents Chemother. 2007;51(8):2784–92. doi:10.1128/AAC.00412-07.
232. Thulin S, Olcen P, Fredlund H, Unemo M. Total variation in the penA gene of Neisseria meningitidis: correlation between susceptibility to beta-lactam antibiotics and penA gene heterogeneity. Antimicrob Agents Chemother. 2006;50(10):3317–24. doi:10.1128/AAC.00353-06.
233. Allen VG, Farrell DJ, Rebbapragada A, Tan J, Tijet N, Perusini SJ, et al. Molecular analysis of antimicrobial resistance mechanisms in Neisseria gonorrhoeae isolates from Ontario. Canada Antimicrob Agents Chemother. 2011;55(2):703–12. doi:10.1128/AAC.00788-10.
234. Camara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, et al. Molecular characterization of two high-level ceftriaxone-resistant Neisseria gonorrhoeae isolates detected in Catalonia, Spain. J Antimicrob Chemother. 2012;67(8):1858–60. doi:10.1093/jac/dks162.
235. Ito M, Deguchi T, Mizutani KS, Yasuda M, Yokoi S, Ito S, et al. Emergence and spread of Neisseria gonorrhoeae clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. Antimicrob Agents Chemother. 2005;49(1):137–43.
236. Liao M, Gu WM, Yang Y, Dillon JA. Analysis of mutations in multiple loci of Neisseria gonorrhoeae isolates reveals effects of PIB, PBP2 and MtrR on reduced susceptibility to ceftriaxone. J Antimicrob Chemother. 2011;66(5):1016–23. doi:10.1093/jac/dkr021.
237. Lindberg R, Fredlund H, Nicholas R, Unemo M. Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in penA, mtrR, porB1b, and ponA. Antimicrob Agents Chemother. 2007;51(6):2117–22. doi:10.1128/AAC.01604-06.
238. Pandori M, Barry PM, Wu A, Ren A, Whittington WL, Liska S, et al. Mosaic penicillin-binding protein 2 in Neisseria gonorrhoeae isolates collected in 2008 in San Francisco, California. Antimicrob Agents Chemother. 2009;53(9):4032–4. doi:10.1128/AAC.00406-09.
239. Takahata S, Senju N, Osaki Y, Yoshida T, Ida T. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of Neisseria gonorrhoeae. Antimicrob Agents Chemother. 2006;50(11):3638–45. doi:10.1128/AAC.00626-06.
240. Whiley DM, Limnios EA, Ray S, Sloots TP, Tapsall JW. Diversity of penA alterations and subtypes in Neisseria gonorrhoeae strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob Agents Chemother. 2007;51(9):3111–6. doi:10.1128/AAC.00306-07.
241. Heymans R, Bruisten SM, Golparian D, Unemo M, de Vries HJ, van Dam AP. Clonally related Neisseria gonorrhoeae isolates with decreased susceptibility to the extended-spectrum cephalosporin cefotaxime in Amsterdam, the Netherlands. Antimicrob Agents Chemother. 2012;56(3):1516–22. doi:10.1128/AAC.05481-11.
242. Fussenegger M, Rudel T, Barten R, Ryll R, Meyer TF. Transformation competence and type-4 pilus biogenesis in Neisseria gonorrhoeae—a review. Gene. 1997;192(1):125–34.
243. Ohnishi M, Watanabe Y, Ono E, Takahashi C, Oya H, Kuroki T, et al. Spread of a chromosomal cefixime-resistant penA gene among different Neisseria gonorrhoeae lineages. Antimicrob Agents Chemother. 2010;54(3):1060–7. doi:10.1128/AAC.01010-09.
244. Ameyama S, Onodera S, Takahata M, Minami S, Maki N, Endo K, et al. Mosaic-like structure of penicillin-binding protein 2 Gene (penA) in clinical isolates of Neisseria gonorrhoeae with reduced susceptibility to cefixime. Antimicrob Agents Chemother. 2002;46(12):3744–9.
245. Bowler LD, Zhang QY, Riou JY, Spratt BG. Interspecies recombination between the penA genes of Neisseria meningitidis and commensal Neisseria species during the emergence of penicillin resistance in N. meningitidis: natural events and laboratory simulation. J Bacteriol. 1994;176(2):333–7.
246. Spratt BG, Bowler LD, Zhang QY, Zhou J, Smith JM. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal Neisseria species. J Mol Evol. 1992;34(2):115–25.
247. Lee SG, Lee H, Jeong SH, Yong D, Chung GT, Lee YS, et al. Various penA mutations together with mtrR, porB and ponA mutations in Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime or ceftriaxone. J Antimicrob Chemother. 2010;65(4):669–75. doi:10.1093/jac/dkp505.
248. Brannigan JA, Tirodimos IA, Zhang QY, Dowson CG, Spratt BG. Insertion of an extra amino acid is the main cause of the low affinity of penicillin-binding protein 2 in penicillin-resistant strains of Neisseria gonorrhoeae. Mol Microbiol. 1990;4(6):913–9.
249. Powell AJ, Tomberg J, Deacon AM, Nicholas RA, Davies C. Crystal structures of penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of Neisseria gonorrhoeae reveal an unexpectedly subtle mechanism for antibiotic resistance. J Biol Chem. 2009;284(2):1202–12. doi:10.1074/jbc.M805761200.
250. Tomberg J, Temple B, Fedarovich A, Davies C, Nicholas RA. A highly conserved interaction involving the middle residue of the SXN active-site motif is crucial for function of class B penicillin-binding proteins: mutational and computational analysis of PBP 2 from N. gonorrhoeae. Biochemistry. 2012;51(13):2775–84. doi:10.1021/bi2017987.
251. Osaka K, Takakura T, Narukawa K, Takahata M, Endo K, Kiyota H, et al. Analysis of amino acid sequences of penicillin-binding protein 2 in clinical isolates of Neisseria gonorrhoeae with reduced susceptibility to cefixime and ceftriaxone. J Infect Chemother. 2008;14(3):195–203. doi:10.1007/s10156-008-0610-7.
252. Tomberg J, Unemo M, Davies C, Nicholas RA. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in Neisseria gonorrhoeae: role of epistatic mutations. Biochemistry. 2010;49(37):8062–70. doi:10.1021/bi101167x.
253. Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. High-level cefixime- and ceftriaxone-resistant Neisseria gonorrhoeae in France: novel penA mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother. 2012;56(3):1273–80. doi:10.1128/AAC.05760-11.
254. Whiley DM, Goire N, Lambert SB, Ray S, Limnios EA, Nissen MD, et al. Reduced susceptibility to ceftriaxone in Neisseria gonorrhoeae is associated with mutations G542S, P551S and P551L in the gonococcal penicillin-binding protein 2. J Antimicrob Chemother. 2010;65(8):1615–8. doi:10.1093/jac/dkq187.
255. Lujan R, Zhang QY, Saez Nieto JA, Jones DM, Spratt BG. Penicillin-resistant isolates of Neisseria lactamica produce altered forms of penicillin-binding protein 2 that arose by interspecies horizontal gene transfer. Antimicrob Agents Chemother. 1991;35(2):300–4.
256. Saez-Nieto JA, Lujan R, Martinez-Suarez JV, Berron S, Vazquez JA, Vinas M, et al. Neisseria lactamica and Neisseria polysaccharea as possible sources of meningococcal beta-lactam resistance by genetic transformation. Antimicrob Agents Chemother. 1990;34(11):2269–72.
257. Antignac A, Boneca IG, Rousselle JC, Namane A, Carlier JP, Vazquez JA, et al. Correlation between alterations of the penicillin-binding protein 2 and modifications of the peptidoglycan structure in Neisseria meningitidis with reduced susceptibility to penicillin G. J Biol Chem. 2003;278(34):31529–35.
258. Ropp PA, Hu M, Olesky M, Nicholas RA. Mutations in ponA, the gene encoding penicillin-binding protein 1, and a novel locus,

penC, are required for high-level chromosomally mediated penicillin resistance in Neisseria gonorrhoeae. Antimicrob Agents Chemother. 2002;46(3):769–77.
259. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of Neisseria gonorrhoeae to antimicrobial hydrophobic agents is modulated by the mtrRCDE efflux system. Microbiology. 1995;141(Pt 3):611–22.
260. Gill MJ, Simjee S, Al-Hattawi K, Robertson BD, Easmon CS, Ison CA. Gonococcal resistance to beta-lactams and tetracycline involves mutation in loop 3 of the porin encoded at the penB locus. Antimicrob Agents Chemother. 1998;42(11):2799–803.
261. Markowitz SM. Isolation of an ampicillin-resistant, non-beta-lactamase-producing strain of Haemophilus influenzae. Antimicrob Agents Chemother. 1980;17(1):80–3.
262. Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, et al. Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistant Haemophilus influenzae type b in patients with meningitis. Antimicrob Agents Chemother. 2004;48(5):1509–14.
263. Mendelman PM, Chaffin DO, Stull TL, Rubens CE, Mack KD, Smith AL. Characterization of non-beta-lactamase-mediated ampicillin resistance in Haemophilus influenzae. Antimicrob Agents Chemother. 1984;26(2):235–44.
264. Mendelman PM, Chaffin DO, Krilov LR, Kalaitzoglou G, Serfass DA, Onay O, et al. Cefuroxime treatment failure of nontypable Haemophilus influenzae meningitis associated with alteration of penicillin-binding proteins. J Infect Dis. 1990;162(5):1118–23.
265. Mendelman PM, Chaffin DO, Musser JM, De Groot R, Serfass DA, Selander RK. Genetic and phenotypic diversity among ampicillin-resistant, non-beta-lactamase-producing, nontypeable Haemophilus influenzae isolates. Infect Immun. 1987;55(11):2585–9.
266. Clairoux N, Picard M, Brochu A, Rousseau N, Gourde P, Beauchamp D, et al. Molecular basis of the non-beta-lactamase-mediated resistance to beta-lactam antibiotics in strains of Haemophilus influenzae isolated in Canada. Antimicrob Agents Chemother. 1992;36(7):1504–13.
267. Malouin F, Schryvers AB, Bryan LE. Cloning and expression of genes responsible for altered penicillin-binding proteins 3a and 3b in Haemophilus influenzae. Antimicrob Agents Chemother. 1987;31(2):286–91.
268. Parr Jr TR, Bryan LE. Mechanism of resistance of an ampicillin-resistant, beta-lactamase-negative clinical isolate of Haemophilus influenzae type b to beta-lactam antibiotics. Antimicrob Agents Chemother. 1984;25(6):747–53.
269. Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant Haemophilus influenzae. Antimicrob Agents Chemother. 2001;45(6):1693–9.
270. Kaczmarek FS, Gootz TD, Dib-Hajj F, Shang W, Hallowell S, Cronan M. Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant Haemophilus influenzae with unusually high resistance to ampicillin. Antimicrob Agents Chemother. 2004;48(5):1630–9.
271. Barbosa AR, Giufre M, Cerquetti M, Bajanca-Lavado MP. Polymorphism in ftsI gene and {beta}-lactam susceptibility in Portuguese Haemophilus influenzae strains: clonal dissemination of beta-lactamase-positive isolates with decreased susceptibility to amoxicillin/clavulanic acid. J Antimicrob Chemother. 2011;66(4):788–96. doi:10.1093/jac/dkq533.
272. Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S, et al. Diversity of beta-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of Haemophilus influenzae. Antimicrob Agents Chemother. 2002;46(7):2208–18.
273. Garcia-Cobos S, Campos J, Lazaro E, Roman F, Cercenado E, Garcia-Rey C, et al. Ampicillin-resistant non-beta-lactamase-producing Haemophilus influenzae in Spain: recent emergence of clonal isolates with increased resistance to cefotaxime and cefixime. Antimicrob Agents Chemother. 2007;51(7):2564–73. doi:10.1128/AAC.00354-07.
274. Sanbongi Y, Suzuki T, Osaki Y, Senju N, Ida T, Ubukata K. Molecular evolution of beta-lactam-resistant Haemophilus influenzae: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. Antimicrob Agents Chemother. 2006;50(7):2487–92. doi:10.1128/AAC.01316-05.
275. Shuel ML, Tsang RS. Canadian beta-lactamase-negative Haemophilus influenzae isolates showing decreased susceptibility toward ampicillin have significant penicillin binding protein 3 mutations. Diagn Microbiol Infect Dis. 2009;63(4):379–83. doi:10.1016/j.diagmicrobio.2008.12.015.
276. Takahata S, Ida T, Senju N, Sanbongi Y, Miyata A, Maebashi K, et al. Horizontal gene transfer of ftsI, encoding penicillin-binding protein 3, in Haemophilus influenzae. Antimicrob Agents Chemother. 2007;51(5):1589–95. doi:10.1128/AAC.01545-06.
277. Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A. Role of inter-species recombination of the ftsI gene in the dissemination of altered penicillin-binding-protein-3-mediated resistance in Haemophilus influenzae and Haemophilus haemolyticus. J Antimicrob Chemother. 2014;69(6):1501–9. doi:10.1093/jac/dku022.
278. Osaki Y, Sanbongi Y, Ishikawa M, Kataoka H, Suzuki T, Maeda K, et al. Genetic approach to study the relationship between penicillin-binding protein 3 mutations and Haemophilus influenzae beta-lactam resistance by using site-directed mutagenesis and gene recombinants. Antimicrob Agents Chemother. 2005;49(7):2834–9.
279. Kishii K, Chiba N, Morozumi M, Hamano-Hasegawa K, Kurokawa I, Masaki J, et al. Diverse mutations in the ftsI gene in ampicillin-resistant Haemophilus influenzae isolates from pediatric patients with acute otitis media. J Infect Chemother. 2010;16(2):87–93. doi:10.1007/s10156-009-0011-6.
280. Cerquetti M, Giufre M, Cardines R, Mastrantonio P. First characterization of heterogeneous resistance to imipenem in invasive nontypeable Haemophilus influenzae isolates. Antimicrob Agents Chemother. 2007;51(9):3155–61. doi:10.1128/AAC.00335-07.
281. Okabe T, Yamazaki Y, Shiotani M, Suzuki T, Shiohara M, Kasuga E, et al. An amino acid substitution in PBP-3 in Haemophilus influenzae associate with the invasion to bronchial epithelial cells. Microbiol Res. 2010;165(1):11–20. doi:10.1016/j.micres.2008.03.003.
282. Bozdogan B, Tristram S, Appelbaum PC. Combination of altered PBPs and expression of cloned extended-spectrum beta-lactamases confers cefotaxime resistance in Haemophilus influenzae. J Antimicrob Chemother. 2006;57(4):747–9. doi:10.1093/jac/dkl039.
283. Matic V, Bozdogan B, Jacobs MR, Ubukata K, Appelbaum PC. Contribution of beta-lactamase and PBP amino acid substitutions to amoxicillin/clavulanate resistance in beta-lactamase-positive, amoxicillin/clavulanate-resistant Haemophilus influenzae. J Antimicrob Chemother. 2003;52(6):1018–21.
284. San Millan A, Giufre M, Escudero JA, Hidalgo L, Gutierrez B, Cerquetti M, et al. Contribution of ROB-1 and PBP3 mutations to the resistance phenotype of a beta-lactamase-positive amoxicillin/clavulanic acid-resistant Haemophilus influenzae carrying plasmid pB1000 in Italy. J Antimicrob Chemother. 2011;66(1):96–9. doi:10.1093/jac/dkq392.
285. Tristram SG, Littlejohn R, Bradbury RS. blaROB-1 presence on pB1000 in Haemophilus influenzae is widespread, and variable cefaclor resistance is associated with altered penicillin-binding proteins. Antimicrob Agents Chemother. 2010;54(11):4945–7. doi:10.1128/AAC.00263-10.

286. Garcia-Cobos S, Arroyo M, Campos J, Perez-Vazquez M, Aracil B, Cercenado E, et al. Novel mechanisms of resistance to beta-lactam antibiotics in Haemophilus parainfluenzae: beta-lactamase-negative ampicillin resistance and inhibitor-resistant TEM beta-lactamases. J Antimicrob Chemother. 2013;68(5):1054–9. doi:10.1093/jac/dks525.
287. Krishnamurthy P, Parlow MH, Schneider J, Burroughs S, Wickland C, Vakil NB, et al. Identification of a novel penicillin-binding protein from Helicobacter pylori. J Bacteriol. 1999;181(16):5107–10.
288. Dore MP, Osato MS, Realdi G, Mura I, Graham DY, Sepulveda AR. Amoxycillin tolerance in Helicobacter pylori. J Antimicrob Chemother. 1999;43(1):47–54.
289. Dore MP, Graham DY, Sepulveda AR. Different penicillin-binding protein profiles in amoxicillin-resistant Helicobacter pylori. Helicobacter. 1999;4(3):154–61.
290. Gerrits MM, Schuijffel D, van Zwet AA, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG. Alterations in penicillin-binding protein 1A confer resistance to beta-lactam antibiotics in Helicobacter pylori. Antimicrob Agents Chemother. 2002;46(7):2229–33.
291. Han SR, Bhakdi S, Maeurer MJ, Schneider T, Gehring S. Stable and unstable amoxicillin resistance in Helicobacter pylori: should antibiotic resistance testing be performed prior to eradication therapy? J Clin Microbiol. 1999;37(8):2740–1.
292. Okamoto T, Yoshiyama H, Nakazawa T, Park ID, Chang MW, Yanai H, et al. A change in PBP1 is involved in amoxicillin resistance of clinical isolates of Helicobacter pylori. J Antimicrob Chemother. 2002;50(6):849–56.
293. van Zwet AA, Vandenbroucke-Grauls CM, Thijs JC, van der Wouden EJ, Gerrits MM, Kusters JG, et al. Stable amoxicillin resistance in Helicobacter pylori. Lancet. 1998;352(9140):1595.
294. Kwon DH, Dore MP, Kim JJ, Kato M, Lee M, Wu JY, et al. High-level beta-lactam resistance associated with acquired multidrug resistance in Helicobacter pylori. Antimicrob Agents Chemother. 2003;47(7):2169–78.
295. Matteo MJ, Granados G, Olmos M, Wonaga A, Catalano M. Helicobacter pylori amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates. J Antimicrob Chemother. 2008;61(3):474–7. doi:10.1093/jac/dkm504.
296. Kim BJ, Kim JG. Substitutions in penicillin-binding protein 1 in amoxicillin-resistant Helicobacter pylori strains isolated from Korean patients. Gut Liver. 2013;7(6):655–60. doi:10.5009/gnl.2013.7.6.655.
297. Qureshi NN, Morikis D, Schiller NL. Contribution of specific amino acid changes in penicillin binding protein 1 to amoxicillin resistance in clinical Helicobacter pylori isolates. Antimicrob Agents Chemother. 2011;55(1):101–9. doi:10.1128/AAC.00545-10.
298. Co EM, Schiller NL. Resistance mechanisms in an in vitro-selected amoxicillin-resistant strain of Helicobacter pylori. Antimicrob Agents Chemother. 2006;50(12):4174–6. doi:10.1128/AAC.00759-06.
299. DeLoney CR, Schiller NL. Characterization of an In vitro-selected amoxicillin-resistant strain of Helicobacter pylori. Antimicrob Agents Chemother. 2000;44(12):3368–73.
300. Paul R, Postius S, Melchers K, Schafer KP. Mutations of the Helicobacter pylori genes rdxA and pbp1 cause resistance against metronidazole and amoxicillin. Antimicrob Agents Chemother. 2001;45(3):962–5.
301. Rimbara E, Noguchi N, Kawai T, Sasatsu M. Mutations in penicillin-binding proteins 1, 2 and 3 are responsible for amoxicillin resistance in Helicobacter pylori. J Antimicrob Chemother. 2008;61(5):995–8. doi:10.1093/jac/dkn051.
302. Spratt BG. Escherichia coli resistance to beta-lactam antibiotics through a decrease in the affinity of a target for lethality. Nature. 1978;274(5672):713–5.
303. Hedge PJ, Spratt BG. Amino acid substitutions that reduce the affinity of penicillin-binding protein 3 of Escherichia coli for cephalexin. Eur J Biochem. 1985;151(1):111–21.
304. Hedge PJ, Spratt BG. Resistance to beta-lactam antibiotics by remodelling the active site of an E. coli penicillin-binding protein. Nature. 1985;318(6045):478–80.
305. Neuwirth C, Siebor E, Duez JM, Pechinot A, Kazmierczak A. Imipenem resistance in clinical isolates of Proteus mirabilis associated with alterations in penicillin-binding proteins. J Antimicrob Chemother. 1995;36(2):335–42.
306. Bellido F, Veuthey C, Blaser J, Bauernfeind A, Pechere JC. Novel resistance to imipenem associated with an altered PBP-4 in a Pseudomonas aeruginosa clinical isolate. J Antimicrob Chemother. 1990;25(1):57–68.
307. Gotoh N, Nunomura K, Nishino T. Resistance of Pseudomonas aeruginosa to cefsulodin: modification of penicillin-binding protein 3 and mapping of its chromosomal gene. J Antimicrob Chemother. 1990;25(4):513–23.
308. Bellido F, Vladoianu IR, Auckenthaler R, Suter S, Wacker P, Then RL, et al. Permeability and penicillin-binding protein alterations in Salmonella muenchen: stepwise resistance acquired during beta-lactam therapy. Antimicrob Agents Chemother. 1989;33(7):1113–5.
309. Obara M, Nakae T. Mechanisms of resistance to beta-lactam antibiotics in Acinetobacter calcoaceticus. J Antimicrob Chemother. 1991;28(6):791–800.
310. Gehrlein M, Leying H, Cullmann W, Wendt S, Opferkuch W. Imipenem resistance in Acinetobacter baumanii is due to altered penicillin-binding proteins. Chemotherapy. 1991;37(6):405–12.
311. Vashist J, Tiwari V, Das R, Kapil A, Rajeswari MR. Analysis of penicillin-binding proteins (PBPs) in carbapenem resistant Acinetobacter baumannii. Indian J Med Res. 2011;133:332–8.
312. Cayo R, Rodriguez MC, Espinal P, Fernandez-Cuenca F, Ocampo-Sosa AA, Pascual A, et al. Analysis of genes encoding penicillin-binding proteins in clinical isolates of Acinetobacter baumannii. Antimicrob Agents Chemother. 2011;55(12):5907–13. doi:10.1128/AAC.00459-11.
313. Gutkind GO, Ogueta SB, de Urtiaga AC, Mollerach ME, de Torres RA. Participation of PBP 3 in the acquisition of dicloxacillin resistance in Listeria monocytogenes. J Antimicrob Chemother. 1990;25(5):751–8.
314. Pierre J, Boisivon A, Gutmann L. Alteration of PBP 3 entails resistance to imipenem in Listeria monocytogenes. Antimicrob Agents Chemother. 1990;34(9):1695–8.
315. Wexler HM, Halebian S. Alterations to the penicillin-binding proteins in the Bacteroides fragilis group: a mechanism for non-beta-lactamase mediated cefoxitin resistance. J Antimicrob Chemother. 1990;26(1):7–20.
316. Nordmann P, Nicolas MH, Gutmann L. Penicillin-binding proteins of Rhodococcus equi: potential role in resistance to imipenem. Antimicrob Agents Chemother. 1993;37(7):1406–9.
317. Paradkar AS, Aidoo KA, Wong A, Jensen SE. Molecular analysis of a beta-lactam resistance gene encoded within the cephamycin gene cluster of Streptomyces clavuligerus. J Bacteriol. 1996;178(21):6266–74.
318. Coque JJ, Liras P, Martin JF. Genes for a beta-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in Nocardia lactamdurans. Embo J. 1993;12(2):631–9.
319. Zervosen A, Lu WP, Chen Z, White RE, Demuth Jr TP, Frere JM. Interactions between penicillin-binding proteins (PBPs) and two novel classes of PBP inhibitors, arylalkylidene rhodanines and arylalkylidene iminothiazolidin-4-ones. Antimicrob Agents Chemother. 2004;48(3):961–9.

Aminoglycosides: Mechanisms of Action and Resistance

14

Alisa W. Serio, Maria L. Magalhães, John S. Blanchard, and Lynn E. Connolly

1 Introduction

Aminoglycosides have been an important part of the antimicrobial armamentarium since their introduction into clinical use in the 1940s. The spectrum of activity, rapid bactericidal activity, and favorable chemical and pharmacokinetic properties of aminoglycosides make them a clinically useful class of drugs. Although the introduction of efficacious and less toxic agents such as the broad-spectrum β-lactam antimicrobials led to a shift away from the use of aminoglycosides, the recent emergence of multi- and extensively drug-resistant Gram-negative pathogens has led to renewed interest in the aminoglycoside class, including the development of new molecules with potent activity against otherwise highly resistant pathogens.

Aminoglycosides bind to the bacterial ribosome, leading to inhibition of protein synthesis by promoting mistranslation and elimination of proofreading. Resistance to aminoglycosides results from mutations or enzymatic alteration of the target in the ribosome, efflux of the drug out of the cell, or enzymatic modification of the drug—all of which lead, indirectly or directly, to reduced binding to the ribosome. Although multiple resistance mechanisms to this important class of drugs have emerged over decades of widespread use, resistance rates have not increased at the rapid pace observed for other antibiotic classes.

Aminoglycosides continue to be used as both empirical and definitive therapy for a broad range of indications [1, 2]. Due to their rapidly bactericidal activity and relatively low rates of resistance compared to other antibiotic classes, they are often combined with a second agent for the empirical treatment of severe sepsis and certain nosocomial infections in patients with a high risk of mortality or when there is concern that the causative pathogen may be resistant to more commonly used agents. Aminoglycosides remain the preferred therapy for certain zoonotic infections such as plague and tularemia and are an important component of combination therapy for many mycobacterial pathogens. Inhaled tobramycin is associated with statistically significant improvement in lung function and reduced density of *Pseudomonas aeruginosa* in cystic fibrosis patients and has now become a standard therapy for these patients. Due to their poor oral bioavailability, aminoglycosides are often a key element of oral gut decolonization/decontamination regimens, including those targeting multidrug-resistant (MDR) pathogens [3, 4]. The ability of paromomycin to bind to eukaryotic ribosomes has led to the use of this agent in the treatment of protozoal infections, most notably cutaneous and visceral leishmaniasis [5, 6].

Our understanding of both the safety and efficacy of aminoglycosides has advanced considerably over the past few decades. Clinical studies have demonstrated low rates of nephrotoxicity with once-daily dosing [7], consistent with pharmacodynamic principles suggesting that less frequent but higher doses reduce the risk of toxicity while maintaining and possibly improving efficacy [8]. These advantages of once-daily dosing of aminoglycosides are now widely accepted and, for many infection types, once-daily dosing has become the standard of care [1].

A.W. Serio, Ph.D.
Achaogen, Inc., 7000 Shoreline Court, Suite 371, South San Francisco, CA 94080, USA

M.L. Magalhães
Department of Food and Animal Production, State University of Santa Catarina, Lages, SC 88520-000, Brazil

J.S. Blanchard, Ph.D.
Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

L.E. Connolly, M.D., Ph.D. (✉)
Achaogen, Inc., 7000 Shoreline Court, Suite 371, South San Francisco, CA 94080, USA

Department of Medicine, Division of Infectious Diseases, University of California, San Francisco, CA 94143, USA
e-mail: lconnolly@achaogen.com

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_14

In this chapter we describe mechanisms of resistance to the aminoglycosides and also discuss new members of this class that have the potential to overcome the predominant resistance mechanisms currently encountered in key human pathogens.

2 Antimicrobial Mechanism of Action

Aminoglycosides are bactericidal antibiotics originally isolated from Actinomycetes species including *Streptomyces* and *Micromonospora* [9, 10]. The general mechanism of action of aminoglycosides is inhibition of protein synthesis by promotion of mistranslation and elimination of proofreading [11]. Aminoglycosides are pseudo-polysaccharides containing amino sugars and are polycationic. Therefore, at physiological pH values, aminoglycosides are highly positively charged and have a high binding affinity for nucleic acids, especially for certain portions of the prokaryotic ribosomal RNA (rRNA). As discussed below, different classes of aminoglycosides bind to different sites on rRNA.

Most of the commonly used aminoglycosides consist of an aminocyclitol 2-deoxystreptamine (DOS) ring linked to amino sugars saturated with amino and hydroxyl substitutions (Fig. 14.1). Aminoglycosides are classified based on their structure into four classes: (1) no deoxystreptamine (DOS) ring (e.g., streptomycin, which has a streptidine ring), (2) mono-substituted DOS (e.g., apramycin), (3) 4,5-disubstituted DOS (e.g., neomycin, ribostamycin), and (4) 4,6-disubstituted DOS (e.g., gentamicin, amikacin, tobramycin) [10, 12]. The structure, along with the various amino and hydroxyl substitutions have a direct impact on the type of bacterial resistance mechanisms that can block or inactivate a given aminoglycoside (Sect. 3.4).

Aminoglycoside uptake by bacterial cells occurs in three phases and the process itself is thought to play a role in the bactericidal activity of this class of antibiotics [11, 13]. The initial step involves electrostatic interactions between the polycationic antibiotic and the negatively charged components of the Gram-negative outer membrane, including lipopolysaccharide (LPS) [11, 14]. The polycationic aminoglycoside competitively displaces essential divalent cations (magnesium) that cross-bridge and stabilize adjacent LPS molecules. Disruption of the outer membrane by this mechanism has been proposed to enhance permeability and initiate aminoglycoside uptake [11, 14–16]. Aminoglycoside transport across the cytoplasmic membrane involves an initial lag phase followed by a second phase in which the drug is rapidly taken up. Transport across the cytoplasmic membrane requires energy from the electron transport system in an oxygen-dependent process [13, 17–19]. Thus, the intrinsic resistance of anaerobic bacteria to aminoglycosides can be explained by the failure to transport the drug inside the cell. Once inside the cell, the drug binds to the 30S ribosomal subunit at the *a*minoacyl-tRNA (aa-tRNA) acceptor site (*A*) on the 16S ribosomal RNA (rRNA), affecting protein synthesis by induction of codon misreading and inhibition of translocation [20, 21].

Some aminoglycosides, like spectinomycin and kasugamycin, were found to have no effect on chain elongation (codon misreading) but block initiating ribosomes completely. Streptomycin and other aminoglycosides similarly block the initiation complex but act later, decreasing the accuracy of translation [22].

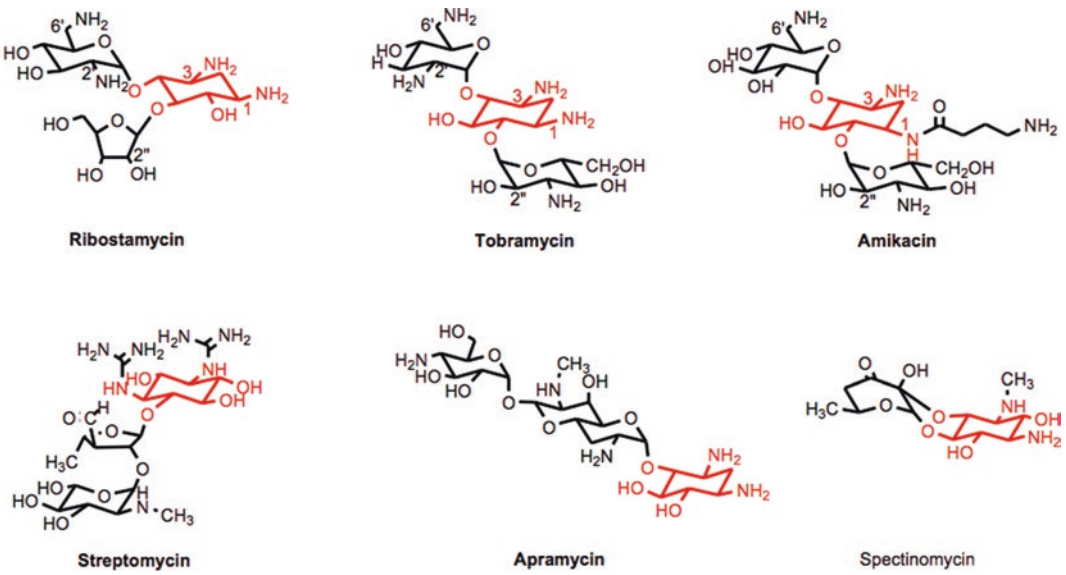


Fig. 14.1 Chemical structures of diverse aminoglycosides illustrating the common aminocyclitol 2-deoxystreptamine (DOS) ring in *red*

It is believed that fidelity of translation depends on two steps—initial recognition between the codon on the mRNA and the anticodon of the charged aa-tRNA and subsequent proofreading. During the initial selection, the cognate codon is recognized, inducing GTP hydrolysis and the release of elongation factors from aa-tRNA [23]. The aminoacyl end of aa-tRNA is free to move into the peptidyl transferase center on the 50S subunit, where peptide bond formation occurs [24]. A similar sequence of events happens when a non-cognate codon is recognized. However, in such cases, following GTP hydrolysis and release of additional factors, non-cognate aa-tRNAs dissociate from the ribosome rather than enter the peptidyl transferase center, due to the lower stability of the codon–anticodon complex [23].

Although the precise mechanism of aminoglycoside-induced miscoding is not completely understood, it has been shown that aminoglycosides enhance the binding stability of cognate aa-tRNAs to the small ribosomal subunit [25]. It has been proposed that such stability enhancement would allow non-cognate tRNAs to enter the peptidyl transferase site, being incorporated into the nascent polypeptide chain.

High-resolution crystal structures of the 30S ribosomal subunit [26, 27] as well as nuclear magnetic resonance (NMR)-derived structures [28] of ribosomal constituents bound to aminoglycoside molecules have provided valuable information about the molecular mechanisms of aminoglycoside binding and action. The NMR structure of the complex between an A site-mimicking RNA molecule and the aminoglycoside paromomycin revealed that the antibiotic binds in the major groove of the A-site in an L-shaped conformation [28]. Critical nucleotides for binding include A^{1492} and U^{1495} as well as the C^{1407}–G^{1494} and A^{1408}–A^{1493} base pairs. The 2-deoxystreptamine and 2,6-dideoxy-2,6-diamino-glucose rings contribute the most important intermolecular contacts. The N1 and N3 amino groups of the central deoxystreptamine ring, found in all typical aminoglycosides, are required for specific binding to the 16S rRNA.

A high-resolution crystal structure of the 30S subunit from *Thermus thermophilus* in complex with different antibiotics was reported in 2000, providing important insights into the molecular mechanisms of translation as well as the mode of action of aminoglycosides [26]. In this work, Carter et al. proposed a model to address how typical aminoglycoside molecules increase the affinity of the aa-tRNA for the A-site. During translation, the selection of aa-tRNA occurs by formation of a mini-helix between the codon of mRNA and the anticodon of the cognate tRNA. They propose that when this tRNA–mRNA complex is formed, two adenines (A^{1492} and A^{1493}) from 16S rRNA flip out from their intrahelical positions and form a hydrogen bonding network with the 2′-OH groups on both sides of the codon–anticodon helix. The two adenines would sense the width of the minor groove, allowing for discrimination of distortions arising from mispairing. In the absence of any aminoglycoside molecule, some energy would be required to flip out these two adenine bases but presumably this energetic cost would be compensated by formation of favorable interactions with the cognate aa-tRNA. By binding to the A site, the aminoglycoside stabilizes the flipped out structure, thus reducing the energetic cost of both cognate and non-cognate aa-tRNA binding and increasing aa-tRNA affinity for the A-site [26, 29]. Therefore, typical aminoglycosides like paromomycin induce miscoding by mimicking the conformational change in the 16S rRNA induced by a correct codon–anticodon pair.

Indeed, it has been reported that aminoglycosides stabilize aa-tRNA binding about six-fold [25]. In contrast, the rate of aminoglycoside-induced misreading ranges from 20- to 200-fold [30, 31] (depending on the codon and the antibiotic), suggesting the existence of additional mechanisms by which binding of aminoglycosides induces codon misreading.

The structure of the atypical aminoglycoside streptomycin bound to the 30S subunit has also been reported [26]. The data reveal that the drug makes interactions with residues from four different domains of the 16S rRNA, including U^{14} in helix 1, C^{526} and G^{527} from helix 18, A^{913} and A^{914} from helix 27 and 28, respectively, and C^{1490} and G^{1491} from helix 44. It also makes contacts with K45 from protein S12. This data offers a structural rationale for the observed properties of streptomycin. It had previously been reported that there are two alternative base pairing schemes in *Escherichia coli* rRNA during translation [32]: one which leads to a ribosomal ambiguity (*ram*) conformation, with high affinity for tRNA, which results in increased miscoding, and a second that leads to a restrictive state with low tRNA affinity. The balance of these two states could be involved in the proofreading process [32, 33]. The structural data from the streptomycin complex indicate that this aminoglycoside preferentially stabilizes the *ram* state [26], providing an explanation for the error-prone translation induced by this drug. By stabilizing the *ram* state, streptomycin would increase initial binding of non-cognate tRNAs as well as make the transition to the restrictive state more difficult, thereby affecting the proposed balance of such states and hence, proofreading.

Although the mechanism of action of aminoglycosides at the translational level has been extensively clarified by the data above, the connection between protein misreading and bactericidal activity remains unclear. In addition to codon misreading, early studies on streptomycin have revealed an additional effect: membrane damage. Several studies showed that the treatment of *E. coli* cells with streptomycin led to the loss of intracellular nucleotides [34], amino acids [35], and potassium [36]. Later studies [22, 37, 38] have proposed that misreading would play an indirect, but essential and determinant role in the bactericidal action of aminoglycosides. The

following model has been proposed: (1) small amounts of the antibiotic penetrate the cell by a mechanism that is not completely understood and bind to the A site in ribosomes that are actively elongating proteins, causing a small degree of misreading; (2) the misread proteins are misfolded and are incorporated into the membrane where they create channels that permit a larger influx of antibiotic; (3) the intracellular antibiotic concentration rises and the drug is trapped inside the cell [39], resulting in the complete inhibition of protein synthesis, which causes bacterial death.

The long-held hypothesis that aminoglycoside-induced cell death is a direct result of inhibition and/or modulation of ribosome function has recently been challenged. An alternative hypothesis was proposed based on the observation that exposure of bacterial cells to aminoglycosides but not bacteriostatic inhibitors of the ribosome such as chloramphenicol, tetracycline, or erythromycin induced the formation of reactive oxygen species (ROS) and hydroxyl radicals [40]. Other bactericidal drugs with different primary cellular targets (β-lactams and quinolones) were also able to induce hydroxyl radical formation, suggesting a common mechanism of killing for bactericidal agents via the simulation of the production of lethal doses of hydroxyl radicals. This hypothesis, in turn, has been questioned by the finding that there was no difference in bacterial killing by various agents, including high dose kanamycin, under aerobic conditions and anaerobic/anoxic conditions, where ROS are not formed [41, 42]. Further, in these experiments, treatment of *E. coli* with bactericidal antibiotics, including kanamycin, did not accelerate the formation of ROS as determined by both indirect and direct measurements of hydrogen peroxide levels [42]. However, the efficacy of antibiotics under anaerobic conditions can be enhanced by exposure to molecular oxygen or alternative electron acceptors [43]. Furthermore, overexpression of proteins, such as catalase, which protect the cell from oxidative damage and the preatment of cells with antioxidants diminishes antibiotic lethality [43]. Although differences in experimental technique may account, in part, for the discrepant results across these studies [44], these latter findings suggest that ROS generated as a downstream consequence of an antibiotic inhibition or modulation of a cellular target may contribute to bacterial killing under some circumstances.

The only difference in sequence of the 16S rRNA between prokaryotes and eukaryotes is at position 1408, which is an adenosine in all prokaryotic and eukaryotic mitochondrial sequences and a guanosine in cytoplasmatic eukaryotic sequences. The A^{1408}–A^{1493} base pair in the bacterial ribosome creates a binding pocket for the primed ring that does not occur in the eukaryotic structure, explaining the relative specificity of aminoglycosides for the bacterial target [28, 45].

3 Mechanisms of Aminoglycoside Resistance

3.1 Ribosomal Mutations

Modification of the primary bacterial target via mutation is a common mechanism of resistance for most antibiotics. However, clinical aminoglycoside resistance is generally not manifested by mutations in genes encoding the structural components of the ribosome. Most species of bacteria have multiple copies of the genes encoding rRNA, and thus every copy of these genes would have to be mutated to enable resistance. The probability of such an occurrence is virtually nonexistent. However, *Mycobacterium* and *Borrelia* are genera that contain either a single copy of the 16S rRNA or a single copy of the entire ribosomal operon [46, 47]. Accordingly, clinical resistance due to ribosomal mutations has relevance in these bacterial genera.

In *Mycobacterium tuberculosis*, high-level resistance to streptomycin can result from mutations in the genes encoding two components of the ribosome, the 16S rRNA [48–50] and the S12 protein [48, 51]. The most frequently occurring mutations are point mutations in the *rpsL* gene encoding the ribosomal S12 protein. Mapping of these mutations revealed that all occurred in highly conserved regions of the gene encoding one of the two critical lysine residues (K43 and K88) [48, 52, 53]. Although structural studies have revealed that streptomycin makes direct contacts with S12 [26], mutations in this protein appear to affect streptomycin binding by perturbing the overall structure of 16S rRNA [54]. An A to G change at nucleotide 1408 in the 16S rRNA has also been associated with high-level resistance to amikacin and other 2-deoxystreptamine aminoglycosides in *M. abscessus* and *M. chelonae* clinical isolates [55].

Interestingly, certain mutations in genes encoding structural components of the ribosome can lead to streptomycin dependence in *M. tuberculosis*. Mutations in *rpsL* are associated with this phenotype and also exhibit extremely low translational error rates [53, 56]. In addition, certain mutations in the conserved 530 stem-loop of 16S rRNA, typically responsible for streptomycin resistance or susceptibility phenotypes, can also result in a streptomycin dependence phenotype [49].

High-level resistance to spectinomycin and other aminoglycosides resulting from mutation in the 16S rRNA and the ribosomal S12 protein has also been reported in vitro in *Borrelia burgdorferi* isolates [57]. Aminoglycosides are not commonly used to treat infections due to this pathogen and the clinical implications of these mechanisms of resistance are unknown.

3.2 16S rRNA Methylation

Aminoglycoside-producing organisms possess a number of mechanisms to defend themselves against the antibiotics that they produce and are thus intrinsically resistant to aminoglycosides. These resistance mechanisms include target modification and enzymatic inactivation of the drug. Target modification in these organisms occurs via 16S rRNA methyltransferases, which confer aminoglycoside resistance by modification of key nucleotide residues, preventing binding of the aminoglycoside to its target in the 16S rRNA [12, 58, 59].

A number of genes encoding S-adenosylmethionine (SAM)-dependent 16S rRNA methylases have been identified from several aminoglycoside-producing Actinomycetes, including *Streptomyces* and *Micromonospora* spp. [60–65]. The 16S rRNA methyltransferases can be separated into two groups based on the site of modification: (1) methylation of the N7 position of nucleotide G^{1405}, which results in resistance to 4,6-disubstituted DOS aminoglycosides, including gentamicin and amikacin [58, 65], and (2) methylation of the N1 position of nucleotide A^{1408}, which results in resistance not only to 4,6-disubstituted DOS aminoglycosides but also to 4,5-disubstituted and monosubstituted DOS aminoglycosides, including neomycin and apramycin [58, 64, 66]. Methylation of these nucleotides abolishes important intermolecular contacts between rRNA and the aminoglycoside molecule.

Until fairly recently, ribosomal protection by methylation of 16S rRNA had been restricted to the chromosomes of aminoglycoside-producing Actinomycetes. However, an aminoglycoside-resistant *P. aeruginosa* clinical isolate from Japan containing a plasmid-encoded 16S rRNA methyltransferase, coined RmtA for *r*ibosomal *m*ethyltransferase *A* was described in 2003 [67]. Subsequently, several additional plasmid-encoded 16S rRNA methylases, encoded by the genes *armA*, *rmtB1*, *rmtB2*, *rmtC*, *rmtD*, *rmtD2*, *rmtE*, *rmtF*, *rmtG*, and *rmtH*, have emerged in clinical isolates that exhibit high-level resistance to multiple aminoglycosides [67–71]. These enzymes modify the G^{1405} nucleotide and thus impact the activity of all 4,6-disubstituted aminoglycosides (i.e., amikacin, gentamicin, and tobramycin). In 2007, the enzyme NpmA was discovered encoded on a plasmid in an aminoglycoside-resistant *E. coli* clinical isolate, also from Japan [72]. This methyltransferase modifies the A^{1408} nucleotide and thus impacts 4,6- and 4-5-disubstituted as well as monosubstituted aminoglycosides, conferring pan-aminoglycoside resistance. This type of methyltransferase appears to be rare at this time, as NpmA remains the only report of this type of enzyme occurring in a clinical isolate [72].

3.3 Efflux-Mediated Resistance

Bacteria are capable of extruding antibiotics and other structurally unrelated molecules via efflux systems, and thus have the ability to reduce the intracellular accumulation of an antibiotic necessary for target inhibition. As a result, these systems often play a key role in conferring multidrug resistance in a number of diverse pathogens. The resistance-nodulation-division (RND) family of efflux systems plays the most prominent role in this type of antibiotic resistance in Gram-negative bacteria [73].

RND transporters are localized in the cytoplasmic membrane of Gram-negative bacteria and use membrane proton motive force as an energy source. A membrane fusion protein (MFP) localized in the periplasmic space connects the RND transporter to the outer membrane pore (OMP) forming a continuous tripartite channel able to export substrates efficiently out of the cell [74, 75].

Several RND systems have been shown to be involved in intrinsic aminoglycoside resistance in various pathogens [76–81]. Intrinsic resistance is characterized by the constitutive expression of efflux pumps causing a natural low-level resistance to various antibiotics [73, 82]. Further, mutations in the regulatory genes of the pumps, or induction of expression in the presence of substrate, can lead to overexpression of the pump itself and high-level antibiotic resistance [10, 82].

In the opportunistic pathogen *P. aeruginosa*, intrinsic low-level resistance to aminoglycosides, tetracycline, and erythromycin is mediated by the expression of the Mex (*m*ultiple *e*fflux) XY-OprM system. This efflux system plays an important role in the intrinsic aminoglycoside resistance observed in *P. aeruginosa* strains associated with chronic lung infections in cystic fibrosis (CF) patients [83]. The system is composed of a transmembrane protein (MexY), an outer membrane channel (OprM), and a periplasmic membrane fusion lipoprotein (MexX) [76, 77]. Auxiliary OprM-like proteins such as OpmG, OpmH, and OpmI may also interact with MexX and MexY to form a tripartite functional pump [84].

MexXY orthologs are found in other species of bacteria. For example, *Burkholderia cenopacia* complex (BCC) species are opportunistic pathogens that also cause chronic lung infections in CF patients and are often intrinsically resistant to antibiotics, including aminoglycosides, due to RND efflux pumps [85]. Some *Burkholderia* species can acquire aminoglycoside resistance due to changes in pump expression. Acquired aminoglycoside resistance in *B. vietnameinsis*, a BCC species that is atypical in that it is initially susceptible to aminoglycosides, was found to be due to mutations that result in overexpression of the AmrAB-OprM efflux system [86].

The AmrAB-OprA efflux system of *Burkholderia pseudomallei*, the causative agent of meliodosis, is orthologous to the *P. aeruginosa* MexXY system and results in intrinsic aminoglycoside and macrolide resistance in this pathogen [87].

The *E. coli* genome contains several genes that encode RND transporters. The AcrD transporter was first identified based on amino acid sequence similarity with MexY. Further investigation demonstrated that AcrD participates in efflux of aminoglycosides after a mutant with a disrupted *acrD* gene was shown to be hypersusceptible to the class [80]. Interestingly, a later study demonstrated that AcrD not only captures aminoglycoside molecules from the cytoplasm, but also from the periplasmic space, followed by the active efflux of the drug out of the cell [88].

RND efflux systems also play a major role in multidrug resistance in the opportunistic pathogen *Acinetobacter baumannii*. Overexpression of the Ade (*A*cinetobacter *d*rug *e*fflux) ABC efflux system has been demonstrated to confer aminoglycoside resistance in clinical isolates [79]. This system extrudes not only aminoglycosides, but also a number of antibiotics in diverse classes including β-lactams, fluoroquinolones, tetracyclines, macrolides, chloramphenicol, and trimethoprim [89]. The AdeDE system was also demonstrated to efflux aminoglycosides and other classes of antibiotics [90]. There were no outer membrane protein genes found associated with the *adeDE* gene cluster, suggesting that an unknown outer membrane protein is recruited to complete formation of the pump [89, 90].

In Mycobacteria, the majority of drug efflux pumps identified thus far belong to the major facilitator superfamily (MFS). Genome sequence analysis revealed 16 open reading frames encoding putative drug efflux pumps belonging to the MFS class in *M. tuberculosis* [91]. These efflux pumps could account for streptomycin-resistant clinical isolates of *M. tuberculosis* that cannot be assigned to any other mechanism to date. Expressson of the *tap* gene, encoding a putative MFS family pump, from *M. fortuitum* or *M. tuberculosis* in the nonpathogenic *M. smegmatis* conferred low-level aminoglycoside and tetracycline resistance [92]. The gene encoding the P55 pump from *M. bovis* similarly conferred aminoglycoside and tetracycline resistance when expressed in *M. smegatis* [93]. These data suggest that MFS family pumps may contribute to aminoglycoside resistance in Mycobacteria.

3.4 Enzymatic Drug Modification

The most common mechanism of aminoglycoside resistance encountered clinically is direct modification of the aminoglycoside molecule via aminoglycoside-modifying enzymes (AMEs). These intracellular bacterial enzymes catalyze the covalent modification of specific amino or hydroxyl groups of the aminoglycoside molecule. The chemically modified drug exhibits diminished binding to the A site of bacterial 16S rRNA, resulting in loss of antibacterial activity and a resistant phenotype in organisms that harbor these enzymes [94]. Structural studies of aminoglycosides complexed to the 16S rRNA have highlighted the importance of several amino and hydroxyl groups for the proper binding of aminoglycoside molecules [26, 28]. The N1 and N3 amino groups of the deoxystreptamine ring hydrogen bond to nucleotides U^{1495} and G^{1494} while the 3′ and 4′-hydroxyl groups of the primed ring contact the A^{1493} and A^{1492} phosphates, respectively. Additionally, the 2′-amino position forms an internal hydrogen bond with the doubly primed ring, which is important for correct positioning of the primed ring, and the amino and hydroxyl groups of the triply primed ring make electrostatic interactions with the phosphate backbone of several rRNA residues. Therefore, it is clear that modifications of these conserved or semi-conserved positions would lead to deleterious effects on aminoglycoside-binding properties and thus the antibacterial activity of the drug.

There are three classes of aminoglycoside-modifying enzymes (Fig. 14.2): aminoglycoside nucleotidyltransferases (ANTs) (also called adenylyltransferases), aminoglycoside phosphotransferases (APHs), and aminoglycoside acetyltransferases (AACs). These classes are further divided into subtypes according to the position on the aminoglycoside that the enzyme modifies. For instance, APH(3′) modifies the 3′-hydroxyl of susceptible aminoglycosides. The enzymes are further classified on the basis of the pattern of resistance designated by a Roman numeral and, in some cases, a letter designating a specific gene (e.g., APH(3′)-IVa) [11, 95]. An alternative nomenclature also exists, in which the genes are designated based on a three letter code (*aac* = acetyltransferase; *aad* = adenylyltransferase; *aph* = acetylphosphotransferase), with a capital letter specifying the site of modification [96]. Under this nomenclature, *aacA*, *aacB*, and *aacC* signify aminoglycoside 6′-*N*-acetyltransferase, aminoglycoside 2′-*N*-acetyltransferase, and aminoglycoside 3-*N*-acetyltransferase, respectively. A number can also be included to provide a unique identifier to each gene [11]. Experts in the field have discussed choosing one convention, but a consensus has not been reached.

An extraordinary number of AME-encoding genes have been identified. A recently published "representative rather than comprehensive" review of these enzymes lists over 100 enzymes and their respective genes and species [11]. AMEs can be either plasmid or chromosomally encoded, the former being associated with transposable elements, facilitating the rapid spread of resistance not only within a given species but also among a large variety of bacterial species.

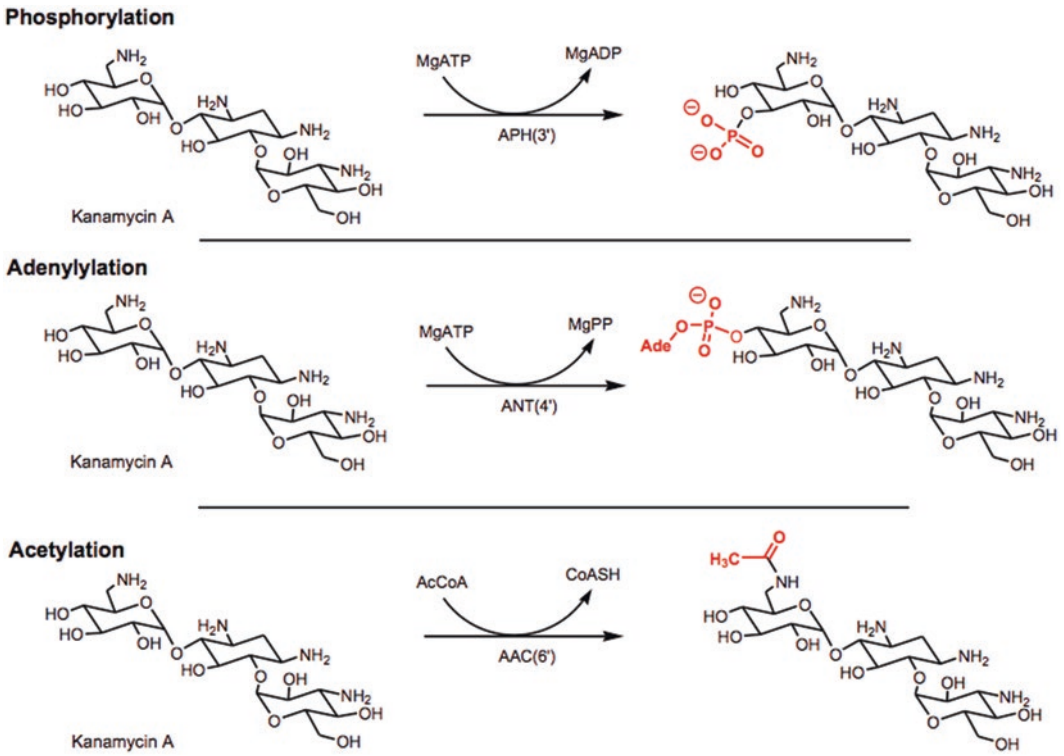


Fig. 14.2 Enzymatic modification of kanamycin by phosphorylation, adenylylation, and acetylation

3.4.1 Aminoglycoside Acetyltransferases

Aminoglycoside acetyltransferases are the largest group of AMEs and catalyze the acetyl-CoA-dependent *N*-acetylation of amino groups of typical aminoglycoside molecules. This class of enzymes includes four major subclasses: the enzymes that modify the amino groups of positions 1 and 3 of the central DOS ring (AAC(1) and AAC(3), respectively), and the enzymes that modify the 2′ and 6′ amino groups of the 2,6-dideoxy-2,6-diamino-glucose ring (AAC(2) and AAC(6), respectively) [10, 66, 97, 98]. The AAC(6′) subclass are the most common AMEs and are found in diverse bacterial species as well as on diverse mobile elements [11].

The first AME reported in bacteria was kanamycin 6′-*N*-acetyltransferase IV (AAC(6′)-IV), identified in 1965 by Okamoto and Suzuki [99]. This enzyme was the second example (after the discovery of penicillinase) of a bacterial enzyme causing antibiotic resistance by drug inactivation or modification. AAC(6′)-IV was the subject of the development of new kinetic diagnostics of enzymatic mechanisms by Radika and Northrop [100], who used these methods to establish that AAC(6′)-IV follows a rapid equilibrium random kinetic mechanism [101].

A chromosomally encoded aminoglycoside 6′-*N*-acetyltransferase (AAC(6′)-Iy) has been identified in clinical isolates of aminoglycoside-resistant *Salmonella enterica* [102]. The *aac(6′)-Iy* gene was located at the end of a long operon in sensitive strains, however, a massive 60 kbp deletion placed the constitutive *nmp* promoter directly upstream of the gene, resulting in the observed resistance phenotype. The deduced AAC(6′)-Iy sequence of 145 amino acids showed significant primary sequence homology with the Gcn5-related *N*-acetyltransferases (GNAT) superfamily. This is an enormous superfamily of enzymes (>100,000 identified to date from published sequenced genomes), whose members show sequence homology to the histone acetyltransferases (HAT) [103]. To date, over three dozen members of the GNAT family have been structurally characterized, revealing a conserved fold. The kinetic characterization of AAC(6′)-Iy has shown that the enzyme presents narrow acyl-donor specificity, but very broad specificity with respect to aminoglycosides containing a 6′-amino functionality. Both substrates must bind to the enzyme before catalysis occurs and the order of substrate binding was proposed to be random [104]. The structural characterization of this enzyme in 2004 confirmed that AAC(6′)-Iy is a member of the GNAT superfamily and revealed strong structural similarities with the *Saccharomyces cerevisiae Hpa*2-encoded histone acetyltransferase [105]. The authors also demonstrated that AAC(6′)-Iy catalyzes acetylation of eukaryotic histone proteins. Such structural and catalytic similarities suggest that bacterial aminoglycoside acetyltransferases and eukaryotic histone acetyltransferases may be evolutionarily linked.

The *aacA29b* gene was identified from an MDR clinical isolate of *P. aeruginosa* exhibiting high-level resistance to various aminoglycosides. On the basis of amino acid sequence homology, it was proposed that this gene encoded a 6′-*N*-acetyltransferase. Surprisingly, this enzyme was found to confer aminoglycoside resistance not by acetylating the drug, but by sequestering aminoglycoside molecules as a result of tight binding, thus preventing the molecule from reaching its target in the ribosome [106].

High-level aminoglycoside resistance in *Enterococcus faecalis* is often due to the plasmid-mediated expression of the bifunctional AAC(6′)-APH(2″) [107]. In *E. faecium*, intrinsic, low-level resistance to aminoglycosides is mediated by the expression of the chromosomally encoded *aac*(*6′*)-*Ii* gene [108].

Kinetic studies have shown that AAC(6′)-Ii follows an ordered Bi-Bi mechanism in which acetyl-CoA binds first to the enzyme followed by the aminoglycoside [109]. Chemistry is not rate-limiting, as evidenced by very small solvent isotope effects and a large dependence of the maximum velocity on the solvent micro viscosity, suggesting that a physical step, likely product dissociation, governs the overall rate of catalysis [109]. The molecular mechanism of this enzyme was investigated by mutagenesis studies and the role of several potential catalytic residues on the active site of the Enterococcal AAC(6′)-Ii were explored [110]. These studies indicate that Glu72 is critical for the proper positioning and orientation of aminoglycoside substrates in the active site. In addition, the amide NH group of Leu 76 is implicated in important interactions with acetyl-CoA and transition state stabilization. The three-dimensional structure of the *E. faecium* AAC(6′)-Ii was solved at 2.7 Å resolution, reveling a compact GNAT fold [111].

AAC(2′) is a class of aminoglycoside acetyltransferases that thus far have only been found encoded on the chromosomes of *Mycobacterium* spp. and *Providencia stuartii* [11, 112–114]. The *aac*(*2′*)-*Ic* gene of *M. tuberculosis* was cloned and expressed in *E. coli* and the purified enzyme acetylated all aminoglycoside substrates tested in vitro. Dead end inhibition studies as well as alternative substrate diagnostic studies supported an ordered sequential mechanism with a degree of randomness, where binding of acyl-CoA is preferred followed by the aminoglycoside. The enzyme is able to perform both *N*-acetyl and *O*-acetyl transfer [115]. Despite this broad activity in vitro, the *aac*(*2′*) genes have not been implicated in clinical aminoglycoside resistance in mycobacteria. In *P. stuartii*, the AAC(2′)-Ia enzyme is thought to play an important role in cell wall turnover via acetylation of peptidoglycan [113, 114]. Expression of the gene is complex, tightly controlled, and dependent on environmental conditions. As such, strains are typically aminoglycoside susceptible and the enzyme is not induced by aminoglycosides. Mutations in a number of regulatory genes are required to confer elevated aminoglycoside minimum inhibitory concentrations (MICs) [113, 114].

The AAC(3) family of aminoglycoside acetyltransferases selectively modify the 3-amino group of the deoxystreptamine ring. At present, this family includes five major types, I–V, based on the pattern of aminoglycoside resistance that they confer. As previously discussed, the 3-amino group is found in all aminoglycosides and is required for specific binding of these molecules to the A site of the rRNA. Acetylation at this position would disrupt crucial interactions required for specific binding, resulting in poor binding to the ribosome. The AAC(3)-I and -II isoenzymes preferentially modify the gentamicin group of aminoglycosides [116, 117]. Initial velocity, product, dead-end, and substrate inhibition studies have revealed that this enzyme follows a random Bi-Bi kinetic mechanism where both substrates must bind to the enzyme active site before catalysis can occur [118]. AAC(3)-III enzymes catalyze the covalent acetylation of a wide variety of aminoglycosides including gentamicin, tobramycin, and neomycin [119].

The AAC(3)-IV enzyme was the first aminoglycoside-modifying enzyme identified as capable of modifying the novel aminoglycoside apramycin [120]. This enzyme was originally found in *E. coli* and *Salmonella typhimurium* isolates from animals [120, 121] but was quickly identified in human clinical isolates from hospitalized patients [122], representing a serious concern given the activity of this enzyme against essentially all therapeutically useful aminoglycosides [123]. Kinetic characterization of the enzyme from *E. coli* revealed the broadest aminoglycoside specificity range of all AAC(3) enzymes [123]. Dead-end inhibition and isothermal titration calorimetry (ITC) experiments revealed that the enzyme follows a sequential, random, Bi-Bi kinetic mechanism. Substrate specificity studies showed that acetylation at the 1-*N* position sterically interferes with 3-*N* acetylation. Similar results have been observed with other AAC(3) enzymes, including AAC(3)-III and AAC(3)-I. Sequence alignment studies indicate that this enzyme is not a member of the GNAT superfamily, but currently no structural data have been reported to confirm such findings.

The last member of the AAC(3) class of enzymes identified to date was AAC(3)-V, isolated from a clinical isolate of *P. aeruginosa* resistant to kanamycin, gentamicin, tobramycin, and sisomicin [124]. The only member of the AAC(3) class of enzymes to be structurally characterized to date is the *Serratia marcescens* AAC(3)-I [125]. The monomer fold was typical of the GNAT superfamily, with the characteristic central antiparallel β-sheet containing two amino-terminal helices on one side of the sheet and the two carboxy-terminal helices on the other.

3.4.2 Aminoglycoside Phosphotransferases

The APH class of enzymes is the second largest group of aminoglycoside-modifying enzymes. These enzymes catalyze the transfer the γ-phosphoryl group from ATP to hydroxyl groups on aminoglycoside molecules. The APH class enzymes have also been referred to as "aminoglycoside kinases" and share some mechanistic and structural similarity with eukaryotic serine-threonine and tyrosine kinases, suggesting an evolutionary connection [126]. As a consequence of the γ-phosphoryl group transfer, favorable electrostatic interactions that formerly existed between the hydroxyl group and specific residues on the rRNA are abolished, resulting in poor binding of the drug on its ribosome target. The majority of these enzymes belong to the APH(3′) subfamily, which is also the most widespread among pathogenic organisms [127].

The *aph*(*3′*)*-IIIa* gene is found primarily in Gram-positive cocci and confers resistance to a wide range of aminoglycoside antibiotics, including kanaymcin, amikacin, neomycin, and butirosin [127]. The three-dimensional structure of the APH(3′)-IIIa enzyme has been solved to 2.2 Å and shows significant structural similarity to eukaryotic serine/threonine (Ser/Thr) and tyrosine protein kinases (EPK) [128]. In addition to structural similarities, APH(3′)-IIIa is inhibited by specific EPK inhibitors [129] and is able to phosphorylate several EPK substrates [130].

APH(3′)-IIIa operates by a Theorell-Chance mechanism, where ATP binds first, prior to the aminoglycoside. After binding and enzymatic activity, the modified drug is the first product to leave, followed by the rate-limiting dissociation of ADP [131].

In Gram-positive organisms, the expression of a bifunctional enzyme 6′-*N*-acetyltransferase and 2″-*O*-phosphotransferase is responsible for high-level resistance to most aminoglycosides currently used in clinical practice [107]. Both activities can be separately expressed and the kinetic properties of the bifunctional enzyme do not differ from its monofunctional counterparts [132].

Streptomycin resistance due to aminoglycoside phosphotransferases is the result of two enzymes, the APH(3″) and the APH(6) [133]. Both enzymes are found in the streptomycin producer *Streptomyces griseus* and the *aph*(*6*)-encoding gene is clustered with streptomycin biosynthetic genes. The reason for such redundancy in aminoglycoside self-defense is not known at the present time.

APH(4) and APH(9) are responsible for resistance to hygromycin and spectinomycin, respectively, by phosphorylation of the 4- and 9-hydroxyl positions on the respective aminoglycoside molecules [98].

3.4.3 Aminoglycoside Nucleotidyltransferases

Aminoglycoside nucleotidyltransferases (or adenylyltransferases) catalyze the reaction between Mg-ATP and aminoglycoside molecules to form the *O*-adenylated aminoglycoside and the magnesium chelate of inorganic pyrophosphate. These enzymes adenylate hydroxyl groups on the positions 2″, 3″, 4′, 6, and 9, where the most relevant reactions, from a clinical perspective, are catalyzed by ANT(2″) and ANT(4′) [9, 10].

ANT(3″) confers resistance to the atypical aminoglycosides streptomycin and spectinomycin, by modifying the 3″-hydroxyl position of streptomycin and 9-hydroxyl group of spectinomycin [134]. ANT(6) and ANT(9) adenylate 6-hydroxyl and 9-hydroxyl groups of streptomycin and spectinomycin, respectively, in Gram-positive organisms [135, 136].

ANT(2″) was first identified in a clinical isolate of *Klebsiella pneumoniae* [137]. This enzyme catalyzes the *O*-adenylylation of the 2″-hydroxyl group of 4,6-substituted aminoglycoside molecules and causes resistance to multiple aminoglycosides because it adenylylates a broad range of substrate molecules [138]. Mechanistic studies have shown that the enzyme follows a Theorell-Chance kinetic mechanism in which the nucleotide binds first then the aminoglycoside. Following binding, pyrophosphate is released prior to the nucleotidylated aminoglycoside and turnover is controlled by the rate-limiting release of the final product [138]. The nucleoside monophosphate is transferred directly to the hydroxyl group of the antibiotic in one step, and the reaction proceeds through inversion of the stereochemistry about the α-phosphorous [139]. Substrate specificity studies confirmed the importance of the 2′-substitution on 2″-*O*-adenylation, where molecules containing 2′-amino groups, instead of a 2′-hydroxyl, favor adenylation to occur [138, 140].

The ANT(4′) kanamycin nucleotidyltransferase was originally isolated from clinical isolates of *Staphylococcus aureus*. This enzyme adenylylates the 4′-hydroxyl group of kanamycin, utilizing ATP, GTP, or UTP as the nucleotide substrate. It can inactivate a wide range of aminoglycosides including kanamycins A, B, and C, gentamicin A, amikacin, tobramycin, and neomycins B and C [141].

4 Mechanism of the Spread of Aminoglycoside Resistance

The rapid dissemination of aminoglycoside resistance among pathogenic organisms has been largely attributed to conjugation of plasmids and non-replicative transposons among bacteria [142–145]. A clinical example of the ongoing importance of conjugative plasmid transfer on resistance to aminoglycosides is the strong association of aminoglycoside resistance determinants with β-lactamases, leading to widespread dissemination of difficult to treat multi- and extensively drug-resistant Gram-negative pathogens (see Sect. 5).

Although aminoglycosides are not first-line therapy for staphylococcal infections, the recent increase in nosocomial infections caused by aminoglycoside-resistant strains is

worrisome because it is often associated with resistance to drugs commonly used to treat staphylococcal infections [146]. In addition, aminoglycoside resistance plasmids residing in avirulent *S. epidermidis* strains present in skin flora of ill patients represent a reservoir that can be further transferred to virulent strains via conjugative transfer [147, 148]. Recent studies have shown that 80 % of hospital-associated methicillin-resistant *S. aureus* (MRSA) infections showed resistance to multiple-aminoglycosides including gentamicin, tobramycin, kanamycin, amikacin, astromicin, and arbekacin, where 56 % of such cases carried a transferable plasmid encoding a bifunctional aminoglycoside-modifying enzyme AAC(6′)-APH(2″) [149]. The gene *aac*(*6′*)-*aph*(*2″*) is present in the Tn 4100-like transposon, which is inserted in both the R plasmid and chromosome of aminoglycoside-resistant isolates [107].

The *armA* gene confers high-level resistance to essentially all clinically important aminoglycosides by methylation of the 16S rRNA. The *armA* gene is part of the functional transposon Tn-1548 together with an *ant*(*3″*) gene [150, 151]. The reported data suggest that the *armA* gene is spread by conjugation followed by transposition. This finding accounts for the observation that the *armA* gene is encountered among Enterobacteriaceae and *A. baumannii* isolated from a variety of sources and geographic locations [12].

The fact that bacteria produce a remarkable array of tools to overcome the bactericidal effects of antimicrobials is alarming. Moreover, the fact that such genetic information is located on mobile DNA elements, which can be easily and rapidly disseminated between most diverse bacteria, is particularly worrisome. The increased incidence of MDR bacteria combined with the rising evidence of resistance transfer from one organism to another may lead to an increasing emergence of nosocomial pathogens for which there is no therapy.

5 Cross-Resistance

5.1 Extended Spectrum β-Lactamases (ESBLs)

Regardless of the bacterial species or enzyme type, many extended spectrum β-lactamase (ESBL) producers are also resistant to aminoglycosides [152] either due to the co-expression of AMEs or RMTs. An analysis of 100 strains of ESBL-producing *K. pneumoniae* isolates from several hospitals in Chile showed that 65 % were resistant to gentamicin and 47 % were resistant to amikacin [153]. The most frequent AMEs genes detected were the *aac*(*6′*)-*Ib* gene in 69 % of strains, conferring resistance to amikacin, kanamycin, and tobramycin and the *aac*(*3*)-*IIa* gene in 36 % of strains, conferring resistance to gentamicin. High rates of aminoglycoside co-resistance in ESBL-producers have also been associated with the co-production of ribosomal methyltransferases [154]. Among 235 ESBL-producing *K. pneumoniae* isolates from a nationwide surveillance performed in Taiwan, 102 (43.4 %) were resistant to amikacin. Ninety-two of these 102 isolates (90.2 %) possessed CTX-M-type β-lactamases, either alone or with SHV-type or CMY-2 β-lactamases and the *armA* and *rmtB* genes were individually detected in 44 and 37 of the 92 CTX-M positive isolates, respectively.

5.2 Carbapenemases

Similar to ESBL-producing strains, carbapenemase-producing Enterobacteriaceae are also commonly co-resistant to aminoglycosides. In a study of 50 carbapenem-resistant *K. pneumoniae* clinical isolates from two medical centers in the USA, 90 % of the isolates produced KPC and 98 % possessed AMEs [155]. Ninety-eight percent had AAC(6′)-Ib, 56 % had APH(3′)-Ia, 38 % had AAC(3)-IV, and 2 % had ANT(2″)-Ia. In many cases, more than one AME was present. Of these isolates, 40 % were non-susceptible to gentamicin, 98 % were non-susceptible to tobramycin, and 16 % were non-susceptible to amikacin according to CLSI criteria. A clear association between the New Delhi metallo-β-lactamase (NDM-1) and the 16S rRNA methyltransferases ArmA, RmtC, and RmtB has also been demonstrated [156–159]. In most regions of the world, both of these resistance mechanisms remain relatively rare with reports of their isolation often linked to travel to the Balkans, Asia, or Southeast Asia [12, 160, 161], where NDM-1- and RMT-producing organisms are endemic.

5.3 Fluoroquinolones

A variant of gene *aac*(6′)-Ib in clinical isolates of Gram-negative bacteria that has acquired the ability to modify fluoroquinolones was recently identified [162]. This enzyme was shown to reduce the activity of ciprofloxacin by *N*-acetylation of the secondary amino nitrogen of its piperazinyl substituent without significantly altering its activity against aminoglycosides [163]. The modified *aac*(*6′*)-*Ib* gene is encoded in an integron cassette with an associated *attC* site. It is found in various integrons including on IncF11 plasmids expressing the extended spectrum β-lactamase CTX-M-15 and has been reported worldwide [164].

6 New Agents

Structural modification of amikacin by acylation of the 1-amino group of the 2-deoxystreptamine ring with 2-hydroxy-4-aminobutyric acid renders this molecule less susceptible to the action of many aminoglycoside-modifying enzymes [66, 165]. Based on the success achieved with amikacin in circumventing drug inactivation by modifying enzymes through the addition of a 2-hydroxy-4-aminobutyric acid 1-amino group, other 1-*N* substituted derivatives, like isepamicin and arbekacin, were synthesized. In these derivatives, the 1-amino substitution protects against modification at 2″-hydroxyl and 3-amino positions, most likely by steric hindrance. This valuable feature explains the broad success and utility of the 1-amino substituted derivatives in situations of resistance to kanamycin, gentamicin, or tobramycin. In a relatively recent study examining isepamicin activity against a large collection of Enterobacteriaceae from a tertiary care center in Greece, susceptibility to isepamicin was observed for 73.2 % of 683 isolates that were non-susceptible to all other aminoglycosides tested, including amikacin, gentamicin, and tobramycin [166]. Isepamicin and arbekacin, however, are still largely susceptible to ANT(4′) enzymes [141] and are not widely available commercially.

Dibekacin (a 3′,4′-dideoxykanaymcin B derivative) was rationally designed to circumvent inactivation by the APH(3′) and ANT(4′) enzymes. Further modification of this drug by addition of a 4-amino-2-hydroxybutyryl group on the 1-amino group produced arbekacin. Arbekacin is particularly successful against MRSA, and has been used in Japan since 1990 [165, 167]. However, strains of *S. aureus* resistant to arbekacin have been isolated, where a mutation in the *aac*(6′)-*aph*(2″) gene permits arbekacin acetylation at the 4″ position [168].

Plazomicin, a semisynthetic molecule derived from sisomicin, was rationally designed to provide protection from the majority of known AMEs (Fig. 14.3) [169]. Sisomicin, and thus plazomicin, naturally lack the 3′- and 4′-OH groups, providing protection against the APH(3′)-III, -VI, and -VII and ANT(4′) enzymes that generate resistance to amikacin. Introduction of a hydroxyl-aminobutyric acid substituent at the C-1 amino group provides protection from the AAC(3), ANT(2″), and APH(2″) enzymes. Finally, the hydroxyethyl substituent at the 6′ position blocks the AAC(6′) AMEs without reducing potency as has been associated with other efforts to protect this position [170]. AAC(2)-I, an AME encoded in the chromosome of *P. stuartii* and some mycobacterial species (Sect. 3.4.1), is associated with decreased plazomicin potency. As with other 4,6-linked aminoglycosides, plazomicin is not active in the presence of 16S rRNA methylases.

Because of the modifications introduced, plazomicin retains potent activity against Enterobacteriaceae that are resistant to currently available aminoglycosides, including MDR strains. The in vitro activity of plazomicin was evaluated against 300 MDR (carbapenemase and/or ESBL-producing) isolates from four hospitals in Athens [171]. Only 6.7 %, 17.7 %, and 37.3 % of the strains tested were susceptible to tobramycin, amikacin, and gentamicin, respectively, according to CLSI criteria. In contrast, plazomicin demonstrated potent activity against this otherwise highly resistant collection of strains, with an MIC range of 0.25–4 mg/L, and MIC_{50} and MIC_{90} values of 1 and of 2 mg/L, respectively, which is substantially lower than those for the comparator aminoglycosides. Plazomicin is currently in development for the treatment of serious infections due to MDR Enterobacteriaceae, including carbapenem-resistant strains.

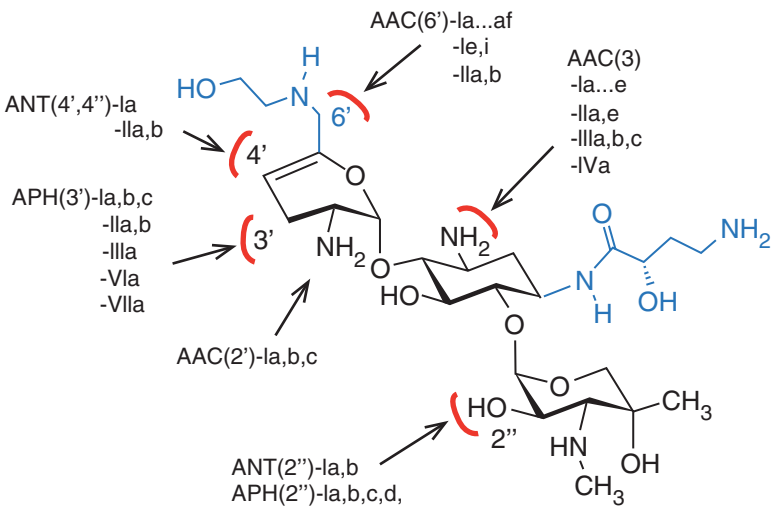


Fig. 14.3 Chemical structure of plazomicin illustrating structural modifications in blue leading to reduced susceptibility (*red curve*) to a wide range of aminoglycoside-modifying enzymes [ANT(4′4″), APH(3′), ANT(2″), APH(2″), AAC(6′), and AAC(3)], with the exception of AAC(2′)

7 Conclusion

Despite the emergence and spread of enzymatic and efflux-mediated aminoglycoside resistance mechanisms, this class of agents continues to be a critical member of the antibiotic armamentarium, particularly for the treatment of life-threatening infections due to Gram-negative pathogens. The emergence and global spread of MDR Gram-negative pathogens over the last decade has led to renewed interest in the aminoglycoside class, including the development of new molecules with potent activity against otherwise highly resistant pathogens.

References

1. Avent ML, Rogers BA, Cheng AC, Paterson DL. Current use of aminoglycosides: indications, pharmacokinetics and monitoring for toxicity. Intern Med J. 2011;41(6):441–9. doi:10.1111/j.1445-5994.2011.02452.x.
2. Jackson J, Chen C, Buising K. Aminoglycosides: how should we use them in the 21st century? Curr Opin Infect Dis. 2013;26(6):516–25. doi:10.1097/QCO.0000000000000012.
3. Silvestri L, van Saene HK, Milanese M, Gregori D, Gullo A. Selective decontamination of the digestive tract reduces bacterial bloodstream infection and mortality in critically ill patients. Systematic review of randomized, controlled trials. J Hosp Infect. 2007;65(3):187–203. doi:10.1016/j.jhin.2006.10.014.
4. Tascini C, Sbrana F, Flammini S, Tagliaferri E, Arena F, Leonildi A, Ciullo I, Amadori F, Di Paolo A, Ripoli A, Lewis R, Rossolini GM, Menichetti F, GENGUT Study Group. Oral gentamicin gut decontamination for prevention of KPC-producing Klebsiella pneumoniae infections: relevance of concomitant systemic antibiotic therapy. Antimicrob Agents Chemother. 2014;58(4):1972–6. doi:10.1128/AAC.02283-13.
5. Ben Salah A, Ben Messaoud N, Guedri E, Zaatour A, Ben Alaya N, Bettaieb J, Gharbi A, Belhadj Hamida N, Boukthir A, Chlif S, Abdelhamid K, El Ahmadi Z, Louzir H, Mokni M, Morizot G, Buffet P, Smith PL, Kopydlowski KM, Kreishman-Deitrick M, Smith KS, Nielsen CJ, Ullman DR, Norwood JA, Thorne GD, McCarthy WF, Adams RC, Rice RM, Tang D, Berman J, Ransom J, Magill AJ, Grogl M. Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. N Engl J Med. 2013;368(6):524–32. doi:10.1056/NEJMoa1202657.
6. Sundar S, Agrawal N, Arora R, Agarwal D, Rai M, Chakravarty J. Short-course paromomycin treatment of visceral leishmaniasis in India: 14-day vs 21-day treatment. Clin Infect Dis. 2009;49(6):914–8. doi:10.1086/605438.
7. Nicolau DP, Belliveau PP, Nightingale CH, Quintiliani R, Freeman CD. Implementation of a once-daily aminoglycoside program in a large community-teaching hospital. Hosp Pharm. 1995;30(8):674–6. 679–680.
8. Drusano GL, Ambrose PG, Bhavnani SM, Bertino JS, Nafziger AN, Louie A. Back to the future: using aminoglycosides again and how to dose them optimally. Clin Infect Dis. 2007;45(6):753–60. doi:10.1086/520991.
9. Kotra LP, Haddad J, Mobashery S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother. 2000;44(12):3249–56.
10. Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. Chem Rev. 2005;105(2):477–98. doi:10.1021/cr0301088.
11. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. Drug Resist Updat. 2010;13(6):151–71. doi:10.1016/j.drup.2010.08.003.
12. Wachino J, Arakawa Y. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. Drug Resist Updat. 2012;15(3):133–48. doi:10.1016/j.drup.2012.05.001.
13. Schlessinger D. Failure of aminoglycoside antibiotics to kill anaerobic, low-pH, and resistant cultures. Clin Microbiol Rev. 1988;1(1):54–9.
14. Hancock RE. Alterations in outer membrane permeability. Annu Rev Microbiol. 1984;38:237–64. doi:10.1146/annurev.mi.38.100184.001321.
15. Hancock RE, Farmer SW, Li ZS, Poole K. Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of Escherichia coli. Antimicrob Agents Chemother. 1991;35(7):1309–14.
16. Hancock RE, Raffle VJ, Nicas TI. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 1981;19(5):777–85.
17. Bryan LE, Kowand SK, Van Den Elzen HM. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: clostridium perfringens and Bacteroides fragilis. Antimicrob Agents Chemother. 1979;15(1):7–13.
18. Mates SM, Patel L, Kaback HR, Miller MH. Membrane potential in anaerobically growing Staphylococcus aureus and its relationship to gentamicin uptake. Antimicrob Agents Chemother. 1983;23(4):526–30.
19. Miller MH, Edberg SC, Mandel LJ, Behar CF, Steigbigel NH. Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of Staphylococcus aureus. Antimicrob Agents Chemother. 1980;18(5):722–9.
20. Davies J, Gorini L, Davis BD. Misreading of RNA codewords induced by aminoglycoside antibiotics. Mol Pharmacol. 1965;1(1):93–106.
21. Davies J, Jones DS, Khorana HG. A further study of misreading of codons induced by streptomycin and neomycin using ribopolynucleotides containing two nucleotides in alternating sequence as templates. J Mol Biol. 1966;18(1):48–57.
22. Davis BD. Mechanism of bactericidal action of aminoglycosides. Microbiol Rev. 1987;51(3):341–50.
23. Green R, Noller HF. Ribosomes and translation. Annu Rev Biochem. 1997;66:679–716. doi:10.1146/annurev.biochem.66.1.679.
24. Moazed D, Noller HF. Intermediate states in the movement of transfer RNA in the ribosome. Nature. 1989;342(6246):142–8. doi:10.1038/342142a0.
25. Karimi R, Ehrenberg M. Dissociation rate of cognate peptidyl-tRNA from the A-site of hyper-accurate and error-prone ribosomes. Eur J Biochem. 1994;226(2):355–60.
26. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature. 2000;407(6802):340–8. doi:10.1038/35030019.
27. Vicens Q, Westhof E. Crystal structure of paromomycin docked into the eubacterial ribosomal decoding A site. Structure. 2001;9(8):647–58.
28. Fourmy D, Recht MI, Blanchard SC, Puglisi JD. Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science. 1996;274(5291):1367–71.

29. Fourmy D, Recht MI, Puglisi JD. Binding of neomycin-class aminoglycoside antibiotics to the A-site of 16 S rRNA. J Mol Biol. 1998;277(2):347–62. doi:10.1006/jmbi.1997.1552.
30. Bilgin N, Ehrenberg M. Mutations in 23 S ribosomal RNA perturb transfer RNA selection and can lead to streptomycin dependence. J Mol Biol. 1994;235(3):813–24. doi:10.1006/jmbi.1994.1041.
31. Davies J, Davis BD. Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. The effect of drug concentration. J Biol Chem. 1968;243(12):3312–6.
32. Lodmell JS, Dahlberg AE. A conformational switch in Escherichia coli 16S ribosomal RNA during decoding of messenger RNA. Science. 1997;277(5330):1262–7.
33. Puglisi JD, Blanchard SC, Green R. Approaching translation at atomic resolution. Nat Struct Biol. 2000;7(10):855–61. doi:10.1038/79603.
34. Roth H, Amos H, Davis BD. Purine nucleotide excretion by Escherichia coli in the presence of streptomycin. Biochim Biophys Acta. 1960;37:398–405.
35. Anand N, Davis BD. Damage by streptomycin to the cell membrane of Escherichia coli. Nature. 1960;185:22–3.
36. Dubin DT, Davis BD. The effect of streptomycin on potassium flux in Escherichia coli. Biochim Biophys Acta. 1961;52:400–2.
37. Busse HJ, Wöstmann C, Bakker EP. The bactericidal action of streptomycin: membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of Escherichia coli and subsequent caging of the antibiotic inside the cells due to degradation of these proteins. J Gen Microbiol. 1992;138(3):551–61.
38. Davis BD, Chen LL, Tai PC. Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. Proc Natl Acad Sci U S A. 1986;83(16):6164–8.
39. Nichols WW, Young SN. Respiration-dependent uptake of dihydrostreptomycin by Escherichia coli. Its irreversible nature and lack of evidence for a uniport process. Biochem J. 1985; 228(2):505–12.
40. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. Cell. 2007;130(5):797–810. doi:10.1016/j.cell.2007.06.049.
41. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. Killing by bactericidal antibiotics does not depend on reactive oxygen species. Science. 2013;339(6124):1213–6. doi:10.1126/science.1232688.
42. Liu Y, Imlay JA. Cell death from antibiotics without the involvement of reactive oxygen species. Science. 2013;339(6124):1210–3. doi:10.1126/science.1232751.
43. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobritz MA, Braff D, Schwarz EG, Ye JD, Pati M, Vercruysse M, Ralifo PS, Allison KR, Khalil AS, Ting AY, Walker GC, Collins JJ. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci U S A. 2014;111(20):E2100–9. doi:10.1073/pnas.1401876111.
44. Fang FC. Antibiotic and ROS linkage questioned. Nat Biotechnol. 2013;31(5):415–6. doi:10.1038/nbt.2574.
45. Lynch SR, Puglisi JD. Structural origins of aminoglycoside specificity for prokaryotic ribosomes. J Mol Biol. 2001;306(5):1037–58. doi:10.1006/jmbi.2000.4420.
46. Bercovier H, Kafri O, Sela S. Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem Biophys Res Commun. 1986;136(3): 1136–41.
47. Schwartz JJ, Gazumyan A, Schwartz I. rRNA gene organization in the Lyme disease spirochete, Borrelia burgdorferi. J Bacteriol. 1992;174(11):3757–65.
48. Honoré N, Cole ST. Streptomycin resistance in mycobacteria. Antimicrob Agents Chemother. 1994;38(2):238–42.
49. Honoré N, Marchal G, Cole ST. Novel mutation in 16S rRNA associated with streptomycin dependence in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1995;39(3):769–70.
50. Meier A, Kirschner P, Bange FC, Vogel U, Böttger EC. Genetic alterations in streptomycin-resistant Mycobacterium tuberculosis: mapping of mutations conferring resistance. Antimicrob Agents Chemother. 1994;38(2):228–33.
51. Finken M, Kirschner P, Meier A, Wrede A, Böttger EC. Molecular basis of streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol. 1993;9(6):1239–46.
52. Blanchard JS. Molecular mechanisms of drug resistance in Mycobacterium tuberculosis. Annu Rev Biochem. 1996;65:215–39. doi:10.1146/annurev.bi.65.070196.001243.
53. Toivonen JM, Boocock MR, Jacobs HT. Modelling in Escherichia coli of mutations in mitoribosomal protein S12: novel mutant phenotypes of rpsL. Mol Microbiol. 1999;31(6):1735–46.
54. Allen PN, Noller HF. Mutations in ribosomal proteins S4 and S12 influence the higher order structure of 16 S ribosomal RNA. J Mol Biol. 1989;208(3):457–68.
55. Prammananan T, Sander P, Brown BA, Frischkorn K, Onyi GO, Zhang Y, Böttger EC, Wallace RJ. A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in Mycobacterium abscessus and Mycobacterium chelonae. J Infect Dis. 1998;177(6): 1573–81.
56. Björkman J, Samuelsson P, Andersson DI, Hughes D. Novel ribosomal mutations affecting translational accuracy, antibiotic resistance and virulence of Salmonella typhimurium. Mol Microbiol. 1999;31(1):53–8.
57. Criswell D, Tobiason VL, Lodmell JS, Samuels DS. Mutations conferring aminoglycoside and spectinomycin resistance in Borrelia burgdorferi. Antimicrob Agents Chemother. 2006;50(2): 445–52. doi:10.1128/AAC.50.2.445-452.2006.
58. Beauclerk AA, Cundliffe E. Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. J Mol Biol. 1987;193(4):661–71.
59. Cundliffe E. How antibiotic-producing organisms avoid suicide. Annu Rev Microbiol. 1989;43:207–33. doi:10.1146/annurev.mi.43.100189.001231.
60. Holmes DJ, Cundliffe E. Analysis of a ribosomal RNA methylase gene from Streptomyces tenebrarius which confers resistance to gentamicin. Mol Gen Genet. 1991;229(2):229–37.
61. Holmes DJ, Drocourt D, Tiraby G, Cundliffe E. Cloning of an aminoglycoside-resistance-encoding gene, kamC, from Saccharopolyspora hirsuta: comparison with kamB from Streptomyces tenebrarius. Gene. 1991;102(1):19–26.
62. Kelemen GH, Cundliffe E, Financsek I. Cloning and characterization of gentamicin-resistance genes from Micromonospora purpurea and Micromonospora rosea. Gene. 1991;98(1):53–60.
63. Ohta T, Hasegawa M. Analysis of the nucleotide sequence of fmrT encoding the self-defense gene of the istamycin producer, Streptomyces tenjimariensis ATCC 31602; comparison with the squences of kamB of Streptomyces tenebrarius NCIB 11028 and kamC of Saccharopolyspora hirsuta CL102. J Antibiot (Tokyo). 1993;46(3):511–7.
64. Skeggs PA, Thompson J, Cundliffe E. Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of Streptomyces lividans carrying DNA from Streptomyces tenjimariensis. Mol Gen Genet. 1985;200(3):415–21.
65. Thompson J, Skeggs PA, Cundliffe E. Methylation of 16S ribosomal RNA and resistance to the aminoglycoside antibiotics gentamicin and kanamycin determined by DNA from the gentamicin-producer, Micromonospora purpurea. Mol Gen Genet. 1985;201(2):168–73.

66. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: activity and resistance. Antimicrob Agents Chemother. 1999;43(4):727–37.
67. Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, Yagi T, Kato H, Arakawa Y. Acquisition of 16S rRNA methylase gene in Pseudomonas aeruginosa. Lancet. 2003;362(9399):1888–93. doi:10.1016/S0140-6736(03)14959-8.
68. Doi Y, Yokoyama K, Yamane K, Wachino J, Shibata N, Yagi T, Shibayama K, Kato H, Arakawa Y. Plasmid-mediated 16S rRNA methylase in Serratia marcescens conferring high-level resistance to aminoglycosides. Antimicrob Agents Chemother. 2004;48(2):491–6.
69. Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, Doi Y, Kimura K, Ike Y, Arakawa Y. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a proteus mirabilis isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob Agents Chemother. 2006;50(1):178–84. doi:10.1128/AAC.50.1.178-184.2006.
70. Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y. Global spread of multiple aminoglycoside resistance genes. Emerg Infect Dis. 2005;11(6):951–3. doi:10.3201/eid1106.040924.
71. Yan JJ, Wu JJ, Ko WC, Tsai SH, Chuang CL, Wu HM, Lu YJ, Li JD. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in Escherichia coli and Klebsiella pneumoniae isolates from two Taiwanese hospitals. J Antimicrob Chemother. 2004;54(6):1007–12. doi:10.1093/jac/dkh455.
72. Wachino J, Shibayama K, Kurokawa H, Kimura K, Yamane K, Suzuki S, Shibata N, Ike Y, Arakawa Y. Novel plasmid-mediated 16S rRNA m1A1408 methyltransferase, NpmA, found in a clinically isolated Escherichia coli strain resistant to structurally diverse aminoglycosides. Antimicrob Agents Chemother. 2007;51(12):4401–9. doi:10.1128/AAC.00926-07.
73. Poole K. Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect. 2004;10(1):12–26.
74. Murakami S, Nakashima R, Yamashita E, Yamaguchi A. Crystal structure of bacterial multidrug efflux transporter AcrB. Nature. 2002;419(6907):587–93. doi:10.1038/nature01050.
75. Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. Mol Microbiol. 2000;37(2):219–25.
76. Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides. Antimicrob Agents Chemother. 1999;43(11):2624–8.
77. Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O, Plésiat P. MexXY-OprM efflux pump is necessary for a adaptive resistance of Pseudomonas aeruginosa to aminoglycosides. Antimicrob Agents Chemother. 2003;47(4):1371–5.
78. Islam S, Jalal S, Wretlind B. Expression of the MexXY efflux pump in amikacin-resistant isolates of Pseudomonas aeruginosa. Clin Microbiol Infect. 2004;10(10):877–83. doi:10.1111/j.1469-0691.2004.00991.x.
79. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. Antimicrob Agents Chemother. 2001;45(12):3375–80. doi:10.1128/AAC.45.12.3375-3380.2001.
80. Rosenberg EY, Ma D, Nikaido H. AcrD of Escherichia coli is an aminoglycoside efflux pump. J Bacteriol. 2000;182(6):1754–6.
81. Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P, Nguyen LY, Shawar RM, Folger KR, Stover CK. Characterization of a Pseudomonas aeruginosa efflux pump contributing to aminoglycoside impermeability. Antimicrob Agents Chemother. 1999;43(12):2975–83.
82. Poole K. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother. 2005;56(1):20–51. doi:10.1093/jac/dki171.
83. Morita Y, Tomida J, Kawamura Y. MexXY multidrug efflux system of Pseudomonas aeruginosa. Front Microbiol. 2012;3:408. doi:10.3389/fmicb.2012.00408.
84. Jo JT, Brinkman FS, Hancock RE. Aminoglycoside efflux in Pseudomonas aeruginosa: involvement of novel outer membrane proteins. Antimicrob Agents Chemother. 2003;47(3):1101–11.
85. Buroni S, Pasca MR, Flannagan RS, Bazzini S, Milano A, Bertani I, Venturi V, Valvano MA, Riccardi G. Assessment of three resistance-nodulation-cell division drug efflux transporters of Burkholderia cenocepacia in intrinsic antibiotic resistance. BMC Microbiol. 2009;9:200. doi:10.1186/1471-2180-9-200.
86. Jassem AN, Forbes CM, Speert DP. Investigation of aminoglycoside resistance inducing conditions and a putative AmrAB-OprM efflux system in Burkholderia vietnamiensis. Ann Clin Microbiol Antimicrob. 2014;13:2. doi:10.1186/1476-0711-13-2.
87. Moore RA, DeShazer D, Reckseidler S, Weissman A, Woods DE. Efflux-mediated aminoglycoside and macrolide resistance in Burkholderia pseudomallei. Antimicrob Agents Chemother. 1999;43(3):465–70.
88. Aires JR, Nikaido H. Aminoglycosides are captured from both periplasm and cytoplasm by the AcrD multidrug efflux transporter of Escherichia coli. J Bacteriol. 2005;187(6):1923–9. doi:10.1128/JB.187.6.1923-1929.2005.
89. Coyne S, Courvalin P, Périchon B. Efflux-mediated antibiotic resistance in Acinetobacter spp. Antimicrob Agents Chemother. 2011;55(3):947–53. doi:10.1128/AAC.01388-10.
90. Chau SL, Chu YW, Houang ET. Novel resistance-nodulation-cell division efflux system AdeDE in Acinetobacter genomic DNA group 3. Antimicrob Agents Chemother. 2004;48(10):4054–5. doi:10.1128/AAC.48.10.4054-4055.2004.
91. De Rossi E, Arrigo P, Bellinzoni M, Silva PA, Martín C, Aínsa JA, Guglierame P, Riccardi G. The multidrug transporters belonging to major facilitator superfamily in Mycobacterium tuberculosis. Mol Med. 2002;8(11):714–24.
92. Aínsa JA, Blokpoel MC, Otal I, Young DB, De Smet KA, Martín C. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in Mycobacterium fortuitum and Mycobacterium tuberculosis. J Bacteriol. 1998;180(22):5836–43.
93. Silva PE, Bigi F, Santangelo MP, Romano MI, Martín C, Cataldi A, Aínsa JA. Characterization of P55, a multidrug efflux pump in Mycobacterium bovis and Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2001;45(3):800–4. doi:10.1128/AAC.45.3.800-804.2001.
94. Llano-Sotelo B, Azucena EF, Kotra LP, Mobashery S, Chow CS. Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. Chem Biol. 2002;9(4):455–63.
95. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev. 1993;57(1):138–63.
96. Novick RP, Clowes RC, Cohen SN, Curtiss R, Datta N, Falkow S. Uniform nomenclature for bacterial plasmids: a proposal. Bacteriol Rev. 1976;40(1):168–89.
97. Azucena E, Mobashery S. Aminoglycoside-modifying enzymes: mechanisms of catalytic processes and inhibition. Drug Resist Updat. 2001;4(2):106–17. doi:10.1054/drup.2001.0197.
98. Wright GD. Aminoglycoside-modifying enzymes. Curr Opin Microbiol. 1999;2(5):499–503.
99. Okamoto S, Suzuki Y. Chloramphenicol-, dihydrostreptomycin-, and kanamycin-inactivating enzymes from multiple drug-resistant Escherichia coli carrying episome 'R'. Nature. 1965;208(5017):1301–3.
100. Radika K, Northrop D. A new kinetic diagnostic for enzymatic mechanisms using alternative substrates. Anal Biochem. 1984;141(2):413–7.

101. Radika K, Northrop DB. The kinetic mechanism of kanamycin acetyltransferase derived from the use of alternative antibiotics and coenzymes. J Biol Chem. 1984;259(20):12543–6.
102. Magnet S, Courvalin P, Lambert T. Activation of the cryptic aac(6′)-Iy aminoglycoside resistance gene of Salmonella by a chromosomal deletion generating a transcriptional fusion. J Bacteriol. 1999;181(21):6650–5.
103. Vetting MW, S de Carvalho LP, Yu M, Hegde SS, Magnet S, Roderick SL, Blanchard JS. Structure and functions of the GNAT superfamily of acetyltransferases. Arch Biochem Biophys. 2005; 433(1):212–26. doi:10.1016/j.abb.2004.09.003.
104. Magnet S, Lambert T, Courvalin P, Blanchard JS. Kinetic and mutagenic characterization of the chromosomally encoded Salmonella enterica AAC(6′)-Iy aminoglycoside N-acetyltransferase. Biochemistry. 2001;40(12):3700–9.
105. Vetting MW, Magnet S, Nieves E, Roderick SL, Blanchard JS. A bacterial acetyltransferase capable of regioselective N-acetylation of antibiotics and histones. Chem Biol. 2004;11(4):565–73. doi:10.1016/j.chembiol.2004.03.017.
106. Magnet S, Smith TA, Zheng R, Nordmann P, Blanchard JS. Aminoglycoside resistance resulting from tight drug binding to an altered aminoglycoside acetyltransferase. Antimicrob Agents Chemother. 2003;47(5):1577–83.
107. Culebras E, Martínez JL. Aminoglycoside resistance mediated by the bifunctional enzyme 6′-N-aminoglycoside acetyltransferase-2″-O-aminoglycoside phosphotransferase. Front Biosci. 1999;4: D1–8.
108. Costa Y, Galimand M, Leclercq R, Duval J, Courvalin P. Characterization of the chromosomal aac(6′)-Ii gene specific for Enterococcus faecium. Antimicrob Agents Chemother. 1993; 37(9):1896–903.
109. Draker KA, Northrop DB, Wright GD. Kinetic mechanism of the GCN5-related chromosomal aminoglycoside acetyltransferase AAC(6′)-Ii from Enterococcus faecium: evidence of dimer subunit cooperativity. Biochemistry. 2003;42(21):6565–74. doi:10.1021/bi034148h.
110. Draker KA, Wright GD. Molecular mechanism of the enterococcal aminoglycoside 6′-N-acetyltransferase': role of GNAT-conserved residues in the chemistry of antibiotic inactivation. Biochemistry. 2004;43(2):446–54. doi:10.1021/bi035667n.
111. Wybenga-Groot LE, Draker K, Wright GD, Berghuis AM. Crystal structure of an aminoglycoside 6′-N-acetyltransferase: defining the GCN5-related N-acetyltransferase superfamily fold. Structure. 1999;7(5):497–507.
112. Aínsa JA, Pérez E, Pelicic V, Berthet FX, Gicquel B, Martín C. Aminoglycoside 2′-N-acetyltransferase genes are universally present in mycobacteria: characterization of the aac(2′)-Ic gene from Mycobacterium tuberculosis and the aac(2′)-Id gene from Mycobacterium smegmatis. Mol Microbiol. 1997;24(2):431–41.
113. Macinga DR, Rather PN. The chromosomal 2′-N-acetyltransferase of Providencia stuartii: physiological functions and genetic regulation. Front Biosci. 1999;4:D132–40.
114. Rather PN, Orosz E, Shaw KJ, Hare R, Miller G. Characterization and transcriptional regulation of the 2′-N-acetyltransferase gene from Providencia stuartii. J Bacteriol. 1993;175(20):6492–8.
115. Hegde SS, Javid-Majd F, Blanchard JS. Overexpression and mechanistic analysis of chromosomally encoded aminoglycoside 2′-N-acetyltransferase (AAC(2′)-Ic) from Mycobacterium tuberculosis. J Biol Chem. 2001;276(49):45876–81. doi:10.1074/jbc.M108810200.
116. Le Goffic F, Martel A, Witchitz J. 3-N enzymatic acetylation of gentamicin, tobramycin, and kanamycin by Escherichia coli carrying an R factor. Antimicrob Agents Chemother. 1974; 6(6):680–4.
117. Williams JW, Northrop DB. Purification and properties of gentamicin acetyltransferase I. Biochemistry. 1976;15(1):125–31.
118. Williams JW, Northrop DB. Kinetic mechanisms of gentamicin acetyltransferase I. Antibiotic-dependent shift from rapid to nonrapid equilibrium random mechanisms. J Biol Chem. 1978; 253(17):5902–7.
119. Biddlecome S, Haas M, Davies J, Miller GH, Rane DF, Daniels PJ. Enzymatic modification of aminoglycoside antibiotics: a new 3-N-acetylating enzyme from a Pseudomonas aeruginosa isolate. Antimicrob Agents Chemother. 1976;9(6):951–5.
120. Davies J, O'Connor S. Enzymatic modification of aminoglycoside antibiotics: 3-N-acetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. Antimicrob Agents Chemother. 1978;14(1):69–72.
121. Chaslus-Dancla E, Martel JL, Carlier C, Lafont JP, Courvalin P. Emergence of aminoglycoside 3-N-acetyltransferase IV in Escherichia coli and Salmonella typhimurium isolated from animals in France. Antimicrob Agents Chemother. 1986;29(2): 239–43.
122. Chaslus-Dancla E, Pohl P, Meurisse M, Marin M, Lafont JP. High genetic homology between plasmids of human and animal origins conferring resistance to the aminoglycosides gentamicin and apramycin. Antimicrob Agents Chemother. 1991;35(3):590–3.
123. Magalhaes ML, Blanchard JS. The kinetic mechanism of AAC3-IV aminoglycoside acetyltransferase from Escherichia coli. Biochemistry. 2005;44(49):16275–83. doi:10.1021/bi051777d.
124. Coombe RG, George AM. Purification and properties of an aminoglycoside acetyltransferase from Pseudomonas aeruginosa. Biochemistry. 1982;21(5):871–5.
125. Wolf E, Vassilev A, Makino Y, Sali A, Nakatani Y, Burley SK. Crystal structure of a GCN5-related N-acetyltransferase: Serratia marcescens aminoglycoside 3-N-acetyltransferase. Cell. 1998;94 (4):439–49.
126. Wright GD, Thompson PR. Aminoglycoside phosphotransferases: proteins, structure, and mechanism. Front Biosci. 1999;4:D9–21.
127. Kim C, Mobashery S. Phosphoryl transfer by aminoglycoside 3′-phosphotransferases and manifestation of antibiotic resistance. Bioorg Chem. 2005;33(3):149–58. doi:10.1016/j.bioorg.2004.11.001.
128. Hon WC, McKay GA, Thompson PR, Sweet RM, Yang DS, Wright GD, Berghuis AM. Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell. 1997;89(6):887–95.
129. Daigle DM, McKay GA, Wright GD. Inhibition of aminoglycoside antibiotic resistance enzymes by protein kinase inhibitors. J Biol Chem. 1997;272(40):24755–8.
130. Daigle DM, McKay GA, Thompson PR, Wright GD. Amino_glycoside antibiotic phosphotransferases are also serine protein kinases. Chem Biol. 1999;6(1):11–8. doi:10.1016/S1074-5521(99)80016-7.
131. McKay GA, Wright GD. Kinetic mechanism of aminoglycoside phosphotransferase type IIIa. Evidence for a Theorell-Chance mechanism. J Biol Chem. 1995;270(42):24686–92.
132. Martel A, Masson M, Moreau N, Le Goffic F. Kinetic studies of aminoglycoside acetyltransferase and phosphotransferase from Staphylococcus aureus RPAL. Relationship between the two activities. Eur J Biochem. 1983;133(3):515–21.
133. Heinzel P, Werbitzky O, Distler J, Piepersberg W. A second streptomycin resistance gene from Streptomyces griseus codes for streptomycin-3″-phosphotransferase. Relationships between antibiotic and protein kinases. Arch Microbiol. 1988;150(2):184–92.
134. Hollingshead S, Vapnek D. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenylyltransferase. Plasmid. 1985;13(1):17–30.
135. Murphy E. Nucleotide sequence of a spectinomycin adenyltransferase AAD(9) determinant from Staphylococcus aureus and its relationship to AAD(3″) (9). Mol Gen Genet. 1985;200(1):33–9.

136. Ounissi H, Derlot E, Carlier C, Courvalin P. Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci. Antimicrob Agents Chemother. 1990;34(11):2164–8.
137. Benveniste R, Davies J. R-factor mediated gentamicin resistance: a new enzyme which modifies aminoglycoside antibiotics. FEBS Lett. 1971;14(5):293–6.
138. Gates CA, Northrop DB. Substrate specificities and structure-activity relationships for the nucleotidylation of antibiotics catalyzed by aminoglycoside nucleotidyltransferase 2″-I. Biochemistry. 1988;27(10):3820–5.
139. Van Pelt JE, Iyengar R, Frey PA. Gentamicin nucleotidyltransferase. Stereochemical inversion at phosphorus in enzymatic 2′-deoxyadenylyl transfer to tobramycin. J Biol Chem. 1986; 261(34):15995–9.
140. Wright E, Serpersu EH. Enzyme-substrate interactions with an antibiotic resistance enzyme: aminoglycoside nucleotidyltransferase(2″)-Ia characterized by kinetic and thermodynamic methods. Biochemistry. 2005;44(34):11581–91. doi:10.1021/bi050797c.
141. Le Goffic F, Baca B, Soussy CJ, Dublanchet A, Duval J. ANT(4′) I: a new aminoglycoside nucleotidyltransferase found in "staphylococcus aureus" (author's transl). Ann Microbiol (Paris). 1976;127(3):391–9.
142. Courvalin P. Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. Antimicrob Agents Chemother. 1994;38(7):1447–51.
143. Dzidic S, Bedeković V. Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. Acta Pharmacol Sin. 2003; 24(6):519–26.
144. Feizabadi MM, Asadi S, Zohari M, Gharavi S, Etemadi G. Genetic characterization of high-level gentamicin-resistant strains of Enterococcus faecalis in Iran. Can J Microbiol. 2004;50(10):869–72. doi:10.1139/w04-069.
145. Waters VL. Conjugative transfer in the dissemination of beta-lactam and aminoglycoside resistance. Front Biosci. 1999;4:D433–56.
146. Allen JR, Hightower AW, Martin SM, Dixon RE. Secular trends in nosocomial infections: 1970–1979. Am J Med. 1981;70(2): 389–92.
147. Archer GL, Johnston JL. Self-transmissible plasmids in staphylococci that encode resistance to aminoglycosides. Antimicrob Agents Chemother. 1983;24(1):70–7.
148. Forbes BA, Schaberg DR. Transfer of resistance plasmids from Staphylococcus epidermidis to Staphylococcus aureus: evidence for conjugative exchange of resistance. J Bacteriol. 1983;153(2): 627–34.
149. Udou T. Dissemination of nosocomial multiple-aminoglycoside-resistant Staphylococcus aureus caused by horizontal transfer of the resistance determinant (aacA/aphD) and clonal spread of resistant strains. Am J Infect Control. 2004;32(4):215–9. doi:10.1016/j.ajic.2003.11.002.
150. Galimand M, Sabtcheva S, Courvalin P, Lambert T. Worldwide disseminated armA aminoglycoside resistance methylase gene is borne by composite transposon Tn1548. Antimicrob Agents Chemother. 2005;49(7):2949–53. doi:10.1128/AAC.49.7.2949-2953.2005.
151. González-Zorn B, Catalan A, Escudero JA, Domínguez L, Teshager T, Porrero C, Moreno MA. Genetic basis for dissemination of armA. J Antimicrob Chemother. 2005;56(3):583–5. doi:10.1093/jac/dki246.
152. Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Ayala J, Coque TM, Kern-Zdanowicz I, Luzzaro F, Poirel L, Woodford N. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother. 2007;59(2):165–74. doi:10.1093/jac/dkl483.
153. Díaz P, Bello H, Domínguez M, Trabal N, Mella S, Zemelman R, González G. Resistance to gentamicin, amikacin and ciprofloxacin among nosocomial isolates of klebsiella pneumoniae subspecie pneumoniae producing extended spectrum beta-lactamases. Rev Med Chil. 2004;132(10):1173–8.
154. Ma L, Lin CJ, Chen JH, Fung CP, Chang FY, Lai YK, Lin JC, Siu LK, Taiwan Surveillance of Antimicrobial Resistance Project. Widespread dissemination of aminoglycoside resistance genes armA and rmtB in Klebsiella pneumoniae isolates in Taiwan producing CTX-M-type extended-spectrum beta-lactamases. Antimicrob Agents Chemother. 2009;53(1):104–11. doi:10.1128/AAC.00852-08.
155. Almaghrabi R, Clancy CJ, Doi Y, Hao B, Chen L, Shields RK, Press EG, Iovine NM, Townsend BM, Wagener MM, Kreiswirth B, Nguyen MH. Carbapenem-resistant Klebsiella pneumoniae strains exhibit diversity in aminoglycoside-modifying enzymes, which exert differing effects on plazomicin and other agents. Antimicrob Agents Chemother. 2014;58(8):4443–51. doi:10.1128/AAC.00099-14.
156. Berçot B, Poirel L, Nordmann P. Updated multiplex polymerase chain reaction for detection of 16S rRNA methylases: high prevalence among NDM-1 producers. Diagn Microbiol Infect Dis. 2011;71(4):442–5. doi:10.1016/j.diagmicrobio.2011.08.016.
157. Livermore DM, Mushtaq S, Warner M, Zhang JC, Maharjan S, Doumith M, Woodford N. Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. J Antimicrob Chemother. 2011;66(1):48–53. doi:10.1093/jac/dkq408.
158. Mushtaq S, Irfan S, Sarma JB, Doumith M, Pike R, Pitout J, Livermore DM, Woodford N. Phylogenetic diversity of Escherichia coli strains producing NDM-type carbapenemases. J Antimicrob Chemother. 2011;66(9):2002–5. doi:10.1093/jac/dkr226.
159. Poirel L, Savov E, Nazli A, Trifonova A, Todorova I, Gergova I, Nordmann P. Outbreak caused by NDM-1- and RmtB-producing Escherichia coli in Bulgaria. Antimicrob Agents Chemother. 2014;58(4):2472–4. doi:10.1128/AAC.02571-13.
160. Berrazeg M, Diene S, Medjahed L, Parola P, Drissi M, Raoult D, Rolain J. New Delhi Metallo-beta-lactamase around the world: an eReview using Google Maps. Euro Surveill. 2014;19(20).
161. Bushnell G, Mitrani-Gold F, Mundy LM. Emergence of New Delhi metallo-β-lactamase type 1-producing enterobacteriaceae and non-enterobacteriaceae: global case detection and bacterial surveillance. Int J Infect Dis. 2013;17(5):e325–33. doi:10.1016/j.ijid.2012.11.025.
162. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12(1):83–8. doi:10.1038/nm1347.
163. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev. 2009;22(4):664–89. doi:10.1128/CMR.00016-09.
164. Cattoir V, Nordmann P. Plasmid-mediated quinolone resistance in gram-negative bacterial species: an update. Curr Med Chem. 2009;16(8):1028–46.
165. Smith CA, Baker EN. Aminoglycoside antibiotic resistance by enzymatic deactivation. Curr Drug Targets Infect Disord. 2002; 2(2):143–60.
166. Maraki S, Samonis G, Karageorgopoulos DE, Mavros MN, Kofteridis D, Falagas ME. In vitro antimicrobial susceptibility to isepamicin of 6,296 Enterobacteriaceae clinical isolates collected at a tertiary care university hospital in Greece. Antimicrob Agents Chemother. 2012;56(6):3067–73. doi:10.1128/AAC.06358-11.
167. Yoshikawa Y, Morikawa K, Nonaka M, Torii I. Effect of arbekacin on a methicillin-resistant Staphylococcus aureus-induced biofilm in a rat model. J Infect Chemother. 2004;10(5):268–73. doi:10.1007/s10156-004-0336-0.
168. Fujimura S, Tokue Y, Takahashi H, Kobayashi T, Gomi K, Abe T, Nukiwa T, Watanabe A. Novel arbekacin- and amikacin-modifying

enzyme of methicillin-resistant Staphylococcus aureus. FEMS Microbiol Lett. 2000;190(2):299–303.
169. Armstrong ES, Miller GH. Combating evolution with intelligent design: the neoglycoside ACHN-490. Curr Opin Microbiol. 2010;13(5):565–73. doi:10.1016/j.mib.2010.09.004.
170. Nagabhushan T, Miller G, Weinstein M. Structure–activity relationships in aminoglycoside-aminocyclitol antibiotics. In: Whelton A, Neu HC, editors. The aminoglycosides: microbiology, clinical use and toxicology. New York: Marcel Dekker; 1982. p. 3–27.
171. Galani I, Souli M, Daikos GL, Chrysouli Z, Poulakou G, Psichogiou M, Panagea T, Argyropoulou A, Stefanou I, Plakias G, Giamarellou H, Petrikkos G. Activity of plazomicin (ACHN-490) against MDR clinical isolates of Klebsiella pneumoniae, Escherichia coli, and Enterobacter spp. from Athens, Greece. J Chemother. 2012; 24(4):191–4. doi:10.1179/1973947812Y.0000000015.

Tetracycline and Chloramphenicol Resistance Mechanisms

15

Marilyn C. Roberts and Stefan Schwarz

1 Introduction

1.1 Tetracycline Resistance

Tetracyclines are broad-spectrum antibiotics that bind to the elongating ribosome and inhibit delivery of the ternary complex EF-Tu, GTP, and aminoacylated-tRNA to the A-site [1–3]. The primary binding site of tetracycline is located in the helix 34 (h34) of the 16S rRNA in the 30S subunit which overlaps the anticodon stem-loop of the A-site tRNA [1–3]. Over the last 60 years there has been widespread use of tetracycline in both animals and humans which has led to an increase in tetracycline resistance. Tetracycline resistance (Tc^r) occurs most often as a result of the acquisition of new genes that code for energy-dependent efflux of tetracyclines ($n=29$ different genes), a protein that protects bacterial ribosomes from the action of tetracyclines ($n=12$ different genes) or enzymatic inactivation ($n=3$ different genes) and one with unknown mechanism of action (Table 15.1). Many of these genes are associated with mobile plasmids, transposons, and conjugative transposons. Often these elements code for their own transfer, which may influence their distribution among various genera and partially explain the wide differences in host range among the various genes (Table 15.2). Integrons, which are gene-capture systems and allow for multiple antibiotic resistance genes to be linked, are found in many of the genera listed in Table 15.2, but *tet* genes are not generally associated with these elements [1, 2]. For an update of the number of tetracycline resistance genes please see http://faculty.washington.edu/marilynr/. This website gets updated at least twice each year

The antibiotic resistance genes can be distinguished from each other using molecular methods including DNA-DNA hybridization with oligonucleotide probes and DNA sequencing, microarrays, or more recently after the entire genome has been sequenced. Two genes are considered related, i.e., of the same class, and given the same gene designation if the gene products share ≥80 % of the amino acid sequences in common with each other. Two genes are considered different from each other if their gene products share ≤79 % amino acid sequence identity [6]. This comparison can now be done using GenBank sequence information. However over the last 10 years mosaic tetracycline-resistant genes are interclass hybrids between two or three different ribosomal protection proteins [7, 8]. The genes show different patterns of mosaicism but their final size remains the same [9]. No other types of interclass hybrid genes have yet been identified. To identify a mosaic *tet* gene, the complete gene needs to be sequenced since a probe will not normally be able to distinguish a mosaic from a normal ribosomal protection gene using probes alone [7–9].

A total of 45 different genes have been identified and characterized as conferring tetracycline resistance and most are identified as *tet* genes (Table 15.1). However, the innate genes found in *Streptomyces* in general have a different nomenclature because they were the genes first identified in oxytetracycline-producing organisms, and thus the nomenclature reflects the organisms first shown to carry the particular gene. A total of 126 different genera including 76 Gram-negative and 50 Gram-positive have been identified as carrying known acquired tetracycline resistance genes (Table 15.2). A limited number of bacteria acquire Tc^r by mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, change regulation of innate efflux systems, or alter the 16S rRNA [2]. Tetracycline resistance genes are often linked to other known genes and thus, when tetracycline resistance is acquired, the host may become multidrug resistant [1, 2].

M.C. Roberts, Ph.D. (✉)
Department of Environmental and Occupational Health Sciences, School of Public Health, University of Washington, Box 357238, Seattle, WA 98195, USA
e-mail: marilynr@uw.edu

S. Schwarz, Ph.D.
Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute (FLI), Neustadt-Mariensee, Germany

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_15

Table 15.1 Mechanism of resistance for characterized *tet* and *otr* genes

Efflux (29)	Ribosomal Protection (12)	Enzymatic (3)	Unknown[a]
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32),	*tet*(X)	*tet*(U)
tet(G), *tet*(H), *tet* (J), *tet*(V), *tet*(Y)	*tet*(Q), *tet* (T), *tet*(36)	*tet*(34)	
tet(Z), *tet*(30), *tet*(31), *tet*(33)	*otr*(A), *tetB*(P)[b], *tet*[c]	*tet*(37)	
tet(35)[d]	*tet*(44)		
tet(39), *tet*(41)			
tet (K), *tet*(L), *tet*(38), *tet*(45)			
tetA(P), *tet*(40)			
otr(B), *otr*(C)			
tcr3			
tet(42)			
tet(43)			
*tet*AB(46)[e]			

[a]*tet* (U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins
[b]*tetB*(P) is not found alone and *tetA*(P) and *tetB*(P) are counted as one operon
[c]*tet*(X) and *tet*(37) are unrelated but both are NADP-requiring oxidoreductases: *tet*(34) similar to the xanthine-guanine phosphoribosyl transferase genes of *V. cholerae*
[d]Not related to other *tet* efflux genes [4]
[e]Represents two different genes that are both required for resistance [5]

1.2 Chloramphenicol Resistance

Chloramphenicol resistance (Cm^r) is primarily due to the presence of chloramphenicol acetyltransferases (CATs) which inactive chloramphenicol [10]. There are two different types of CAT enzymes which are genetically unrelated (Table 15.3). Cm^r may also be due to the efflux of chloramphenicol via specific membrane-associated transporters [11]. Both, the genes coding for CATs and specific exporters, are often associated with mobile elements such as plasmids, transposons, or gene cassettes and are able to be transferred by conjugation, mobilization, transduction, or transformation between bacteria of different species and genera. Some chromosomal multidrug transporters have also been identified which export chloramphenicol [12]. Cm^r may also occur from mutations which reduce expression of outer membrane proteins [13], mutations in the 23S rRNA [14], inactivation of chloramphenicol by 3-*O*-phosphotransferases [15], or target site modification by a 23S rRNA methylase [16, 17].

In contrast to the tetracycline resistance genes, there is no internationally accepted nomenclature for chloramphenicol resistance genes currently available. However, when using the same criteria as for the classification of *tet* genes, 16 groups of classical chloramphenicol acetyltransferase genes (*cat*), at least six groups of *cat* genes of the second type, which occasionally are referred to as *xat* genes [10], and 11 different groups of genes coding for specific exporters can be distinguished (Table 15.3). In addition, a single gene, *cfr*, is known to code for the aforementioned rRNA methylase and to mediate resistance by target site modification [16, 17]. During whole genome sequencing, *cat*-like genes have been annotated in the genomes of several bacteria, e.g., *Brucella melitensis* (GenBank NC_003317) and *Bacillus cereus* (GenBank NC_004722). However, comparisons on both nucleotide and amino acid level revealed little, if no homology with the known chloramphenicol resistance determinants, and it has not been demonstrated whether these *cat*-like genes actually confer chloramphenicol resistance. More information on phenicol resistance can be found in two reviews [18, 19].

2 Mechanisms of Tetracycline Resistance

2.1 Tetracycline Resistance due to Efflux Proteins

Most of the *tet*, *tcr*, and *otr* genes code for tetracycline resistance efflux proteins and are part of the Major Facilitator Superfamily (MFS) of transports. The proteins are membrane-bound and exchange a proton for a tetracycline-cation complex against a concentration gradient [20]. This reduces the intracellular tetracycline concentration which in turn protects the ribosomes within the cell. These proteins share amino acid and protein structure similarities with other efflux proteins involved in multiple-drug resistance, quaternary ammonium resistance, chloramphenicol, and quinolone resistance [12]. The Gram-negative *tet*(B) gene codes for an efflux protein, which confers resistance to both tetracycline and minocycline, but not to the glycylcyclines [2]. All the other tetracycline efflux proteins confer resistance to tetracycline, but not to minocycline or glycylcyclines.

Table 15.2 Distribution of *tet, and otr* resistance genes

Mechanism	Gene	No. Gram-positive bacteria, *Mycobacterium*, *Mycoplasma*, *Nocardia*, *Streptomyces* and *Ureplasma*	No. Gram-negative	Total No Genera	Genera
		n=52	*n*=77	129	
Efflux *n*=29	*tet*(A)	0	24	24	*Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bordetella*, *Chryseobacterium*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia*, *Flavobacterium. Klebsiella*, *Laribacter*, *Ochrobactrum*, *Plesiomonas*, *Proteus*, *Pseudomonas*, *Rahnella*, *Rhizobium*, *Salmonella*, *Serratia*, *Shigella, Variovorax*, *Veillonella*, *Vibrio*
	tet(B)	0	33	33	*Acinetobacter*, *Actinobacillus*, *Aeromonas*, *Aggregatibacter*, *Brevundimonas*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Gallibacterium*, *Haemophilus*, *Klebsiella*, *Mannheimia*, *Moraxella*, *Neisseria*, *Ochrobactrum*, *Pantoea*, *Pasteurella*, *Plesiomonas*, *Photobacterium*, *Proteus*, *Providencia*, *Pseudoalteromonas*, *Pseudomonas*, *Roseobacter*, *Rhizobium*, *Salmonella*, *Serratia*, *Shewanella*, *Shigella*, *Treponema*, *Vibrio*, *Yersinia*
	tet(C)	0	16	16	*Aeromonas*, *Bordetella*, *Chlamydia*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Francisella*, *Halomonas*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Roseobacter*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*
	tet(D)	0	22	22	*Aeromonas*, *Alteromoas*, *Brevundimonas*, *Burkholderia*, *Chryseobacterium*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia*, *Halomonas*, *Klebsiella*, *Morganella*, *Pasteurella*, *Plesiomonas*, *Photobacterium*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Shewanella*, *Shigella*, *Vibrio*, *Yersinia*
	tet(E)	0	11	11	*Aeromonas*, *Alcaligenes*, *Klebsiella, Roseobacter*, *Escherichia*, *Flavobacterium*, *Proteus*, *Providencia*, *Pseudomonas*, *Serratia*, *Vibrio*
	tet(G)	0	16	16	*Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Enterobacter*, *Escherichia*, *Fusobacterium*, *Mannheimia*, *Ochrobactrum*, *Pasteurella*, *Proteus*, *Providencia*, *Pseudomonas*, *Roseobacter*, *Salmonella*, *Shewanella*, *Vibrio*
	tet(H)	0	12	12	*Acinetobacter*, *Actinobacillus*, *Aeromonas*, *Bibersteinia*, *Gallibacterium*, *Histophilus*, *Mannheimia*, *Moraxella*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Psychrobacter*
	tet(J)	0	3	3	*Escherichia*, *Morganella*, *Proteus*
	tet(K)	12	4	16	*Bacillus*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Gallibacterium*, *Haemophilus*, *Lactobacillus*, *Listeria*, *Mycobacterium*, *Nocardia*, *Peptostreptococcus*, *Sporosarcins*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Vibrio*
	tet(L)	23	19	42	*Acinetobacter*, *Actinobacillus*, *Actinomyces*, *Bacillus*, *Bifidobacterium*, *Bhargavaea*, *Citrobacter*, *Clostridium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Flavobacterium*, *Fusobacterium*, *Gallibacterium*, *Geobacillus*, *Kurthia*, *Lactobacillus*, *Listeria*, *Mannheimia*, *Morganella*, *Mycobacterium*, *Nocardia*, *Oceanobacillus*, *Ochrobactrum*, *Paenibacillus*, *Pasteurella*, *Pediococcus*, *Peptostreptococcus*, *Proteus*, *Pseudomonas*, *Rahnella*, *Robins oniella*, *Salmonella*, *Savagea*, *Sporosarcina*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Vagococcus*, *Variovorax*, *Veillonella*, *Virgibacillus*
	tetA(P)	1	0	1	*Clostridium*
	tet(V)	1	0	1	*Mycobacterium*
	tet(Y)	0	4	4	*Aeromonas*, *Escherichia*, *Photobacterium*
	tet(Z)	2	0	2	*Corynebacterium*, *Lactobacillus*
	tet(30)	0	2	2	*Agrobacterium*
	tet(31)	0	3	3	*Aeromonas*, *Bordetella*, *Gallibacterium*
	tet(33)	2	0	2	*Arthrobacter*, *Corynebacterium*
	tet(35)	0	2	2	*Stenotrophomonas*, *Vibrio*
	tet(38)	1	0	1	*Staphylococcus*
	tet(39)	4	8	12	*Acinetobacter*, *Alcaligenes*, *Bacillus*, *Brevundimonas*, *Cellulosimicrobium*, *Corynebacterium*, *Enterobacter*, *Lysinibacillus*, *Providencia*, *Pseudomonas*, *Psychrobacter*, *Stenotrophomonas*
	tet(40)	2	0	2	*Clostridium*, *Streptococcus*
	tet(41)	0	1	1	*Serratia*
	tet(42)	5	1	6	*Bacillus*, *Microbacterium*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Staphylococcus*
	tet(43)	1	0	1	*Staphylococcus*
	tet(45)	3	0	3	*Bacillus*, *Bhargavaea*, *Staphylococcus*
	*tet*AB (46)	1	0	1	*Streptococcus*
	tcr	1	0	1	*Streptomyces*
	otr(B)	2	0	2	*Mycobacterium*, *Streptomyces*
	otr(C)	1	0	1	*Streptomyces*

(continued)

Table 15.2 (continued)

Mechanism	Gene	No. Gram-positive bacteria, *Mycobacterium*, *Mycoplasma*, *Nocardia*, *Streptomyces* and *Ureplasma*	No. Gram-negative	Total No Genera	Genera
		$n=52$	$n=77$	129	
Ribosomal Protection $n=12$	*tet*(M)	39	38	77	*Abiotrophia*, *Acinetobacter*, *Actinomyces*, *Aerococcus*, *Aeromonas*, *Afipia*, *Alcaligenes*, *Amycolatopsis*, *Anaerococcus*, *Arthrobacter*, *Bacteroides*, *Bacterionema*, *Bacillus*, *Bifidobacterium*, *Bhargavaea*, *Brachybacterium*, *Catenibacterium*, *Cottaibacterium*, *Citrobacter*, *Clostridium*, *Corynebacterium*, *Edwardsiella*, *Eikenella*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Eubacterium*, *Erysipelothrix*, *Finegoldia*, *Flavobacterium*, *Fusobacterium*, *Gardnerella*, *Gemella*, *Granulicatella*, *Hafnia*, *Haemophilus*, *Kingella*, *Klebsiella*, *Kurthia*, *Lawsonia*, *Lactobacillus*, *Lactococcus*, *Leifsonia*, *Listeria*, *Microbacterium*, *Morgonella*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Norcardia*, *Paenibacillus*, *Pantoea*, *Pasteurella*, *Peptostreptococcus*, *Photobacterium*, *Prevotella*, *Proteus*, *Providencia*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Ralstonia*, *Rhanela*, *Rhizobium*, *Savagea*, *Selenomonas*, *Serratia*, *Shewanella*, *Shigella*, *Stenotrophomonas*, *Sporosarcina*, *Staphylococcus*, *Streptococcus*, *Ureaplasma*, *Veillonella*, *Vibrio*
	tet(O)	19	17	36	*Actinobacillus*, *Aerococcus*, *Aeromonas*, *Anaerovibrio*, *Bifidobacterium*, *Brevundimonas*, *Burkholderia*, *Butyrivibrio*, *Campylobacter*, *Citrobacter*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Fusobacterium*, *Gemella*, *Granulicatella*, *Lactobacillus*, *Lactococcus*, *Leifsonia*, *Megasphaera*, *Microbacterium*, *Mobiluncus*, *Mycobacterium*, *Neisseria*, *Nocardia*, *Paenibacillus*, *Pasteurella*, *Peptostreptococcus*, *Pseudomonas*, *Psychrobacter*, *Rhizobium*, *Rhodococcus*, *Shewanella*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*
	tetB(P)	1	0	1	*Clostridium*
	tet(Q)	8	11	19	*Anaerovibrio*, *Bacteroides*, *Capnocytophaga*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Gardnerella*, *Lactobacillus*, *Mitsuokella*, *Mobiluncus*, *Neisseria*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Ruminococcus*, *Selenomonas*, *Streptococcus*, *Subdolgranulum*, *Veillonella*
	tet(S)	6	3	9	*Citrobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Veillonella*
	tet(T)	2	7	9	*Aeromonas*, *Brevundimonas*, *Chryseobacterium*, *Enterococcus*, *Pseudomonas*, *Rhizobium*, *Shewanella*, *Stenotrophomonas*, *Streptococcus*
	tet(W)	10	21	31	*Acidaminococcus*, *Actinomyces*, *Arcanobacterium*, *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Brevundimonas*, *Butyrivibrio*, *Chryseobacterium*, *Citrobacter*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Fusobacterium*, *Klebsiella*, *Lactobacillus*, *Lawsonia*, *Megasphaera*, *Mitsuokella*, *Neisseria*, *Porphyromonas*, *Prevotella*, *Pseudomonas*, *Rhizobium*, *Roseburia*, *Selenomonas*, *Shewanella*, *Staphylococcus*, *Streptococcus*, *Subdolgranulum*, *Veillonella*
	tet(32)	2	0	2	*Eubacterium*, *Streptococcus*
	tet(36)	2	1	3	*Bacteroides*, *Clostridium*, *Lactobacillus*
	tet(44)	2	1	3	*Campylobacter*, *Clostridium*, *Staphylococcus*
	tet	1	0	1	*Streptomyces*
	otr(A)	3	0	3	*Bacillus*, *Mycobacterium*, *Streptomyces*
Enzymatic $n=3$	*tet*(X)	0	12	12	*Comamonas*, *Bacteroides*, *Delftia*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Riemerella*, *Roseobacter*, *Pseudomonas*, *Spingobacterium*, *Serratia*, *Wautersiella*
	tet(34)	0	4	4	*Aeromonas*, *Pseudomonas*, *Serratia*, *Vibrio*
	tet(37)	0	0	0	Uncultured
Unknown $n=1$	*tet*(U)	3	0	3	*Enterococcus*, *Staphylococcus*, *Streptococcus*

Adapted from [4]

Table 15.3 Acquired phenicol resistance genes

Mechanism	Group	Gene	Gene(s) included	Genera
Efflux	Type E-1	*cmlA1*	*cmlA*, *cmlA2*, *cmlA4*, *cmlA5*, *cmlA6*, *cmlA7*, *cmlA8*, *cmlA10*, *cmlB*	*Acinetobacter*, *Aeromonas*, *Arcanobacterium*, *Citrobacter*, *Comamonas*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Laribacter*, *Pantoea*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Staphylococcus*
	Type E-2	*cml*		*Escherichia*
	Type E-3	*floR*	*cmlA*-like, *flo*, *pp-flo*, *cmlA9*	*Acinetobacter*, *Actinobacillus*, *Aeromonas*, *Azospirillum*, *Bibersteinia*, *Bordetella*, *Edwardsiella*, *Enter obacter*, *Escherichia*, *Kaisatia*, *Klebsiella*, *Mannheimia*, *Pasteurella*, *Photobacterium*, *Providencia*, *Riemerella*, *Rhodospirillum*, *Salmonella*, *Shinella*, *Stenotrophomonas*, *Vibrio*
	Type E-4	*fexA*		*Bacillus*, *Enterococcus*, *Exiguobacterium*, *Nosocomiicoccus*, *Staphylococcus*, *Streptococcus*
	Type E-5	*cml*		*Streptomyces*
	Type E-6	*cmlv*		*Streptomyces*
	Type E-7	*cmrA*	*cmr*	*Rhodococcus*
	Type E-8	*cmr*	*cmx*	*Corynebacterium*
	Type E-9	*cmlB1*		*Bordetella*
	Type E-10	*fexB*		*Enterococcus*
	Type E-11	*pexA*		Uncultured
Inactivating enzyme	Type A-1	*catA1*	*cat*, *catI*, *pp-cat*	*Achromobacter*, *Acinetobacter*, *Aeromonas*, *Bifidobacterium*, *Citrobacter*, *Corynebacterium*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Herbaspirillum*, *Klebsiella*, *Morganella*, *Neisseria*, *Parabacteroides*, *Photobacterium*, *Proteus*, *Ruminococcus*, *Salmonella*, *Serratia*, *Shigella*
	Type A-2	*catA2*	*cat*, *catII*	*Aeromonas*, *Agrobacterium*, *Avibacterium*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Gallibacterium*, *Haemophilus*, *Legionella*, *Klebsiella*, *Morganella*, *Photobacterium*, *Proteus*, *Salmonella*, *Vibrio*
	Type A-3	*catA3*	*cat*, *catIII*	*Actinobacillus*, *Bibersteinia*, *Edwardsiella*, *Escherichia*, *Klebsiella*, *Mannheimia*, *Pasteurella*, *Providencia*, *Shigella*
	Type A-4	*cat*		*Proteus*
	Type A-5	*cat*		*Streptomyces*
	Type A-6	*cat86*		*Bacillus*
	Type A-7	*cat*(*pC221*)	*cat*, *catC*	*Bacillus*, *Enterococcus*, *Gallibacterium*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*
	Type A-8	*cat*(*pC223*)	*cat*	*Bacillus*, *Enterococcus*, *Lactococcus*, *Listeria*, *Staphylococcus*, *Streptococcus*
	Type A-9	*cat*(*pC194*)	*cat*, *cat-TC*	*Bacillus*, *Enterococcus*, *Gallibacterium*, *Lactobacillus*, *Paenibacillus*, *Ruminiclostridium*, *Staphylococcus*, *Streptococcus*
	Type A-10	*cat*		*Bacillus*
	Type A-11	*catP*	*catD*	*Clostridium*, *Neisseria*
	Type A-12	*catS*		*Streptococcus*
	Type A-13	*cat*		*Campylobacter*
	Type A-14	*cat*		*Aeromonas*, *Listonella*, *Photobacterium*, *Proteus*, *Vibrio*
	Type A-15	*catB*		*Clostridium*
	Type A-16	*catQ*		*Clostridium*
	Type B-1	*catB1*	*cat*	*Agrobacterium*
	Type B-2	*catB2*		*Acinetobacter*, *Aeromonas*, *Bordetella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Salmonella*
	Type B-3	*catB3*	*catB4*, *catB5*, *catB6*, *catB8*	*Achromobacter*, *Acinetobacter*, *Aeromonas*, *Bordetella*, *Citrobacter*, *Comamonas*, *Delftia*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Morganella*, *Pantoea*, *Proteus*, *Providencia*, *Pseudomonas*, *Raoultella*, *Riemerella*, *Salmonella*, *Serratia*, *Staphylococcus*, *Stenotrophomonas*, *Vogesella*
	Type B-4	*catB7*		*Pseudomonas*
	Type B-5	*catB9*		*Vibrio*
	Type B-6	*catB10*		*Pseudomonas*
rRNA methylase		*cfr*		*Bacillus*, *Enterococcus*, *Escherichia*, *Jeotgalicoccus*, *Macrococcus*, *Proteus*, *Staphylococcus*, *Streptococcus*
hydrolase		*estDL136*		Uncultured

Note: Last update: December 16th 2011. Adapted from; 16, 82 and nucleotide BLAST searches

The majority of the *tet* efflux genes codes for an approximately 46 kDa membrane-bound efflux protein. With the exception of the *tet*AB(46), the Gram-negative efflux genes in general have two functional domains, α and β which correspond to the N- and C-terminal halves of the protein, respectively. Experimental data suggests that residues dispersed across the protein are important for function [21]. Mutations affecting energy coupling have been located in cytoplasmic loops 2–3 and 10–11 of the efflux protein [22].

Sixteen of the *tet* genes [*tet*(A), (B), (C), (D), (E), (G), (H), (J), (Z), (30), (31), (33), (35), (39), (41), and (42)] have repressor genes upstream of the structural gene [20, 21]. Most of these *tet* genes have been linked to other antibiotic resistance genes such as those that confer aminoglycoside, β-lactamase, chloramphenicol/florfenicol, methicillin, streptomycin, sulfamethoxazole, trimethoprim, genes coding for mercury resistance, and/or *Salmonella* genomic island 1 [23]. The repressor genes are read in the opposite direction from the structural genes and regulate expression of the structural gene in the presence versus absence of tetracycline. Most of the repressors for regulation of the structural *tet* genes are associated exclusively with Gram-negative bacteria. However, the *tet*(Z) and *tet*(33) genes are of Gram-positive origin, but have repressors genes upstream from their structural gene, like the Gram-negative efflux genes. These were the first Gram-positive efflux proteins identified that were shown to be controlled by a repressor protein [24, 25]. The *tet*(33) has been linked to *aadA9* encoding for aminoglycoside resistance and IS*6100* [25]. The *tet*(40) gene has a 42 % G+C content and has been linked with the mosaic *tet*(O/32/O) gene [23].

In contrast, the Gram-positive *tet*(K) and *tet*(L) efflux genes, with G+C % of 28–40 %, are not regulated by repressors but by translational attenuators, share 58–59 % amino acid identity with each other, and confer resistance to tetracyclines, but not minocycline. The *tet*(K) and *tet*(L) genes are generally found on small transmissible plasmids in Gram-positive bacteria, which on occasion these plasmids or the *tet* genes have been associated with the chromosome. These genes have been linked to trimethoprim resistance genes (*dfrK*) [23]. A number of plasmid borne *tet*(L) genes have been sequenced and share between 98 and 99 % sequence identity with each other, while the chromosomal *tet*(L) gene from *B. subtilis* has only 81 % amino acid sequence identity with the other *tet*(L) genes [26]. Both *tet* genes were originally isolated in Gram-positive genera, but now the *tet*(K) gene has been isolated in four Gram-negative genera, while 19/42 (45 %) of the genera identified in Table 15.2 carrying the *tet*(L) gene are Gram-negative. The *tet*(K) gene has been linked to mercury resistance genes [23].

The *tet*(39) gene was first isolated in Gram-negative bacteria and has a G+C % of 40 %. It has now been isolated from Gram-positive genera bringing into question the ancestral source of this gene [27, 28]. The *tet*(40) gene has a 42 % G+C content and has been linked with the mosaic *tet*(O/32/O) gene [23]. The *tet*(43) gene also has a higher G+C % of 65 % though it has only been identified in one Gram-positive genus (Table 15.2). The *tet*(45) has a G+C % of 34 % and found in Gram-positive bacteria (Table 15.2).

The TetP operon from *Clostridium perfringens* consists of two overlapping genes, *tetA*(P) (G+C % 30 %) and *tetB*(P) (31 %). The *tetA*(P) gene codes for an efflux protein, but does not have the conserved motifs that are common in the other *tet* efflux proteins [29]. There are some sequenced differences between the *tetA*(P) gene which is found alone vs. those that overlap with the *tetB*(P) gene [30]. The *tetB*(P) gene codes for a protein with amino acid identity of 37–39 % to the Tet(M) ribosomal protection protein. The *tetA*(P) gene has been isolated from C*lostridium* spp. without the *tetB*(P) gene but the reverse has not been found. The recently identified *tet*AB(46) gene codes for two nonidentical proteins and it is suggested that they function as a heterodimeric ABC transporter requiring both proteins for function and tetracycline resistance and have 45 % G+C content. There have not been any studies to verify physical interaction between the two proteins. But in-frame deletions of either *tet*A(46) or *tet*B(46) demonstrated that both proteins are needed for tetracycline resistance in the original host [5].

2.2 Tetracycline Resistance due to Ribosomal Protection Proteins

Twelve ribosomal protection proteins have been described (Table 15.1). These genes code for cytoplasmic proteins that protect the ribosomes from the action of tetracycline in vitro and in vivo and confer resistance to tetracycline, doxycycline, and minocycline. Most studies have used the Tet(M) and/or Tet(O) proteins which share ~75 % sequence similarity and ~45 % identity with elongation factor G (EF-G) [2, 3, 31]. The tetracycline ribosomal protection proteins are grouped together within the translation factor superfamily of GTPases. The Tet(M) and Tet(O) proteins catalyze the release of tetracycline from the ribosomes in a GTP-dependent fashion. Similar mechanism is thought to occur with all the tetracycline ribosome protection proteins. Recently using a cryo-EM structure of the Tet(M)-GDPNP-70S complex the authors suggest that the Tet(M) protein confers resistance to tetracycline by directly interacting and altering the conformation of the nucleotide C1054 within h34 of the 16S rRNA, which is part of the tetracycline binding site. The altered conformation of the C1054 changes the stacking interaction with tetracycline leading to dissociation from the ribosome and prevents rebinding and promotes binding of the ternary complex EF-Tu-GTP-aa-tRNA complex [3]. This mechanism

works in vivo and in vitro while the tetracycline resistance efflux proteins require intact membranes to function. These *tet* genes may be evolutionarily derived from the elongation factors but they cannot substitute for the elongation factors in vivo or in vitro [31].

The Tet(M), Tet(O), Tet(S), Tet(T), Tet(W), Tet(32), Tet(36), Tet(44) share between 78 and 64 % amino acid identity [32, 33]. The G+C % content of seven of these genes varies from 30 % [*tet*(44)] to 40 % and are thought to be of Gram-positive origin. The exception is the *tet*(W) gene which has a 53 % G+C % [34] and it is unclear what is the ancestral source of the *tet*(W) gene. All eight of these *tet* genes are found in both Gram-positive and Gram-negative genera (Table 15.2). The *tetB*(P) gene is less related than the other eight ribosomal protection *tet* genes. When the *tetB*(P) gene was cloned, alone it conferred on the host very low levels of tetracycline resistance (3 μg/mL) [30]. The genes isolated originally from *Streptomyces* [*tet*, *otr*(A)] have higher G+C % >70 % as would be expected (Table 15.2).

To date all mosaic genes are associated with rearrangements of ribosomal protection genes. Combinations of *tet*(O), *tet*(W) and/or *tet*(32) or *tet*(M) and *tet*(S) have been identified in Gram-positive bacteria while combinations of *tet*(O), *tet*(W) have been found in Gram-negative bacteria [4, 35, 36]. Why the mosaic ribosomal protection proteins are found in certain environments are not known nor is the advantages, if any, of carrying mosaic *tet* genes provides to its host.

The *tet*(M) gene is usually associated with conjugative transposons like Tn*916*-Tn*1545* family [37]. This family of transposons includes small elements that just carry the *tet*(M) gene to large elements carrying multiple different antibiotic resistance genes including one or more of the following: *erm*(B) gene, coding for an rRNA methylase which confers macrolide, lincosamide, and streptogramin B resistance, *mef*(A), *msr*(D) coding for macrolide resistance, aminoglycoside phosphotransferase gene *aphA-3* encoding kanamycin resistance (Km^r), *cat* genes coding for chloramphenicol resistance (Cm^r), *mer* operon coding for mercury resistance, *tet*(B) gene and other transposons such as Tn*917* [1, 2, 23]. The combination of *tet*(M) and *erm*(B) genes is often found in Gram-positive bacteria, such as streptococci, staphylococci, and enterococci [1, 2, 23]. The presence of the classical *cat* and *aphA-3* genes within these common transposons may explain why Cm^r and/or Km^r *Streptococcus pneumoniae* strains continue to be isolated in areas were the use of these antibiotics have been stopped [34, 37, 38].

The *tet*(O) gene is linked to the *mef*(A) and *msr*(D) macrolide resistance genes. The *tet*(O) gene has been associated with a conjugative transposon which also carries a *mef*(A) gene, that confers efflux of erythromycin out of the cell, in *Streptococcus pyogenes* isolates from Italy [39]. While the *tet*(Q) gene is associated the *erm*(F) gene and has been found in a number of aerobic and aerobic genera [40]. The *tet*(Q) gene has also been linked to *erm*(B), *erm*(G), *mef*(A), and *msr*(D) macrolide resistance genes in addition to *Bacteroides* conjugative transposons [23].

2.3 Tetracycline Resistance due to Enzymatic Inactivation

The *tet*(X) gene encodes an enzyme, which modifies and inactivates the tetracycline molecule. It was originally found in a strict anaerobe, *Bacteroides*, where oxygen is excluded [41] linked to the *erm*(F) gene which does function in *Bacteroides*. It is unlikely that the *tet*(X) gene functions in *Bacteroides*, while the *erm*(F) gene does. The *tet*(X) gene has a G+C % of 38 % and has now been found in eleven other genera (Table 15.2) where the gene can be expressed. The Tet(X) is a cytoplasmic protein that chemically modifies tetracycline in the presence of both oxygen and NADPH. Sequence analysis indicates that this protein shares amino acid homology with other NADPH-requiring oxidoreductases. A second gene, *tet*(37), has been identified from the oral human microbiome in two studies [42, 43]. This protein also requires oxygen to function and should function in aerobic but not anaerobic species. Unfortunately, no attempt to determine the host(s) of this gene has been done (Table 15.2). The *tet*(34) gene was first identified in the chromosome of *Vibrio* sp. [44]. The protein has homology to xanthine-guanine phosphoribosyl transferase genes which act in purine nucleotide salvage synthesis. The authors suggest that the mechanism of the Tet(34) protein is the activation of Mg^{2+}-dependent purine nucleotide synthesis which protects protein synthesis [44].

2.4 Other/Unknown Mechanisms of Tetracycline Resistance

The *tet*(U) gene confers low level tetracycline resistance [2]. This gene produces a small protein (105 amino acids) which is smaller than the efflux and the ribosomal proteins. There is 21 % similarity over the 105 amino acids between the Tet(U) and Tet(M) proteins beginning close to the carboxy terminus of the latter. These similarities do not include the consensus GTP-binding sequences, important for resistance in the ribosomal protection proteins. Thus, it is unclear what the mechanism of resistance is for the Tet(U) protein. However recently, the *tet*(U) gene has been found in functional genomic assays suggesting that it can confer tetracycline

resistance under these types of laboratory conditions using 20 μg/mL tetracycline selection (Roberts et al., unpublished observations). It has now been identified in three Gram-positive genera (Table 15.2).

2.5 Tetracycline Resistance due to Mutations

Laboratory-derived mutations in the *tet*(A) and the *tet*(B) gene have led to glycylcycline resistance suggesting that bacterial resistance due to mutations in the tetracycline efflux proteins may develop over time and with clinical use to glycylcycline [2]. Mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, can also affect the bacterial host's resistance to tetracycline [45]. Tc^r *Helicobacter pylori* and *Mycobacterium avium* complex and the anaerobic spirochete *Brachyspira hyodysenteriae* also have been identified with chromosomal mutations which increase tetracycline minimal inhibitory concentrations [46, 47]. Mutations that upregulate innate efflux pumps can alter the host's susceptiblity to tetracycline. One example is *Neisseria gonorrhoeae* which has an innate *mtrCDE*-encoded efflux pump. A 1 base pair deletion of an A within the 13 bp inverted repeat sequence of the *mtrR* promoter region leads to a fourfold increased resistance to tetracycline, penicillin, and erythromycin [48]. In *N. gonorrhoeae*, the chromosomally mediated resistance is often more common than plasmid-mediated antibiotic resistance [49]. A new review on efflux pumps and their association with increased resistance to antibiotics including tetracycline can be found at Sun et al. [50].

3 Mechanisms of Chloramphenicol Resistance

3.1 Chloramphenicol Resistance due to Chloramphenicol *O*-Acetyltransferases

Both types of CAT enzymes have a trimeric structure normally composed of three identical monomers which is encoded by the *cat* gene [10]. The classical CAT monomers vary between 207 and 238 amino acids, whereas those of the second type of CATs are smaller with 209–219 amino acids. All CATs transfer an acetyl group from a donor molecule (usually acetyl-CoA) to the C3 position of the chloramphenicol. This acetyl group is then shifted from C3 to C1 and the C3 position is again available for a second acetylation step. Neither the mono- nor di-acetylated chloramphenicol molecules have antimicrobial activity [10]. None of the CAT enzymes are able to inactivate florfenicol, a chloramphenicol derivative that is exclusively licensed for use in animals [51], because the C3 position is fluorinated in the florfenicol molecule. As a result, the C3 position of florfenicol cannot act as acceptor site for acetyl groups making florfenicol resistant to inactivation by these enzymes.

The classical CATs represent a highly diverse group of enzymes which show an overall identity of 44 %. These enzymes have been detected in Gram-positive and Gram-negative, aerobic and anaerobic bacteria (Table 15.3). They can be placed into 22 genetic groups using ≥80 % amino acid identity to define a group. However, 15 of the groups have a single gene from a single species (Table 15.2). The CATI, CATII, and CATIII, which represent members of the first three genetic groups in Table 15.2, are exclusively found in Gram-negative genera and are expressed constitutively. The genes coding for these enzymes have been completely sequenced and the biochemical and enzymatic characteristics of the proteins studied in detail [52]. The CATIII enzyme was the first to be crystallized and provided insight into the folding of the CAT monomers and helped to identify the amino acids that were important for the structure and the function of the CAT enzyme [53].

The next three genetic groups of classical CAT were named according to the plasmids (pC221, pC223/pSCS7, and pC194), on which they were first detected. These have been identified in a variety of Gram-positive genera (Table 15.2). The K_M values for chloramphenicol and acetyl-CoA, the isoelectric point, pH optimum, and thermostability for the CATs associated with the Gram-positive plasmids have been determined [52, 54]. These *cat* genes are induced by chloramphenicol and have translational attenuators located immediately upstream of the respective *cat* genes which resemble those located upstream of the tetracycline resistance genes *tet*(K) and *tet*(L) [55].

The closely related CATP and CATD proteins were first identified in the Gram-positive anaerobe *Clostridium* sp. where they are located on transposons [56]. These genes have also been identified in Cm^r Gram-negative *Neisseria meningitidis* [57, 58]. This group is unusual because members are found in both Gram-positive and Gram-negative genera (Table 15.2). Both genes are expressed constitutively.

The second type of CAT enzymes are only distantly related to the classical CATs and are structurally similar to acetyltransferases involved in streptogramin A resistance [10]. At least five different genetic groups can be distinguished, though all enzymes have approximately 77 % identity with each other. These *cat* genes are often associated with gene cassettes and integrons in Gram-negative bacteria [59]. Some of these *cat* genes have also been identified in transposons. The CAT protein from *Agrobacterium tumefaciens* has different acetylation kinetics when compared to the classical CATIII enzyme [10]. This difference might explain the distinctly lower chloramphenicol MIC mediated by this CAT protein. Because of this lower resistance level, it was speculated that members of this second type of CATs might have a physiological role, other than chloramphenicol resistance in their host bacteria, though little else is known about these enzymes [10].

3.2 Chloramphenicol Resistance due to Specific Exporters

The specific exporters involved in the export of either chloramphenicol or chloramphenicol and florfenicol are members of the Major Facilitator Superfamily of efflux proteins [12] and commonly exhibit 10–14 transmembrane segments (TMS) [11]. There are 11 genetic groups, though nine are found in a single genus including six from soil and environmental bacteria (Table 15.3). The *cmr* and *cmx* genes are found on plasmids, while the *cmx* gene is associated with a transposon [11]. The *Rhodococcus* genes are associated with plasmids, while the *cmrA* is located on transposon Tn*5561*. The *Streptomyces venezuelae* Cmlv protein is thought to play a role in self-defense of the antibiotic producer from its own products. Several closely related *cmlA* genes have been identified on gene cassettes in Gram-negative bacteria and unlike other cassette-borne genes, *cmlA*, is inducibly expressed by a translational attenuator, similar to that of staphylococcal *cat* genes [59, 60]. Another type of chloramphenicol exporter, CmlB1, which shares 74–77 % identity with the known CmlA proteins, was identified on a plasmid from *Bordetella bronchiseptica* [61]. The *cmlB1* gene is also preceded by a translational attenuator and inducibly expressed. Both the CmlA and CmlB1 proteins cannot efficiently export florfenicol from the bacterial cell and bacteria with these genes are florfenicol susceptible.

Resistance to both chloramphenicol and florfenicol is characteristic for the group comprising *floR* and *pp-flo* genes. A review of these genes can be found in Butaye et al. [11]. The *pp-flo* gene was detected in the fish pathogen *Photobacterium damselae* subsp. *piscicida* [62]. The *floR* gene can be found in the chromosome of multiresistant *Salmonella enterica* serovars including Typhimurium DT104, *Vibrio cholerae*, *E. coli*, *B. bronchiseptica*, and *Acinetobacter baumannii*, or on plasmids of *E. coli*, *Salmonella* Newport, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Pasteurella trehalosi*, and *Stenothrophomonas maltophila*. The *floR* gene has been identified as part of the chromosomally located integrative and conjugative element ICE*Pmu1* from *Pasteurella multocida*, which carries and transfers a total of 12 antimicrobial resistance genes [63].

The gene *fexA* is part of transposon Tn*558* and was first identified on a *Staphylococcus lentus* plasmid. Expression of *fexA* is inducible with either chloramphenicol or florfenicol. A translational attenuator similar to those of *cat* genes from *Staphylococcus* sp. and *Bacillus pumilus* was identified immediately upstream of the *fexA* gene [64]. A *fexA* gene variant (*fexAv*), which conferred only chloramphenicol resistance, was recently identified in *Staphylococcus pseudintermedius* [65]. The exporter FexAv exhibited two amino acid substitutions, Gly33Ala and Ala37Val, both of which seem to be important for substrate recognition. Site-directed mutagenesis, that reverted the mutated base pairs to those present in the original *fexA* gene, restored the chloramphenicol/florfenicol resistance phenotype. A novel florfenicol and chloramphenicol resistance gene, designated *pexA*, was discovered in Alaskan soil using a metagenomic approach [66]. The gene *fexB*, which also confers resistance to chloramphenicol and florfenicol, was found on non-conjugative plasmids of *Enterococcus faecium* and *Enterococcus hirae* [67]. It shows 59.7 % and 37.2 % nucleotide identity with the genes *fexA* and *pexA*, respectively, while the deduced FexB protein sequence exhibited 56.1 % and 15.6 % amino acid identity with the FexA and PexA protein sequences, respectively.

3.3 Phenicol Resistance due to Multidrug Transporters, Permeability Barriers, Mutations, Phosphorylation, Hydrolysis, or Target Site Methylation

Multidrug transporter systems assigned to the Resistance/Nodulation/Cell Division family have been reported to export phenicols from the bacterial cell and include the AcrAB-TolC system in *Escherichia coli* [68] and the MexAB-OprM and MexCD-OprJ systems in *Pseudomonas aeruginosa* [12]. In Gram-positive bacteria, several 12-TMS multidrug transporters of the Major Facilitator Superfamily, such as Blt and Bmr proteins from *Bacillus subtilis* and NorA from *Staphylococcus aureus*, have been reported to have a substrate spectrum that includes chloramphenicol [12]. Another two closely related 12-TMS multidrug efflux proteins, MdfA and Cmr, have been identified in *Escherichia coli* [69, 70].

Cm^r Gram-negative bacteria may be due to the loss or a distinct decrease in the expression of outer membrane proteins which serve as the entry for chloramphenicol into the bacterial cell. Examples have been reported in *Haemophilus influenzae* [71] and *Salmonella enterica* serovar Typhi [13]. Activation of the *mar* locus in *Enterobacteriaceae* may also play a role in the decreased expression of the outer membrane protein OmpF by producing an antisense RNA that interferes with the translation of *ompF* transcripts.

Several mutations in the 23S rRNA of *E. coli* [14] are known to confer Cm^r. Deletions of 6 bp in the gene coding for the ribosomal protein L4 in *Streptococcus pneumoniae* have been reported to confer simultaneous resistance to chloramphenicol, oxazolidinones, and macrolides [72]. Inactivation of chloramphenicol by *O*-phosphorylation has only been observed in the chloramphenicol producer *Streptomyces venezuelae* and is believed to contribute to self-defense of the host [73].

The gene *estDL136* from a soil metagenome library was found to specify a hydrolase which, when cloned in *Escherichia coli*, inactivated both chloramphenicol and

florfenicol [74]. A chloramphenicol hydrolase gene from the chloramphenicol producer *Streptomyces venezuelae* has also been identified and is considered to protect the organism from its antibiotic product [75].

4 Distribution of Resistance Genes

4.1 Distribution of Tetracycline Genes

The 29 Gram-negative *tet* efflux genes have been found in aerobic and facultative anaerobic Gram-negative genera and less commonly in anaerobic Gram-negative genera (Table 15.2). Many new studies have been done looking at the presence of tet genes in various environments, and over the last 5 years the number of different genera carrying these genes have greatly increased [4, 23, 27]. Seventeen are found in Gram-negative genera, of which four, *tet*(K) [$n=16$], *tet*(L) [$n=42$], *tet*(39) [$n=12$], and *tet*(42) [$n=6$] efflux genes, are found in both Gram-negative and Gram-positive species. Twelve *tet*/*otr* genes are found only in Gram-positive and/or *Mycobacterium* and/or *Streptomyces* genera (Table 15.2). The *tet*(B) gene has the widest host range among the Gram-negative genera ($n=33$), while the *tet*(L) gene now has the widest host range of all the efflux genes because it has been identified in 23 Gram-positive and 19 Gram-negative genera. The *tet*(A) gene is the next most common being present in 24 Gram-negative genera, followed by the *tet*(D) in 22, the *tet*(C) in 16, the *tet*(G) in 16, the *tet*(E) in 11, the *tet*(H) in 12, the *tet*(J), and *tet*(31) genes in 3 different Gram-negative genera, and the *tet*(35), and *tet*(41) in 1 genus each (Table 15.2). Among the 12 Gram-positive *tet*/*otr* genes, three are found only in *Streptomyces*, one in both *Mycobacterium* and *Streptomyces*, one in *Mycobacterium*, one in *Clostridium*, and the remaining six genes found in 2–3 Gram-positive genera. However, limited surveillance studies have been done with these 12 efflux genes.

A number of different *tet* efflux genes have been identified in *Aeromonas* [$n=13$], *Escherichia* [$n=12$], *Pseudomonas,* [$n=13$ genera], and *Vibrio* [$n=10$ genera] (Table 15.2). One study found a correlation between the plasmid incompatibility group and a particular *tet* gene the plasmid carried [76]. There are also some *tet* efflux genes, such as *tet*(E), that are associated with genera found in the environment rather than people or animals. The *tet*(C) gene has been found in the obligate intracellular bacteria *Chlamydia suis* chromosome [77]. This marks the first description of any acquired antibiotic resistance gene identified in obligate intracellular bacteria. Associated with the *tet*(C) gene was an ISCS605 element similar to those found in *Helicobacter*. In addition, a 10.1 kb fragment shared 99 % identity between the *C. suis* genetic island and the *Aeromonas salmonicida* plasmid pRAS3.2. One can only speculate how the *tet*(C) gene came to be linked to a genetic island that has components from two distantly unrelated genera that inhabit distinctive ecological niches. However, this data suggests that even obligate intracellular bacteria like *Chlamydia* sp. are able to exchange and acquire tetracycline resistance genes.

Twelve ribosomal protection genes have been identified and most are of Gram-positive origin (see above). Eight of these genes have been identified in Gram-positive and Gram-negative genera (Table 15.2). The *tet*(M) and *tet*(Q) genes are generally associated with conjugative chromosomal elements, which code for their own transfer [78] Conjugative transposons appear to have less host specificity than do plasmids, which may explain why the *tet*(M) gene is found naturally in 77 different genera [including 39 Gram-positive and 38 Gram-negative genera]. The *tet*(O) gene has been found in 36 genera [19 Gram-positive and 17 Gram-negative genera], while the *tet*(W) gene has been identified in 31 different genera [10 Gram-positive and 21 Gram-negative genera]. The *tet*(Q) gene has been found in 19 genera [8 Gram-positive and 11 Gram-negative genera], while the *tet*(S) and *tet*(T) genes have been detected in 9 genera each (Table 15.2). The remaining *tet* genes are found in 1–3 Gram-positive genera (Table 15.2).

The *tet*(X) gene is found in anaerobic *Bacteroides* sp. though it is unlikely to have much clinical relevance in this host. It was considered an oddity until the recent identification of a second gene *tet*(37) with the same mechanism of action though genetically unrelated [42]. Unfortunately, the host(s) of the *tet*(37) gene is not known. More work needs to be done to understand the role these two genes may have in nature.

The *tet*(U) gene has now been identified in 3 Gram-positive genera. It also has been found by functional genomics (Dr. Roberts' unpublished observations) that more work with this gene and its associated protein is needed to understand if the protein does code for tetracycline resistance and what the mechanism of resistance is.

4.2 Distribution of Chloramphenicol Resistance Genes

A wide distribution of the classical *cat* genes has been identified for 7 of the 22 groups (Table 15.2). The Tn*9*-borne *catA1* (*catI*) gene has been found in a multiple genera of Gram-negative bacteria. Besides chromosomal locations, the *catA1* gene is often detected on large plasmids that carry additional resistance genes. The plasmid-borne gene *catA2* (*catII*) is frequently associated with Cm^r *Haemophilus* sp. [78], but has also been found on plasmids from *Photobacterium damselae* and *Agrobacterium tumefaciens*. The gene *catA3* (*catIII*) from *Shigella flexneri* has also been detected in bacteria different from *Enterobacteriaceae*. This gene represents part of plasmid-borne multi-resistance gene clusters in

Mannheimia spp. [79] and uncultured eubacteria. The staphylococcal *cat*-carrying plasmids pC221, pC223/pSCS7, and pC194 are small plasmids of <5 kb in size that only mediate Cm^r [80]. These small plasmids have also been isolated in *Bacillus* sp. [80]. Naturally occurring co-integrates between pC221 and pS194, a small staphylococcal streptomycin resistance plasmid, have also been detected. Plasmids similar to pC221 can also recombine with larger plasmids to form new resistance plasmids that have a broader host range, extended transfer abilities, and carry additional resistance genes like the conjugative plasmid pIP501, which has a pC221-like *cat* gene, the macrolide resistance gene *erm*(B) gene, and the Tc^r gene *tet*(M) [81]. The *cat* genes of the pC221 group have been detected in *Staphylococcus* sp., *Streptococcus agalactiae*, *Enterococcus faecalis*, and *Bacillus subtilis*, while the *cat* gene of the pC223 has been found on plasmids from *Staphylococcus* sp., *Listeria monocytogenes*, *Lactococcus lactis*, and *Enterococcus faecium*. The pC194-like *cat* genes have been identified in *Staphylococcus aureus*, *Enterococcus faecium*, *Lactobacillus reuteri*, and *Streptococcus suis*.

The *catB1*gene from *Agrobacterium tumefaciens*, the *catB7* gene from *Pseudomonas aeruginosa*, and the *catB9* gene from *Vibrio cholerae* have been found exclusively on the chromosome. In contrast, the Tn*2424*-borne *catB2* gene has been detected on plasmids from Gram-negative enteric genera and in the chromosome of *Shewanella oneidensis*. The closely related *catB3–catB8* genes are usually located on plasmids and are widespread among various Gram-negative genera (Table 15.2).

The first two groups of specific exporter genes are most widespread among Gram-negative bacteria (Table 15.2). The cassette-borne *cmlA* group is frequently found on multiresistance integrons or associated with transposons located on conjugative and/or nonconjugative plasmids in *Pseudomonas aeruginosa* and various enteric genera. The genes *fexA* and *fexB* are associated with staphylococcal and enterococcal plasmids, respectively. The gene *cfr* has been identified mainly on plasmids, but also in the chromosomal DNA of *Staphylococcus* sp., *Bacillus* sp., *Enterococcus* spp., *Macrococcus caseolyticus*, *Jeotgalicoccus pinnepedialis*, *Streptococcus suis*, *Proteus vulgaris*, and *Escherichia coli* [82, 83]. It should be noted that independent acquisition of mobile elements carrying *cat*, *cmlA*, *floR,* or *cfr* genes can lead to the simultaneous occurrence of more than one type of phenicol resistance gene in the same bacterium. Thus, multiresistant isolates of *Salmonella enterica* servar Typhimurium DT104 var. Copenhagen proved to carry a *catA1* gene in addition to a *floR* gene [84], while *catA2* and *catA3* genes were detected on the same plasmids of *Klebsiella pneumoniae* [85] and *catA3* together with *floR* on a plasmid from *Pasteurella trehalosi* [86]. Plasmids carrying *fexA* and *cfr* or *fexB* and *cfr* have also been described [82].

5 Conclusion

Bacterial resistance to tetracycline and/or chloramphenicol due to acquisition of new genes and/or mutation of existing genes has increased over the last 30 years, especially as environmental sources are examined. The environment may have many new genes that are not found in bacteria traditionally associated with animals or humans. Resistance levels vary by geography and by species, but many pathogenic and opportunistic bacteria are resistant to one or both of these antibiotics and spread between animals, humans and the environment is clearly occurring. Acquired genes are often associated with mobile elements which provide flexibility to host bacteria and help in the spread and distribution of these genes across diverse bacterial populations. Multiple antibiotic resistance genes can be clustered on individual mobile elements, which allows for multi-resistance to be transferred increasing the multidrug-resistant bacterial population. Unless the overall use of antibiotics changes, this trend is likely to continue reducing the usability of current therapies as outlined in recent reports from CDC and WHO [87, 88].

References

1. Roberts MC. MiniReview: update on acquired tetracycline resistance genes. FEMS Microbiol Lett. 2005;245:195–203.
2. Chopra I, Roberts MC. Tetracycline antibiotics: mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol Mol Bio Rev. 2001;65:232–60.
3. Donhofer A, Franckenberg S, Wickles S, et al. Structural basis for TetM-mediated tetracycline resistance. Proc Natl Acad Sci U S A. 2012;109:16900–5.
4. Nomenclature for Tetracycline Genes. http://faculty.washington.edu/marilynr/. Accessed 13 June 2014.
5. Warburton PJ, Ciric L, Lerner A, et al. TetAB(46), a predicted heterodimeric ABC transporter conferring tetracycline resistance in *Streptococcus australis* isolated from the oral cavity. J Antimicrob Chemother. 2013;68:17–22.
6. Levy SB, McMurry LM, Barbosa TM, et al. Nomenclature for new tetracycline resistance determinants. Antimicrob Agents Chemother. 1999;43:1523–4.
7. Levy SB, McMurray L, Roberts MC. Tet protein hybrids. Antimicrob Agents Chemother. 2005;49:3099.
8. Stanton TB, Humphrey SB, Scott KP, et al. Hybrid *tet* genes and *tet* gene nomenclature: request for opinions. Antimicrob Agents Chemother. 2005;49:1265–6.
9. Palmieri C, Maji G, Mingoia M, et al. Characterization of a *Streptococcus suis tet*(O/W/32/O)-carrying element transferable to streptococcal pathogens. Antimicrob Agents Chemother. 2012; 56:4697–702.
10. Murray IA, Shaw WV. *O*-Acetyltransferases for chloramphenicol and other natural products. Antimicrob Agents Chemother. 1997;41: 1–6.
11. Butaye P, Cloeckaert A, Schwarz S. Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. Int J Antimicrob Agents. 2003;22: 205–10.
12. Paulsen IT, Brown MH, Skurray RA. Proton-dependent multidrug efflux systems. Microbiol Rev. 1996;60:575–608.

13. Toro CS, Lobos SR, Calderon I, et al. Clinical isolate of a porin-less *Salmonella typhi* resistant to high levels of chloramphenicol. Antimicrob Agents Chemother. 1990;34:1715–9.
14. Ettayebi M, Prasad SM, Morgan EA. Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*. J Bacteriol. 1985;162:551–7.
15. Izard T, Ellis J. The crystal structures of chloramphenicol phosphotransferase reveal a novel inactivation mechanism. EMBO J. 2000;19:2690–700.
16. Kehrenberg C, Schwarz S, Jacobsen L, et al. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Mol Microbiol. 2005; 57:1064–73.
17. Long KS, Poehlsgaard J, Kehrenberg C, et al. The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. Antimicrob Agents Chemother. 2006;50:2500–5.
18. Schwarz S, Kehrenberg C, Doublet B, et al. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev. 2004;28:19–542.
19. Schwarz S, White DG. Phenicol resistance. In: White DG, Alekshun MN, McDermott PF, editors. Frontiers in antimicrobial resistance: a tribute to Stuart B. Levy. Washington, DC: American Society for Microbiology, ASM Press; 2005. p. 124–47.
20. Yamaguchi A, Ono N, Akasaka T, et al. Metal-tetracycline/H^+ antiporter of *Escherichia coli* encoded by a transposon, Tn*10*. J Biol Chem. 1990;265:15525–30.
21. McNicholas P, McGlynn M, Guay GG, et al. Genetic analysis suggests functional interactions between the N- and C-terminal domains of the TetA(C) efflux pump encoded by pBR322. J Bacteriol. 1995;177:5355–7.
22. McNicholas P, Chopra I, Rothstein DM. Genetic analysis of the TetA(C) gene on plasmid pBR322. J Bacteriol. 1992;174: 7926–33.
23. Roberts MC. Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline resistant bacteria. In: Keen P, Montforts M, editors. Antibiotic resistance in the environment. Hoboken: Wiley; 2012. p. 93–121.
24. Tauch A, Puhler A, Kalinowski J, et al. TetZ, a new tetracycline resistance determinant discovered in gram-positive bacteria, shows high homology to gram-negative regulated efflux systems. Plasmid. 2000;44:285–91.
25. Tauch A, Gotker S, Puhler A, et al. The 27.8-kb R-plasmid pTET3 from *Corynebacterium glutamicum* encodes the aminoglycoside adenyltransferase gene cassette *aadA9* and the regulated tetracycline efflux system Tet 33 flanked by active copies of the widespread insertion sequence IS*6100*. Plasmid. 2002;48:117–29.
26. Stasinopoulos SJ, Farr GA, Bechhofer DH. *Bacillus subtilis tetA*(L) gene expression: evidence for regulation by translational reinitiation. Mol Microbiol. 1998;30:923–32.
27. Adelowo OO, Fagade OE. The tetracycline resistance gene *tet39* is present in both Gram-negative and Gram-positive bacteria from a polluted river, Southwestern Nigeria. Lett Appl Microbiol. 2009; 48:167–72.
28. Roberts MC, No D, Kuchmiy E, Miranda CD. The tetracycline resistant tet(39) gene identified in three new genera of bacteria isolated in 1999 from Chilean Salmon farms. J Antimicrob Chemother. 2015;70:619–20.
29. Johanesen PA, Lyras D, Bannam TL, et al. Transcriptional analysis of the *tet*(P) operon from *Clostridium perfringens*. J Bacteriol. 2001;183:7110–9.
30. Sloan J, McMurry LM, Lyras D, et al. The *Clostridium perfringens* Tet P determinant comprise two overlapping genes: *tetA*(P), which mediates active tetracycline efflux, and *tetB*(P), which is related to the ribosomal protection family of tetracycline-resistance determinants. Mol Microbiol. 1994;11:403–15.
31. Connell SR, Tracz DM, Nierhaus KH, et al. Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother. 2003;47:3675–81.
32. Abril C, Brodard I, Perreten V. Two novel antibiotic resistance genes, *tet*(44) and *ant*(6)-Ib, are located within a transferable pathogencity island in *Campylobacter fetus* subsp. *fetus*. Antimicrob Agents Chemother. 2010;54:3052–5.
33. Melville CM, Scott KP, Mercer DK, et al. Novel tetracycline resistance gene, *tet*(32), in the *Clostridium*-related human colonic anaerobe K10 and its transmission in vitro to the rumen anaerobe *Butyrivibrio fibrisolvens*. Antimicrob Agents Chemother. 2001;45: 3246–9.
34. Roberts MC. Tetracycline resistance due to ribosomal protection proteins. In: White DG, Alekshun MN, McDermott PF, editors. Frontiers in antibiotic resistance: a tribute to Stuart B. Levy. Washington, DC: American Society for Microbiology; 2005. p. 19–28.
35. Stanton TB, Humphrey SB. Isolation of tetracycline-resistant *Megasphaera elsdenii* strains with novel mosaic gene combinations of *tet*(O) and *tet*(W) from swine. Appl Environ Microbiol. 2003; 69:3874–82.
36. Palmieri C, Magi G, Mingoia M, et al. Characterization of a *Streptococcus suis tet*(O/W/32/O)-carrying element transferable to major streptococcal pathogens. Antimicrob Agents Chemother. 2012;56:4697–702.
37. Clewell DB, Flannagan SE, Jaworski D. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. Trends Microbiol. 1995;3:229–36.
38. Luna VA, Roberts MC. The presence of the *tetO* gene in a variety of tetracycline resistant *Streptococcus pneumoniae* serotypes from Washington State. J Antimicrob Chemother. 1998;42:613–9.
39. Giovanetti E, Brenciani A, Lupidi R, et al. The presence of the *tet*(O) gene in erythromycin and tetracycline-resistant strains of *Streptococcus pyogenes*. Antimicrob Agents Chemother. 2003;47: 2844–9.
40. Chung WO, Young K, Leng Z, et al. Mobile elements carrying *ermF* and *tetQ* genes in Gram-positive and Gram-negative bacteria. J Antimicrob Chemother. 1999;44:329–35.
41. Speer BS, Bedzyk L, Salyers AA. Evidence that a novel tetracycline resistance gene found on two *Bacteroides* transposons encodes an NADP-requiring oxidoreductase. J Bacteriol. 1991;173:176–83.
42. Diaz-Torres ML, McNab R, Spratt DA, et al. Novel tetracycline resistance determinate from the oral metagenome. Antimicrob Agents Chemother. 2003;47:1430–2.
43. Diaz-Torres ML, Villedieu A, Hunt N, et al. Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. FEMS Microbiol Lett. 2006;258:257–62.
44. Noaka L, Suzuki S. New Mg^{2+} -dependent oxytetracycline resistance determinant Tet 34 in *Vibrio* isolates from marine fish intestinal contents. Antimicrob Agents Chemother. 2002;46:1550–2.
45. Zhang D-F, Jiang B, Xiang Z-M, et al. Functional characterization of altered outer membrane proteins for tetracycline resistance in *Escherichia coli*. Int J Antimicrob Agents. 2008;32:315–9.
46. Toledo H, Lopez-Solis R. Tetracycline resistance in Chilean clinical isolates of *Helicobacter pylori*. J Antimicrob Chemother. 2010;65:470–3.
47. Pringle M, Fellstrom C, Johansson K-E. Decreased susceptibility to doxycycline associated with 16S rRNA gene mutation in *Brachyspira hyodysenteriae*. Vet Microbiol. 2007;123:245–8.
48. Cousin Jr SL, Whittington WL, Roberts MC. Acquired macrolide resistance genes and the 1 bp deletion in the *mtrR* promoter in *Neisseria gonorrhoeae*. J Antimicrob Chemother. 2003;51: 131–3.
49. Morbidity and Mortality Weekly. Sexually transmitted diseases treatment guidelines. 2002; R51, No RR-6.

50. Sun J, Deng Z, Yan A. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem Biophy Res Comm. 2014;453:254–67.
51. Schwarz S, Chaslus-Dancla E. Use of antimicrobials in veterinary medicine and mechanisms of resistance. Vet Res. 2001;32:201–25.
52. Shaw WV. Chloramphenicol acetyltransferase: enzymology and molecular biology. Crit Rev Biochem. 1983;14:1–46.
53. Leslie AGW, Moody PCE, et al. Structure of chloramphenicol acetyltransferase at 1.75 A resolution. Proc Natl Acad Sci U S A. 1988; 85:4133–7.
54. Cardoso M, Schwarz S. Characterization of the chloramphenicol acetyltransferase variants encoded by the plasmids pSCS6 and pSCS7 from *Staphylococcus aureus*. J Gen Microbiol. 1992;138: 275–81.
55. Lovett PS. Translational attenuation as the regulator of inducible *cat* genes. J Bacteriol. 1990;172:1–6.
56. Lyras D, Rood JI. Transposition of Tn*4451* and Tn*4453* involves a circular intermediate that forms a promoter for the large resolvase, TnpX. Mol Microbiol. 2000;38:588–601.
57. Galimand M, Gerbaud G, Guibourdenche M, et al. High-level chloramphenicol resistance in *Neisseria meningitidis*. N Engl J Med. 1998;339:868–74.
58. Shultz TR, Tapsall JW, White PA, et al. Chloramphenicol-resistant *Neisseria meningitidis* containing *catP* isolated in Australia. J Antimicrob Chemother. 2003;52:856–9.
59. Recchia GD, Hall RM. Gene cassettes: a new class of mobile element. Microbiol. 1995;141:3015–27.
60. Stokes HW, Hall RM. Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn*1696* integron suggests regulation by translational attenuation. Plasmid. 1991;26:10–9.
61. Kadlec K, Kehrenberg C, Schwarz S. Efflux-mediated resistance to florfenicol and/or chloramphenicol in *Bordetella bronchiseptica*: identification of a novel chloramphenicol exporter. J Antimicrob Chemother. 2007;59:191–6.
62. Kim E, Aoki T. Sequence analysis of the florfenicol resistance gene encoded in the transferable R plasmid from a fish pathogen, *Pasteurella piscicida*. Microbiol Immunol. 1996;40:665–9.
63. Michael GB, Kadlec K, Sweeney MT, et al. ICE*Pmu1*, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes. J Antimicrob Chemother. 2012;67:84–90.
64. Kehrenberg C, Schwarz S. *fexA*, a novel *Staphylococcus lentus* gene encoding resistance to florfenicol and chloramphenicol. Antimicrob Agents Chemother. 2004;48:615–8.
65. Gómez-Sanz E, Kadlec K, Feßler AT, et al. A novel *fexA* variant from a canine *Staphylococcus pseudintermedius* isolate that does not confer florfenicol resistance. Antimicrob Agents Chemother. 2013;57:5763–6.
66. Lang KS, Anderson JM, Schwarz S, et al. Novel florfenicol and chloramphenicol resistance gene discovered in Alaskan soil by using functional metagenomics. Appl Environ Microbiol. 2010;76: 5321–6.
67. Liu H, Wang Y, Wu C, et al. A novel phenicol exporter gene, *fexB*, found in enterococci of animal origin. J Antimicrob Chemother. 2012;67:322–5.
68. McMurray LM, George AM, Levy SB. Active efflux of chloramphenicol in susceptible *Escherichia coli* strains and in multiple-antibiotic-resistant (Mar) mutants. Antimicrob Agents Chemother. 1994;38:542–6.
69. Edgar R, Bibi E. MdfA, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. J Bacteriol. 1997;179:2274–80.
70. Nilsen IW, Bakke I, Vader A, et al. Isolation of *cmr*, a novel chloramphenicol resistance gene encoding a putative efflux pump. J Bacteriol. 1996;178:3188–93.
71. Burns JL, Mendelman PM, Levy J, Stull TL, Smith AL. A permeability barrier as a mechanism of chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob Agents Chemother. 1985;27: 46–54.
72. Wolter N, Smith AM, Farrell DJ, et al. Novel mechanism of resistance to oxazolidinones, macrolides, and chloramphenicol in ribosomal protein L4 of the pneumococcus. Antimicrob Agents Chemother. 2005;49:3554–7.
73. Mosher RH, Camp DJ, Yang K, et al. Inactivation of chloramphenicol by O-phosphorylation. J Biol Chem. 1995;27:27000–6.
74. Tao W, Lee MH, Wu J, et al. Inactivation of chloramphenicol and florfenicol by a novel chloramphenicol hydrolase. Appl Environ Microbiol. 2012;78:6295–301.
75. Mosher RH, Ranade NP, Schrempf H, et al. Chloramphenicol resistance in *Streptomyces*: cloning and characterization of a chloramphenicol hydrolase gene from *Streptomyces venezuelae*. J Gen Microbiol. 1990;136:293–301.
76. Jones CS, Osborne DJ, Stanley J. Enterobacterial tetracycline resistance in relation to plasmid incompatibility. Mol Cell Probes. 1992;6:313–7.
77. Anderson AA, Dugan J, Jones L, et al. Stable chlamydial tetracycline resistance associated with a *tet*(C) resistance allele. Antimicrob Agents Chemother. 2004;48:3989–95.
78. Roberts M, Corney A, Shaw WV. Molecular characterization of chloramphenicol acetyltransferases isolated from *Haemophilus influenzae*. J Bacteriol. 1982;151:737–41.
79. Kehrenberg C, Schwarz S. Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin, and chloramphenicol in bacteria of the genera *Pasteurella* and *Mannheimia*. FEMS Microbiol Lett. 2001;205:283–90.
80. Lyon BR, Skurray R. Antimicrobial resistance in *Staphylococcus aureus*: genetic basis. Microbiol Rev. 1987;51:88–137.
81. Macrina FL, Archer GL. Conjugation and broad host range plasmids in streptococci and staphylococci. In: Clewell DB, editor. Bacterial conjugation. New York: Plenum Press; 1993. p. 313–29.
82. Roberts MC, Schwarz S. Tetracycline and chloramphenicol resistance mechanisms. In: Mayers DL, Lerner SA, Ouellette M, Sobel J, editors. Antimicrobial drug resistance, Mechanisms of drug resistance, vol. 1. New York: Humana Press; 2009. p. 183–93.
83. Wang Y, Li D, Song L, et al. First report of the multiresistance gene *cfr* in *Streptococcus suis*. Antimicrob Agents Chemother. 2013;57:4061–3.
84. Frech G, Kehrenberg C, Schwarz S. Resistance phenotypes and genotypes of multiresistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium var. Copenhagen isolates from animal sources. J Antimicrob Chemother. 2003;51:180–2.
85. Soge OO, Adeniyi BA, Roberts MC. New antibiotic resistance genes associated with CTX-M plasmids from uropathogenic Nigerian *Klebsiella pneumoniae*. J Antimicrob Chemother. 2006; 58:1048–53.
86. Kehrenberg C, Meunier D, Targant H, et al. Plasmid-mediated florfenicol resistance in *Pasteurella trehalosi*. J Antimicrob Chemother. 2006;58:13–7.
87. Antibiotic Resistant Threats in the United States, CDC 2013. http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf.
88. WHO Antibiotic Resistance global Report of Surveillance 2014. 2014. http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1. Accessed 13 July 2014.

Fluoroquinolone Resistance in Bacteria

16

Bryan D. Schindler*, Joseph Adrian L. Buensalido*, and Glenn W. Kaatz

1 Introduction

FQs are some of the most widely prescribed antimicrobial agents. Of the total sales of $42 billion from antibiotics worldwide in 2009, FQs represented 17 % of the market, generating $7 billion in global sales (Furiex Pharmaceuticals; http://www.furiex.com). A detailed discussion of structure–activity relationships is beyond the scope of this chapter, but these agents have undergone several iterations, or "generations," which have consisted of structural modifications to improve potency and spectrum of activity. The classification system of quinolones, from first to fourth generation, is based on the improving spectrum of antibacterial activity and potency against pneumococci and anaerobic organisms and is more of a practical classification system for clinical use [1]. The first generation quinolone upon which all subsequent derivatives are based is nalidixic acid (Fig. 16.1), which was isolated as a by-product during chloroquine synthesis [2]. Nalidixic acid actually is a naphthyridone based on the presence of a nitrogen atom at position 8, whereas quinolones generally have a carbon atom at this position. Second generation drugs, all of which have a fluorine at position 6 of the quinolone nucleus (hence the term "fluoroquinolone"), include norfloxacin, ciprofloxacin, enoxacin, ofloxacin, and pefloxacin. These drugs have added antimicrobial activity versus aerobic Gram-positive bacteria and better activity against Gram-negative bacteria compared to the first-generation drugs, but lack activity against anaerobic bacteria [1]. Third generation agents have even greater activity versus Gram-positive bacteria, especially pneumococci, plus good potency against anaerobic bacteria, and include sparfloxacin, grepafloxacin, temafloxacin, and levofloxacin. Garenoxacin (not US FDA approved), gemifloxacin, gatifloxacin, moxifloxacin, and trovafloxacin (discontinued) are considered fourth generation agents, with even greater activity against pneumococci and anaerobes [1]. FQs are widely used in ophthalmology, and the newest and first developed specifically for topical ophthalmologic use is besifloxacin, which is approved for the treatment of bacterial conjunctivitis. Besifloxacin has a C-8 chlorine substituent and thus is a chloro-fluoroquinolone. It has dual-targeting activity versus topoisomerase IV and DNA gyrase (topoisomerase II), resulting in increased potency and a decreased chance of the emergence of bacterial resistance [3]. Tosufloxacin and sitafloxacin are approved for clinical use in Japan. Sitafloxacin has been shown to have favorable susceptibility rates among most bacteria tested (excluding methicillin-resistant *Staphylococcus aureus* [MRSA] and *Enterococcus faecium*), non-inferiority to levofloxacin and tosufloxacin, and a low propensity to cause resistance in *Streptococcus pneumoniae* [4].

Many FQs have been approved by various regulatory agencies worldwide, and some have been withdrawn after widespread use revealed unforeseen toxicities. Examples of this include temafloxacin, which was found to be associated with hypoglycemia and hemolytic-uremic syndrome, and trovafloxacin, found to be associated with severe hepatotoxicity [5, 6]. Although serious adverse events following FQ use are relatively rare, some that have been associated with these drugs include prolongation of the corrected QT interval which can predispose users to serious, life-threatening arrhythmias, rash, seizure, delirium, glucose intolerance, and hepatotoxicity [7–14]. The most commonly used FQs,

*Author contributed equally with all other contributors.

B.D. Schindler, Ph.D.
Microbiologist II, NSF International, Ann Arbor, MI, USA

J.A.L. Buensalido, M.D.
Clinical Associate Professor, Section of Infectious Diseases, Department of Medicine, University of the Philippines—Philippine, General Hospital Manila, Metro Manila, Philippines

G.W. Kaatz, M.D. (✉)
Research and Development, John D. Dingell VA Medical Center, Professor Medicine, Department of Internal Medicine and Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, MI, USA
e-mail: gkaatz@juno.com

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_16

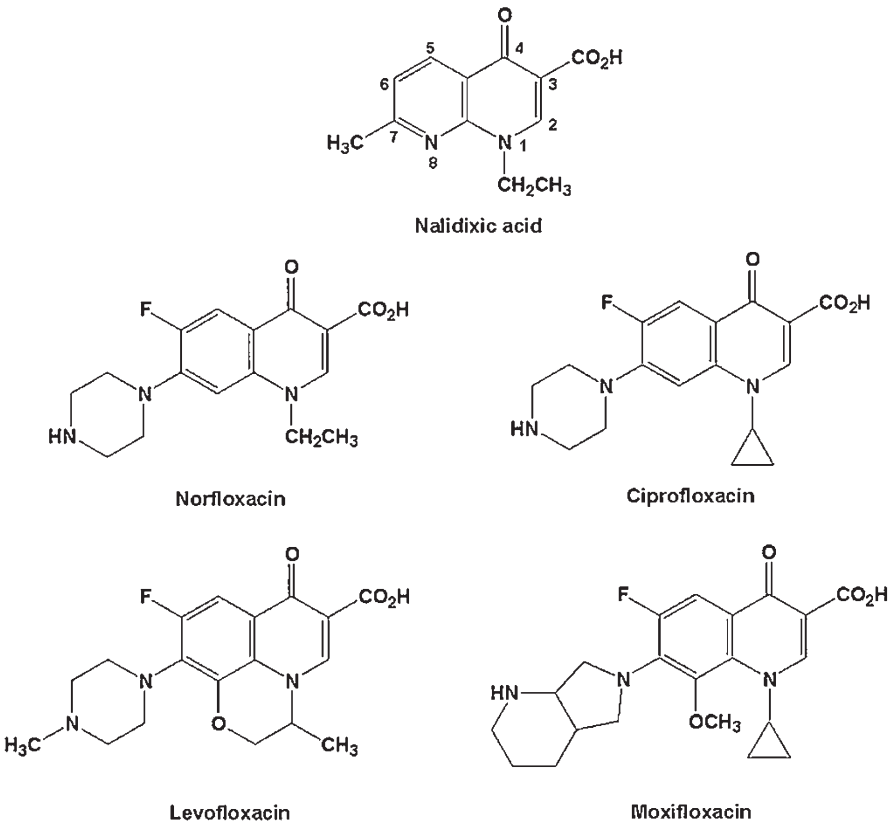


Fig. 16.1 Structures of selected quinolones. The numbering scheme of the quinolone nucleus is given for nalidixic acid

including ciprofloxacin, levofloxacin, and moxifloxacin, are still associated with certain adverse effects such as *Clostridium difficile*-associated diarrhea (as are other antibiotics) and tendinopathy [15]. A 2012 Canadian study showed that the use of a FQ increased the risk of retinal detachment compared to nonusers, but that the absolute risk was very small (4 per 10,000 person-years) [16]. Sitafloxacin generally has the same set of possible adverse effects, but in addition gastrointestinal hemorrhage and leukopenia associated with this drug have been described [17].

FQs are broad-spectrum bactericidal agents active against many Gram-positive and -negative bacteria and target the essential bacterial enzymes DNA gyrase and DNA topoisomerase IV [18, 19]. These enzymes are involved in DNA replication and repair, and in the presence of a quinolone, an intermediate ternary complex consisting of drug, enzyme, and a severed DNA strand is formed. These complexes block further DNA replication leading to cell death. Mutational alterations of the genes encoding DNA gyrase and/or topoisomerase IV in the so-called quinolone resistance determining region, or QRDR, resulting in critical amino acid substitutions reduce quinolone interaction with each enzyme. These mutations are the basis for high-level, target-based resistance and will be discussed in detail subsequently. Another important mechanism of quinolone resistance is overexpression of membrane-based drug efflux pumps, which also will be discussed. Such efflux pumps reduce the effective intracellular drug concentration to either a non-inhibitory or borderline inhibitory level, favoring the emergence of target-based mutations and high-level resistance [20–22]. Target-enzyme and efflux resistance mechanisms are frequently found together in FQ-resistant isolates [23–25].

All clinically relevant bacterial species are capable of developing resistance to FQs, but historically problematic organisms are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. For many FQs these organisms tend to have a narrower therapeutic index than other bacteria in that the minimum inhibitory concentration (MIC) and achievable serum levels are relatively close. In this situation, subtherapeutic drug levels will exist for long periods of time during therapy favoring the emergence of QRDR mutations and reduced FQ susceptibility. Newer agents with increased potency against *S. aureus* have helped to reduce this problem but unfortunately the majority of MRSA strains recovered from clinical specimens are already highly FQ resistant, mainly on the basis of target mutations [26]. Resistance in methicillin-susceptible (MSSA) strains is less problematic, but can be significant in some geographic locales. In 2005, we collected more than 200 bloodstream isolates of *S. aureus* over a 7 month period from different patients hospitalized in Detroit, Michigan. Of these strains, 65 % were MRSA and

35 % were MSSA. Norfloxacin resistance (MIC ≥ 16 μg/mL) was observed in 60 % and 12 % of MRSA and MSSA, respectively (unpublished data). Resistance rates for other areas may differ, but these data illustrate the extent FQ resistance among clinical *S. aureus* strains. These multiple mechanisms of quinolone resistance can severely compromise the clinical utility of this important class of antimicrobial agents, and efforts must be made to reduce selective pressure by using these drugs only when they are clearly indicated.

Fluoroquinolones (FQs) are completely synthetic antimicrobial agents, and as such, it was felt that bacterial resistance would develop slowly, if at all. However, FQs are so widely prescribed that led to the development of a great selective pressure for the emergence of strains with reduced susceptibility to them. Such strains have appeared among nearly all species against which FQs have activity. In this chapter we will discuss FQ resistance in both Gram-negative and Gram-positive bacteria. In the previous edition of this book, we did not address resistance to these agents in *Mycobacterium tuberculosis* since they were not frequently used to treat infections caused by this organism at that time. However, the emergence of multidrug-resistant and then extensively drug-resistant tuberculosis have made this topic very relevant now. We will conclude with a short discussion on means to limit FQ resistance and perhaps to overcome some preexistent resistance by the use of multidrug efflux pump inhibitors.

2 Gram-Negative Bacteria

Gram-negative bacteria are an important cause of morbidity and mortality. Increasing drug resistance observed in many Gram-negative organisms parallels the increasing use and abuse of antimicrobial agents, and this is certainly true for the FQs [27]. Until 1998 it was thought that FQ resistance in Gram-negatives occurred either by way of target alteration or active drug extrusion by membrane-based efflux pumps. A third mechanism described more recently involves the Qnr protein, the gene for which is plasmid-encoded and thus transferable [10, 24, 28–30].

2.1 Target-Mediated Resistance

As already mentioned, the targets of FQs are the essential bacterial enzymes DNA gyrase and topoisomerase IV. DNA gyrase, the major type II topoisomerase in bacteria and initially described by Gellert et al., is a heterotetramer composed of two pairs of subunits (A and B) encoded by the *gyrA* and *gyrB* genes, respectively [31]. The GyrA subunits bind to DNA and the GyrB subunits are ATPases. The main function of this enzyme is to maintain negative supercoiling via DNA strand breakage and rejoining, a function that facilitates the movement of DNA through replication and transcription complexes. Negative supercoiling is essential for initiation of DNA replication, and introduction of supercoils depends on the binding of ATP to gyrase with subsequent ATP hydrolysis [32]. DNA gyrase also helps in removing knots and in the bending and folding of DNA. Following the discovery of DNA gyrase, it was ascertained that this enzyme is a target of FQs [32, 33].

Kato et al. discovered DNA topoisomerase IV, which is also a heterotetrameric enzyme composed of two subunit pairs encoded by the *parC* and *parE* genes [34]. ParC and ParE are homologous with GyrA and GyrB, respectively, with a high degree of amino acid conservation in the QRDR regions. The principle function of topoisomerase IV appears to be its ability to decatenate linked daughter chromosomes at the terminal stages of DNA replication [35]. Despite DNA gyrase and topoisomerase IV sharing considerable amino acid sequence similarity, they have distinct mechanisms of action. One of the important differences seems to be that DNA gyrase wraps DNA around itself, while topoisomerase IV does not [36]. Given the homology between DNA gyrase and topoisomerase IV the latter enzyme was presumed to be a FQ target, which has now been demonstrated clearly [37, 38]. In fact, additional topoisomerases are current subjects of antimicrobial research. Stabilization of covalent complexes formed by them has been shown to result in bacterial cell death in recent studies, and thus they may be promising targets for new antibiotics [39].

As previously mentioned, the mechanism by which FQs act is through binding to DNA-DNA gyrase and DNA-topoisomerase IV complexes causing a conformational change in the enzyme structure [40–42]. They also alter the enzyme-bound DNA itself [40, 43, 44]. In the presence of FQs, the topoisomerases become trapped on DNA and the resultant FQ-enzyme-DNA ternary complex forms a physical barrier at the replication fork, inhibiting further DNA replication which results in cell death [45, 46].

In Gram-negative bacteria the primary target for most FQs is DNA gyrase, with topoisomerase IV being a secondary target [28, 38, 47]. In contrast, in most Gram-positive bacteria and for most FQs, topoisomerase IV is the primary target [47–49]. These differences are thought to be due to the differential affinity of FQs for the two enzymes in each respective background [47, 50]. FQ resistance occurs in a stepwise fashion as a result of the accumulation of mutations mainly in *gyrA* and *parC* that result in amino acid substitutions in the QRDR. Less commonly, mutations occur in *gyrB* and *parE* that can contribute to reduced FQ susceptibility [25, 28, 51, 52]. Additional MIC increases are seen when a "first-step" mutant, having a critical amino acid substitution in the primary target, acquires a "second-step" mutation resulting in an amino acid substitution in the secondary

Table 16.1 Topoisomerase amino acid substitutions associated with reduced FQ susceptibility in *E. coli*. Data are from references [25, 53, 261–263]

GyrA	GyrB	ParC	ParE
Ala51 → Val	Asp426 → Asn	Ser54 → Thr	Leu445 → His
Ala67 → Ser	Lys447 → Glu	Ser57 → Thr	Ser458 → Ala, Pro, Thr, Trp
Gly81 → Cys, Asp		Thr66 → Ile	Glu460 → Asp, Lys
Asp82 → Gly		Asp69 → Glu	Ile464 → Phe
Ser83 → Leu, Trp, Ala, Val		Gly78 → Asp	
Ala84 → Pro, Val		Ser80 → Arg, Ile, Leu	
Asp87 → Ala, Asn, Gly, His, Tyr, Val		Glu84 → Ala,Gly, Lys, Val	
Gln106 → Arg, His		Asn105 → Ser	
		Ala108 → Thr, Val	

Table 16.2 Topoisomerase amino acid substitutions associated with reduced FQ susceptibility in *S. aureus*

GyrA	GyrB	ParC (GrlA)	ParE (GrlB)
Arg33 → Pro[a]	Tyr372 → Asn	Lys23 → Asn	Pro25 → His
Leu35 → Met[a]	Asp437 → Asn	Val41 → Gly	Ser410 → Pro
Arg48 → Leu[a]	Arg458 → Glu	Arg43 → Cys	Glu422 → Asp
Ser84 → Ala, Leu, Lys, Val	Glu477 → Ala	Ile45 → Met	Asp432 → Asn, Gly, Val, His
Ser85 → Pro		Leu46 → Met	Pro451 → Gln, Ser
Glu86 → Lys, Gly		Tyr47 → Asp	Asn470 → Asp
Glu88 → Lys, Val		Ala48 → Thr	Glu472 → Lys, Val
Gly106 → Asp		Met49 → Thr	His478 → Tyr
Ala119 → Pro[a]		Tyr50 → Asn	
Pro165 → Ser		Ser52 → Arg	
Ala169 → Ser[a]		Asp69 → Tyr	
Leu198 → Ser[a]		Gly78 → Cys	
Asn201 → Ile[a]		Ser80 → Phe, Tyr	
Ser205 → Leu[a]		Ser81 → Pro	
Glu211 → Lys[a]		Glu84 → Ala, Gly, Leu,	
		Lys, Tyr, Val	
		His103 → Tyr	
		Ala116 → Glu, Pro	
		Pro157 → Leu	
		Ala176 → Gly, Thr	

Data are from references [25, 53, 264, 265]

[a]GyrA substitution mutations were found in the same clinical isolate and it is likely that all do not contribute to FQ resistance

target enzyme [28]. Many topoisomerase mutations in *E. coli*, as well as many other Gram-negative bacteria, have been shown to correlate with raised FQ MICs (Table 16.1).

Analyses of *gyrA* mutants have revealed that most of the FQ resistance conferring mutations cluster near the 5′ end of the gene in the QRDR region. For *E. coli*, this region includes codons 67–106 and for other species the region homologous to this [25, 51, 53]. Very near to the QRDR is the codon for the active site tyrosine (codon 122). Tyrosine-122 binds covalently to DNA when the enzyme breaks the phosphodiester bonds of DNA, forming a phosphotyrosine linkage [54]. Single *gyrB* mutants appear to be less resistant to FQs than single *gyrA* mutants. In *E. coli*, only two *gyrB* mutations have been recognized (Table 16.2) [25]. Only Asp426 → Asn confers resistance to FQs, whereas, with the exception of resistance to acidic FQs such as nalidixic acid, Lys447 → Glu results in an increase in FQ susceptibility [51, 53].

Within topoisomerase IV, mutations in *parC* occur more frequently than those in *parE*. As mentioned previously, topoisomerase IV generally is the secondary FQ target in *E. coli* and other Gram-negative organisms, including *Salmonella typhi* [55–60]. g*yrA*-*parC* double mutants exhibit a higher level of FQ resistance than *gyrA* single mutants, with the highest levels of resistance found in the mutants with two *gyrA* and two *parC* mutations. The reverse generally is true in Gram-positive organisms, where the first mutations are usually seen in the topoisomerase IV genes with the gyrase genes being the secondary targets.

Multidrug-resistant *Acinetobacter baumanii* has recently emerged as a major pathogen and is now a public health

threat worldwide. Its resistance to FQs, as with most Gram-negative bacteria, is due to mutations in DNA gyrase and topoisomerase IV [61–63], but alterations in cell membrane permeability and active drug efflux have also been described [61, 64]. *A. baumanii* isolates that were fully resistant to ciprofloxacin had been shown to have the following amino acid alterations: Ser83 → Leu in GyrA (considered to be the principal substitution), and Lys59 → Gln plus Ser80 → Leu in ParC. The Ser80 → Leu in ParC was consistently present in all resistant strains, absent in all the susceptible strains, and found in only some intermediately resistant strains. Thus, Ser80 → Leu in ParC may be a prerequisite for *A. baumannii* to develop full resistance. The majority of resistant isolates also had the Ser83 → Leu mutation in GyrA and some had the Lys59 → Gln mutation in ParC [61].

An interesting association between toxin production and QRDR mutations in *P. aeruginosa* was recently identified. In a group of 270 clinical isolates, Agnello and Wong-Beringer found that the presence of the *exoU* gene was highly associated with FQ resistance and the presence of >2 target site mutations (especially the GyrA + ParC combination) compared to *exoS*-positive strains [65]. ExoS and ExoU are two *P. aeruginosa* toxins exported by a type III secretion system. The authors contend that this correlation between FQ resistance mutations and virulence genotype suggest a possible coevolution of the two traits, resulting in a more virulent genotype under FQ-enriched environments.

A new class of drugs, called dual-targeting inhibitors, have been designed to act on both bacterial DNA gyrase and topoisomerase IV at the ATP-binding subunits GyrB/ParE, avoiding GyrA/ParC-mediated FQ resistance [66–68]. The targeting of both enzymes by a unique mechanism is felt to decrease the chances of developing cross-resistance to other antibiotics [66]. This new class is broadly active against many Gram-positive and Gram-negative pathogens, including ciprofloxacin-resistant strains [69].

A recent study assessed the activity of five novel gyrase inhibitors on 303 highly resistant *P. aeruginosa* isolates and found that they had very good potency. The most active inhibitor had MIC_{50} and MIC_{90} values of 1 and 2 μg/mL, respectively, but generally MICs were ≤4 μg/mL for all five compounds. All of them maintained their in vitro potency regardless of the phenotypic profile to other compounds, including FQs, and even in strains that were multidrug resistant [66]. These findings reveal an exciting new area in the research and development for agents that can hopefully overcome target-mediated FQ resistance.

2.2 Decreased Outer Membrane Permeability

FQs must traverse the outer membrane, periplasmic space, cell wall, and cytoplasmic membrane of Gram-negative organisms to reach their topoisomerase targets. The porous bacterial cell wall does not impede the diffusion of small molecules such as FQs. The outer membrane may provide a rather formidable barrier, however, and in conjunction with efflux pumps (see below) can result in significant FQ resistance [25, 53, 70–74]. FQs traverse this structure by two mechanisms, which include diffusion across the lipid bilayer and passage through pore-forming proteins called porins. Porins are protein channels that allow influx and egress of hydrophilic molecules. All FQs may cross the outer membrane through the porins, but diffusion across the lipid bilayer is dependent on the hydrophobicity of the molecule. The more hydrophobic FQs such as nalidixic acid are capable of traversing the lipid bilayer, whereas the more hydrophilic compounds such as ciprofloxacin are more dependent on porins [70–73]. Three main porins are found in *E. coli* and consist of OmpF, OmpC, and OmpA. Loss of porins by mutational inactivation of structural genes often manifests as a decrease in FQ susceptibility, but this effect is significantly amplified in the presence of drug efflux. *E. coli* mutants with reduced amounts of OmpF, the most abundant porin, exhibit low-level FQ resistance [70, 75]. Other unrelated drugs such as tetracyclines, chloramphenicol, and some β-lactams also utilize this porin and hence OmpF-deficient mutants may also demonstrate resistance to these drugs due to decreased accumulation [70, 73, 76, 77].

Chromosomal loci such as *marRAB* and *soxRS* encode transcriptional factors that regulate OmpF expression in *E. coli* [78, 79]. Overexpression of *marA*, *soxS*, and, more recently, *sdiA* result in posttranscriptional repression of OmpF and thus FQ resistance by increasing the expression of *micF*, an antisense regulator [78–80]. Similarly, expression of *ompX*, which encodes another outer membrane protein, has been shown to increase during early exposure to drugs (including nalidixic acid, ciprofloxacin, and norfloxacin) or environmental stresses. This affects *ompF* expression, suggesting that OmpF and OmpX are involved in the control of antibiotic penetration through the outer membrane [81]. Additional information relating to the roles of MarA and SoxS in FQ resistance will be presented in Sect. 2.3.

The permeability of the outer membranes of *P. aeruginosa* and *A. baumanii* may account for some of their intrinsic resistance to various antibiotics, including FQs [82]. The *P. aeruginosa* outer membrane has very poor permeability to hydrophilic molecules, approximately 100-fold less than that of the *E. coli* outer membrane [83], although permeability differences compared to other bacteria such as *P. putida* and *Salmonella typhimurium* are relatively minor [84].

2.3 Efflux-Related Resistance

Gram-negative bacteria tend to be resistant to a wider range of antimicrobial agents compared to Gram-positive species. The outer membrane is one reason for this in that it acts as a

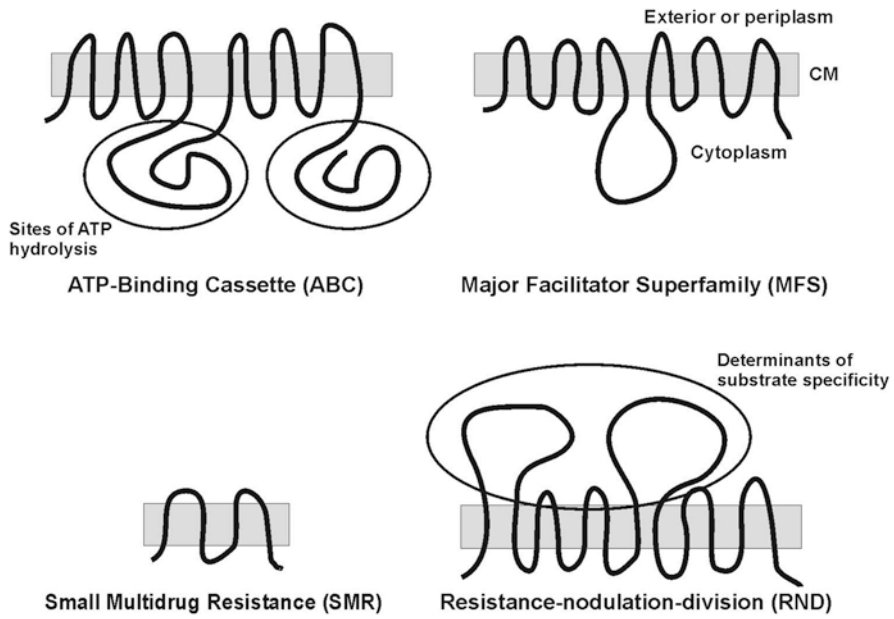


Fig. 16.2 Schematic illustrating the general structural characteristics of each family of bacterial efflux pump. The sites at which ATP hydrolysis occurs in ABC pumps are indicated. MATE pumps do not necessarily have the large central loop that is characteristic of members of the MFS, and some MFS proteins have 14 membrane-spanning segments. Substrate specificity for RND pumps such as AcrB, and perhaps other pumps of this family, lie in the two large periplasmic loops. The cytoplasmic membrane is shown in *gray*, and the cytoplasm and exterior/periplasm are as indicated

barrier to the penetration of hydrophilic molecules. This mechanism generally confers only low-level resistance. Membrane-based efflux pumps contribute more significantly to innate drug insensitivity. Bacterial efflux pumps can be divided into five families based on structural characteristics, mechanisms of action, and source of energy for the transport process. These include primary transporters that depend on ATP hydrolysis for drug export (ATP-binding cassette, or ABC pumps) and secondary transporters that require an intact ion gradient across the cell membrane for their function (major facilitator superfamily [MFS], resistance-nodulation-division [RND], small multidrug resistance [SMR], and the multidrug and toxic compound extrusion [MATE] families) (Fig. 16.2). The most common ion gradient used among these secondary transporters is the H^+ gradient, but MATE family proteins also can utilize the Na^+ gradient [85]. Efflux pumps may be quite specific with respect to substrates transported, with a clinically relevant example being the various MFS tetracycline efflux pumps found in Gram-negative and Gram-positive bacteria [86].

Circumvention of the resistance generated by specific drug pumps is as simple as providing alternative therapy with agents not affected by the pump in question. Multidrug efflux pumps, which can have broad substrate specificity, are capable of extruding numerous structurally dissimilar compounds resulting in a multidrug-resistant (MDR) phenotype. The activity of these pumps can pose a very formidable therapeutic challenge (Table 16.3). In strains with increased expression of pump genes, drug efflux can lead to subtherapeutic intracellular concentrations of an antibiotic substrate resulting in the ideal milieu for the development of chromosomal mutations that confer high-level antibiotic resistance. Overexpression of these pump genes may occur secondary to chromosomal mutations in the promoter region or in the gene encoding the regulator for pump gene expression. Efflux-related resistance can also result from up-function mutations in the coding region of efflux pumps, enhancing their transport efficiency [87]. With respect to FQs, efflux-related resistance has been identified in virtually all medically important Gram-negative organisms including *E. coli*, *P. aeruginosa*, *A. baumanii*, and *M. tuberculosis* [88–90]. No pumps having FQs as sole substrates have been described.

The AcrB pump, an RND family protein, is the predominant FQ efflux system of *E. coli* [91]. The RND family pumps of Gram-negative organisms are composed of three different subunits which include the pump protein itself, which is a transmembrane protein having 12 membrane-spanning alpha helices or transmembrane segments (TMSs), an outer membrane pore-forming channel or porin, and a periplasmic membrane fusion protein (MFP) that links the other two [92–94]. The AcrB pump, the functional unit of which is a homotrimer, utilizes TolC as its outer membrane channel, to which it is associated by the AcrA MFP [95]. AcrB has a broad substrate profile including antibiotics (FQs, tetracyclines, chloramphenicol, β-lactams, nalidixic acid, rifampin, novobiocin, and fusidic acid), dyes, and disinfectants [96]. The expression of *acrAB*, which is transcribed as an operon, is governed by at least two global regulatory systems, the *marRAB* and *soxRS* loci; both sys-

Table 16.3 Selected bacterial multidrug efflux pumps

Pump	Family	Organism	Selected substrates[a]
Gram-negative			
AcrB	RND	*E. coli*	FQ, BL, CM, TCN, TI
MdfA	MFS	*E. coli*	FQ, CM, EM, TCN
MexB	RND	*P. aeruginosa*	FQ, BL, CM, TCN, TI, TM
MexD	RND	*P. aeruginosa*	FQ, CM, EM, TCN, TI, TM
MexF	RND	*P. aeruginosa*	FQ, CM, TM
MexY	RND	*P. aeruginosa*	FQ, AF, AG, EB, EM
SmeE	RND	*S. maltophilia*	FQ, CM, TCN
NorM	MATE	*V. parahaemolyticus*	FQ, EB
Gram-positive			
NorA	MFS	*S. aureus*	FQ, AF, BAC, CT, EB, TPP
PmrA	MFS	*S. pneumoniae*	FQ, EB
PatAB	ABC	*S. pneumoniae*	FQ, AF, EB
Bmr	MFS	*B. subtilis*	FQ, AF, EB, TPP
Blt	MFS	*B. subtilis*	FQ, AF, EB, TPP
MepA	MATE	*S. aureus*	FQ, BAC, DQ, EB, TPP, PT
LmrA	ABC	*L. lactis*	FQ, AG, BL, CM, TCN
Rv1634	MFS	*M. tuberculosis*	FQ
DrrABC	ABC	*M. tuberculosis*	FQ, AG
Rv2686c-Rv2687c-Rv2688c	ABC	*M. tuberculosis*	FQ

[a]*AF* acriflavine, *AG* aminoglycosides, *BAC* benzalkonium chloride, *BL* beta-lactams, *CM* chloramphenicol, *CT* cetrimide. *DQ* dequalinium; *EB* ethidium bromide, *EM* erythromycin, *FQ* fluoroquinolones, *PT* pentamidine, *TCN* tetracycline, *TI*, tigecycline; *TM* trimethoprim, *TPP* tetraphenylphosphonium

tems positively regulate the production of AcrAB. Multiple antibiotic-resistant (Mar-type) mutants of *E. coli* have mutations in the *marRAB* operon [97]. The Mar phenotype is induced following exposure to a variety of chemicals with aromatic rings, including salicylate. The most common location for mutations conferring the Mar phenotype is in *marR*, which encodes for the repressor of the *marRAB* operon. *E.coli soxRS* mutants exhibit a resistance phenotype similar to *marR* mutants. Increased quantities of MarA and SoxS and Rob (a homologue of the first two) upregulate *acrAB* and *tolC* and downregulate the production of the OmpF porin channel [98–100]. These changes lead to multiple antibiotic resistance by these synergistic mechanisms.

The crystal structure of AcrB in the presence and absence of substrates has been solved [101–104]. The pump acquires substrates from both outer leaflet of the cytoplasmic membrane and the cytoplasm [105]. Substrate specificity of AcrB seems to lie in its large periplasmic loops (Fig. 16.2) [106], where the substrate enters a binding pocket that is aromatic, allowing multisite binding, resulting in drug extrusion by a three-step rotating mechanism. Substrate binding in the transmembrane domain is followed by transportation to the binding pocket in the periplasmic region and lastly substrate is released into a funnel-like structure toward TolC, which facilitates drug extrusion from the cell [103, 104, 107, 108]. Further study using site-directed mutagenesis of the hydrophobic binding pocket in AcrB identified phenylalanine at position 610 as essential for transport of many different substrates, including the FQs levofloxacin and ciprofloxacin [89, 109].

In *P. aeruginosa* the main multidrug efflux system is the *mexAB-oprM* operon, which encodes proteins homologous to AcrAB-TolC. *mexCD-oprJ*, *mexEF-oprN*, *mexXY-oprM*, and most recently *mexVW-oprM* are additional multidrug efflux operons found in this organism. Each of these operons encodes for a set of three proteins similar in structure and function to MexAB-OprM and all are RND family efflux pumps that confer resistance to FQs [110–112]. Like AcrAB-TolC, the most striking characteristic of these pump systems is their broad substrate specificity. The substrate profile for MexAB-OprM includes FQs, chloramphenicol, nalidixic acid, trimethoprim, tetracyclines (including tigecycline), dyes, disinfectants, and organic solvents (Table 16.3). Most wild-type strains of *P. aeruginosa* express MexAB-OprM constitutively, which contributes to the intrinsic multidrug-resistant nature of this organism [113]. While all Mex family transporters have FQs as substrates, the degree to which each member confers clinically relevant FQ resistance is still being actively investigated [114–116].

Multidrug efflux pumps having FQs as substrates have been identified in many other Gram-negative bacteria. Examples include the SmeDEF RND pump system of *Stenotrophomonas maltophilia*, the NorM and BexA MATE pumps of *Vibrio parahaemolyticus* and *Bacteroides thetaiotaomicron*, respectively, and the VceAB MFS pump of *V. cholerae* [117–120] (Table 16.3). Overexpression of these

pumps in either their natural or a heterologous background results in increased MICs for a variety of FQs. As mentioned earlier, active efflux has been noted to contribute to FQ resistance in multidrug-resistant *A. baumanii*, an increasingly common cause of nosocomial infections. The three efflux pump systems that are overexpressed in *A. baumanii* are all of the RND family, specifically AdeABC, AdeFGH, and AdeIJK [64, 88, 121–123].

2.4 Plasmid-Mediated FQ Resistance

In 1998, Martínez-Martínez et al. reported FQ resistance to be expressed in the presence of pMG252, a plasmid belonging to incompatibility group IncC [10]. This plasmid mediates low-level resistance to both nalidixic acid and more modern FQs and has a broad host range. Subsequently, the gene responsible for FQ resistance was identified and named *qnr* [124]. Qnr is a member of the pentapeptide repeat protein (PRP) family due to the presence of tandem repeats of the amino acids [S,T,A,V][D,N][L,F][S,T,R][G] [125]. Qnr "protects" both DNA gyrase and topoisomerase IV from FQ inhibition [126, 127].

Surface-exposed loops of the Qnr homodimer interact electrostatically with the GyrA and GyrB proteins, mimicking DNA and thus preventing FQ-mediated DNA strand breakage. Another proposed mechanism by which Qnr proteins act is by binding to and destabilizing the topoisomerase-FQ-DNA complex, which leads to regeneration of the catalytically active form of topoisomerase [124, 126–129].

Prevalence studies have revealed that among FQ-resistant strains of *E. coli* recovered in Shanghai, China, 7.7 % contained the *qnrA* gene [130]. In the United States, *qnrA* was present in 11.1 % of FQ-resistant *Klebsiella pneumoniae* strains but not in any of the tested *E. coli* strains [131]. Further investigation led to the discovery that *qnrA* was present in clinical strains of *Enterobacter* spp. [132]. Thus, the *qnrA* gene is widely distributed and contributes to FQ resistance in Enterobacteriaceae. In the first edition of this book we described a *qnrA*-related gene called *qnrB*, which was discovered in a strain of *K. pneumoniae* that had less than 40 % amino acid sequence identity with *qnrA* [133]. Since then, Qnr protein families have been established according to DNA homology including QnrA, QnrB, QnrS, QnrC, QnrD, and QnrVC [128]. Although *qnr* confers relatively low-level FQ resistance, its presence may facilitate selection of other mutations leading to high-level FQ resistance. Although it has been reported that some *E.coli* isolates having a *qnr* determinant were shown to have low selection of topoisomerase mutations, it is now more recognized that Qnr (or Qnr-like) proteins provide bacteria the enhanced capacity to gain full resistance to FQs [10, 19, 128, 134, 135].

Plasmid-mediated FQ efflux has also been reported in Gram-negative organisms. Hansen et al. reported FQ resistance in *E. coli* harboring plasmid pOLA52 [136]. The genes responsible are *oqxAB*, which encode a MFP and an RND-family efflux pump, and in addition to FQs this pump system is also capable of transporting dyes, detergents, disinfectants, and other antibiotics. When expressed in isogenic *E. coli* strains, OqxAB increased ciprofloxacin and norfloxacin MICs 32- and 64-fold, respectively, above the control strain. Considering that *oqxAB* was isolated from a conjugative plasmid and that OqxAB conferred ciprofloxacin resistance upon additional *Enterobacter*iaceae, the spread of this FQ resistance mechanism is certainly possible.

Recently, a plasmid-associated gene recovered from a clinical *E. coli* strain was found to encode an aminoglycoside acetyltransferase that also could acetylate selected FQs and compromise their antimicrobial activity [137]. The effect of acetylation was relatively small, as exemplified by expressing the gene in question (*aac*[*6′*]-*Ib-cr*) from a plasmid in an *E. coli* background. The MIC increases were not clinically significant; norfloxacin and ciprofloxacin MICs were increased fourfold, whereas those of levofloxacin and gemifloxacin were unaffected. However, given the disparate geographic locales from which this aminoglycoside acetyltransferase enzyme has been found, the existence of a plasmid-based and naturally occurring enzyme capable of modifying FQs is worrisome as widespread dissemination is possible [138–143]. The combination of this resistance mechanism with others forms of resistance (such as efflux pumps or single QRDR mutations) causing borderline MIC increases may eventually result in a clinically relevant fully resistant organism.

2.5 Additional Instances of Enzymatic Modification of FQs

Being synthetic substances, the occurrence of natural FQ-modifying systems in microorganisms seemed unlikely. However, fungi capable of degrading ciprofloxacin and the veterinary FQ enrofloxacin have been identified [144, 145]. A wastewater *Microbacterium* species capable of defluorinating and hydroxylating norfloxacin has been identified after enrichment with *N*-phenylpiperazine, the piperazine moiety of which is known to be important for the antibacterial activity of several FQs [146]. Along with acetylation of FQs by (*aac*[*6′*]-*Ib-cr*)-expressing bacteria discussed in the previous section, the discovery of these novel FQ-modifying activities suggest others may either evolve or perhaps already exist.

3 Gram-Positive Bacteria

Fewer FQ resistance mechanisms are found in Gram-positive bacteria than those identified in Gram-negatives. The lack of an outer membrane results in no permeability issues beyond those posed by the cytoplasmic membrane. Studies done in vitro provide evidence that inhibition of efflux pumps reduces the emergence of topoisomerase mutations in both *S. aureus* and *S. pneumoniae*, suggesting that efflux pumps play a critical role in the evolution of high-level FQ resistance [147–150]. At the time of the first edition of this book, Qnr-like PRPs or FQ-modifying enzymes had not been reported in any Gram-positive organisms. Since then, PRPs have been discovered from *Enterococcus faecalis*, *Enterococcus faecium*, *Listeria monocytogenes*, *Clostridium perfringens*, *C. difficile*, *Bacillus cereus*, and *Mycobacterium tuberculosis* [151–154]. Although these PRPs share only 17–22 % amino acid identity with QnrA1, they still confer resistance to FQs. A recent phylogenetic analysis identified genes encoding Qnr and related proteins in the chromosomes of 34 Gram-positive organisms, in addition to finding the *mfpA* gene, which encodes a Qnr-like PRP, in the genome of at least 19 *Mycobacteria* and 10 *Actinobacteria* species [155].

3.1 Target-Mediated Resistance

Similar to the situation in Gram-negative bacteria, mutations resulting in amino acid substitutions in the QRDR regions of *gyrA* and *parC* (*grlA* in *S. aureus*) are the main mechanism by which FQ resistance is achieved in Gram-positive bacteria. In general, GrlA is the primary target in Gram-positives, and single amino acid substitutions in this enzyme can result in clinically relevant resistance [156, 157]. Accumulation of QRDR mutations first in *parC* and then in *gyrA* typically results in very high MICs. Topoisomerase amino acid substitutions correlating with FQ resistance in *S. aureus* are presented in Table 16.2.

3.2 Efflux-Related Resistance

Examination of genome data available for *Enterococcus faecalis*, *S. aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* reveals numerous coding regions for putative drug transport proteins (http://www.membranetransport.org). Many of these proteins are homologous with known multidrug transporters for which FQs are substrates. Several of the most extensively studied Gram-positive drug pumps will be discussed in this section.

NorA is a chromosomally encoded 12 TMS *S. aureus* multidrug pump having broad substrate specificity that includes biocides as well as FQs [148, 158–160]. As are all MFS pumps, its activity is dependent on the proton motive force [161]. Knockout mutations have revealed that NorA contributes to FQ susceptibility in wild-type strains in that elimination of the gene results in MIC reductions for norfloxacin and ciprofloxacin [149, 162]. Overexpression of *norA*, either by way of a regulatory mutation or expression from a multicopy plasmid in the laboratory, results in modest MIC increases for selected FQs as well as many other structurally unrelated drugs, mainly hydrophobic cations [148, 159].

The understanding of *norA* regulation is incomplete. Past work has identified the MgrA protein, which binds upstream of *norA* altering its expression [163, 164]. MgrA is not a specific regulator of *norA* expression but rather a global regulator that, in addition to affecting *norA* transcription, also affects the transcription of other pump-encoding genes (including *norB* and *norC*; see below), autolytic regulators, murein hydrolases, and virulence factors such as alpha toxin, coagulase, and nuclease [164–167]. MgrA activity is affected by the *sigB* regulon, which is known to respond to certain stresses but also mediate antibiotic resistance [168, 169]. Activation of the *sigB* regulon is controlled in part by RsbU (of the serine/threonine phosphatase family) through phosphorylation and dephosphorylation events [170]. Interestingly, conflicting reports of MgrA acting as a repressor or an activator of *norA* and *norB* expression is likely due to differences in the *rsbU* genotype of the strains used in the studies [163, 167, 171–173]. It has since been shown that phosphorylation of MgrA by PknB, a putative serine/threonine kinase, can be reversed by RbsU [174, 175]. According to the current model, non-phosphorylated MgrA binds to the *norA* promoter, repressing transcription while phosphorylated MgrA loses affinity for the *norA* promoter, allowing *norA* transcription. The opposite mechanism appears to be true for the activity of MgrA in control of *norB* transcription [175]. A thorough review of the currently understood role of MgrA in efflux pump regulation in *S. aureus* is available elsewhere [160].

NorB and NorC both are 12 TMS MFS multidrug transporters that are quite similar to each other with 61 % amino acid sequence homology [171, 176]. The substrate profile of NorB includes a variety of FQs (norfloxacin, ciprofloxacin, sparfloxacin, moxifloxacin, gemifloxacin, garenoxacin, and premafloxacin), tetraphenylphosphonium bromide, cetrimide, and ethidium bromide, many of which also are substrates for NorA. NorC, whose expression is also controlled in part by MgrA, seems capable of effluxing a similar set of FQ substrates with the exception of gemifloxacin [176]. A novel *S. aureus* 14 TMS MFS multidrug efflux pump, MdeA, was described [177], and when overexpressed, confers resistance to an intriguing array of substrates including norfloxacin, ethidium bromide, benzalkonium chloride, virginiamycin, novobiocin, and fusidic acid [177–179], although it has also been shown that FQs are relatively poor substrates of this pump [178]. Expression of *mdeA* in wild-type strains is low,

but spontaneous mutants having increased transcription are selectable in vitro. These mutants, which have reduced susceptibility to MdeA substrates, were found to have mutations in the *mdeA* promoter [160, 177] but further details regarding the regulation of *mdeA* expression are not available.

Many MRSA strains contain plasmids encoding the multidrug efflux pump proteins QacA and QacB, which are 14 TMS proteins of the MFS family [180–186] (Fig. 16.2). QacA and QacB mediate efflux of monovalent and divalent cations such as the biocides benzalkonium chloride and chlorhexidine, respectively [187, 188]. Although the *qacA* and *qacB* nucleotide sequences differ by only 6–9 bases, QacA has greater efflux activity for divalent cations than does QacB relating to a Asp323→Ala substitution in TMS 10 of QacB [188–191]. *qac* genes are highly prevalent in MRSA in Europe and Asia (~40 % of isolates) [180, 181, 186, 188, 190]. To date, several QacA and QacB variants have been identified with QacBIII conferring decreased susceptibility to norfloxacin and ciprofloxacin as well as cationic biocides. A glutamic acid residue at position 320 is responsible for this augmented FQ efflux activity in QacBIII [188].

The MATE family of efflux proteins was the most recently described and the least well characterized. MATE pumps were initially thought to function for the most part via an unusual sodium ion/drug antiport mechanism but some members were later found to use a proton/drug antiport mechanism [192–194]. These pumps have been found mainly in Gram-negative bacteria, with fewer reports in Gram-positives, including *Clostridium difficile*, *Staphylococcus aureus*, and pneumococci [195–198]. MATE family proteins are similar in size to MFS transporters and are typically arranged into 12 TMSs, but they have no sequence similarity to any MFS proteins (Fig. 16.2). Structural and functional information related to MepA, the sole MATE efflux pump in *S. aureus*, is available [87]. Substrate specificity varies among MATE pumps and can include cationic dyes, aminoglycosides, anticancer agents, and FQs. Gene inactivation studies have demonstrated that MATE pump genes can be expressed at sufficient levels to affect MICs for pump substrates in wild-type cells and along with other pumps and alternative resistance mechanisms can contribute to reduced susceptibility to clinically relevant drugs such as FQs [120].

The MepA pump of *S. aureus* is repressed by MepR, a MarR-family protein encoded immediately upstream of *mepA* [197, 199]. MepA substrates bind to MepR, reducing its affinity for the *mepA* promoter resulting in augmented *mepA* expression. MepR also is autoregulatory in that it represses the expression of its own gene. However, relief of *mepR* repression in the presence of MepA substrates is much less than that observed for *mepA*. The mechanism(s) of this apparent paradox are yet to be worked out, but the end result is significant relief of *mepA* and relative maintenance of *mepR* repression, leading to increased MepA protein unimpeded by MepR when the need for detoxification exists. Recently, it was shown that MepR substitution mutations found in clinical isolates, most commonly A103V, result in *mepA* overexpression by reducing MepR repressor activity [200].

Although not considered a human pathogen, several multidrug transporters of *Bacillus subtilis* have been studied extensively and have contributed greatly to our knowledge of the regulation and function of MFS proteins. Bmr is a 12 TMS MFS MDR transporter having 44 % amino acid identity with NorA and a similar substrate profile [201, 202]. The expression of *bmr* is regulated by the binding to its promoter of BmrR, a transcriptional activator protein encoded by a gene immediately downstream from *bmr* [203]. The crystal structure BmrR in the presence and absence of substrates has been solved and has revealed that Bmr substrates bind to BmrR via hydrophobic and electrostatic interactions, which in turn facilitates BmrR binding to the *bmr* promoter and induction of *bmr* transcription. This and other more detailed structures, both DNA-bound and unbound conformations, have been the subject of scrutiny since then [204–207].

Blt is a second 12 TMS MFS MDR transporter of *B. subtilis* that has a similar substrate profile to those of NorA and Bmr [208]. The expression of *blt* is enhanced in a similar manner to that of *bmr* by the binding of the transcriptional activator BltR (encoded by *bltR*, found immediately upstream of *blt*) to the *blt* promoter. This binding is thought to be improved by the interaction of substrates with BltR, although the specific activator substrates have not been identified. Interestingly, *blt* is not expressed in wild-type cells.

In addition to the specific regulators of *bmr* and *blt* transcription just described, the expression of these genes also is affected by MtaN, a global transcriptional regulator that interacts with the *bmr* and *blt* promoters stimulating their transcription [209]. MtaN consists of the N-terminal 109 residues of a larger protein, Mta (257 residues); the intact parent protein does not activate *bmr* or *blt* transcription. It is hypothesized that upon interacting with a still unidentified inducer, the N- and C-terminal domains of Mta are functionally separated allowing it to work as a transcriptional activator.

Bmr3 is a 14 TMS MFS pump that confers reduced susceptibility to only select FQs and puromycin when overexpressed [210, 211]. When *bmr3* is disrupted, the norfloxacin MIC is unchanged from that of a parent strain suggesting it likely is poorly expressed and does not contribute to intrinsic drug resistance in wild-type cells. However, mutations resulting in increased *bmr3* mRNA stability result in resistance to norfloxacin, puromycin, tosufloxacin, daunomycin, and ethidium bromide, but not to levofloxacin, lincomycin, tetraphenylphosphonium chloride, or rhodamine [211].

PmrA is a 12 TMS proton-dependent MFS transporter found in pneumococci [212]. Disruption of *pmrA* results in increased FQ susceptibility and reduced efflux of ethidium bromide, indicating that at least some FQs are substrates for this pump and that it is a multidrug transporter [150, 213]. However, PmrA is unlikely to be the main transporter involved in FQ efflux in clinical strains as overexpression does not necessarily result in any change in FQ susceptibility [150, 213–217].

PatAB, an ABC transporter found in *S. pneumoniae*, confers intrinsic multidrug resistance, including resistance to some FQs [218]. Unlike PmrA, expression of the genes encoding PatAB (*patA* and *patB*) is induced by FQs [219]. Furthermore, *patA* and *patB* overexpression is also found in ciprofloxacin-resistant laboratory-selected mutants and clinical isolates [220]. ABC transporters can function either as homodimers or heterodimers. The functional PatAB transporter exists as a PatA-PatB heterodimer rather than as a homodimer of either PatA or PatB [221, 222].

Recently, MATE family transporters have been identified which may confer FQ resistance to *S. pneumoniae*. PdrM activity was shown to reduce susceptibility to norfloxacin and ciprofloxacin when overexpressed in *E. coli* [223]. Similarly, inactivation of DinF increased susceptibility to moxifloxacin, levofloxacin, and ciprofloxacin [224]. However, these changes in susceptibility were relatively small and more work is needed to clarify the roles these MATE transporters have in conferring clinically relevant FQ resistance.

EmeA is a NorA homologue identified by probing the *Enterococcus faecalis* V583 genome data [222, 225]. It is a multidrug pump capable of norfloxacin and ethidium bromide efflux. When deleted, susceptibility to acriflavine and ciprofloxacin increases suggesting that these compounds also are substrates. The contribution of EmeA to intrinsic FQ susceptibility in clinical isolates of *E. faecalis* is unknown.

Lactococcus lactis is extensively used in the dairy industry and is generally not considered a human pathogen. Nevertheless, like the study of multidrug pumps in *B. subtilis*, the study of such pumps in *L. lactis* has added significantly to our knowledge of how these pumps work. Of the more than 40 genes encoding putative drug transporters identified in *L. lactis*, only one is known to encode a pump capable of FQ efflux. This pump, LmrA, is an ABC transporter homologous to (and functionally interchangeable with) the human multidrug transporter P-glycoprotein [226].

4 Mycobacterium Tuberculosis

In the first edition we did not discuss *Mycobacterium tuberculosis* (the etiologic agent of tuberculosis) because at that time FQs were not commonly used for treatment of tuberculous infections. Now, about one-third of the world's population is latently infected with *M. tuberculosis*, and 10 % of these will develop active disease at some point in their lives. Without an effective vaccine or more potent antituberculosis drugs to shorten duration of therapy, it is still unclear as to how TB can be controlled in countries in which it is endemic [227]. Recent reports have demonstrated that FQ courses as short as 7 days may lead to resistant TB [228, 229]. Thus, there is now a more pressing need to understand mechanisms of resistance in this organism.

Many of the FQ resistance mechanisms found in Gram-positive and Gram-negative bacteria also exist in *M. tuberculosis* [134, 135, 230–232]. The primary resistance mechanism is QRDR substitution mutations within the DNA gyrase GyrA subunit [227, 233–236]. It is recognized that Ala90 and Asp94 are the most frequently mutated positions in GyrA, but Gly88, Ser91, and Ala74 are also possible mutation sites [236–238]. GyrB substitutions in clinical strains of *M. tuberculosis* are increasingly being reported, but the substrate specificity and degree of resistance varies among these mutations [24, 239–243]. Novel DNA gyrase mutations are still being identified [239, 243–245]. A comprehensive analysis of topoisomerase mutations in *M. tuberculosis* is also available [243]. Resistance can also result from DNA gyrase-independent mechanisms, such as overexpression of *mfpA*, the product of which is a protein of the PRP family that causes resistance to FQs by binding to DNA gyrase, inhibiting its activity [154, 246].

Several *M. tuberculosis* efflux pumps confer resistance to FQs when expressed in *M. smegmatis*. Rv1634 (MFS family) conferred a fourfold increase in MICs to ciprofloxacin, lomefloxacin, and norfloxacin [247]. In addition to norfloxacin, DrrAB (ABC family) conferred resistance to a broad range of clinically relevant antibiotics. This activity was reversed by the efflux pump inhibitors reserpine and verapamil [248]. Lastly, Rv2686c-Rv2687c-Rv2688c (ABC family) expression increased ciprofloxacin MICs eightfold. Interestingly, expression of Rv2686c alone was enough to confer a fourfold increase in ciprofloxacin MIC [249]. As with numerous efflux systems described herein, the importance of these *M. tuberculosis* transporters in conferring FQ resistance in clinical isolates is currently unclear.

5 Means to Limit or Overcome FQ Resistance

FQs are among the most frequently prescribed antimicrobial agents. It is not unusual that they are used inappropriately, with an example being the prescription of levofloxacin for viral upper respiratory tract infections. Education of primary care physicians regarding the seriousness of the antimicrobial agent resistance problem in general, and that of FQs in

particular, and encouraging them to not succumb to pressure to prescribe antimicrobial treatment for infections that are most likely viral in nature will help to reduce selective pressure. The dissemination of well-conceived guidelines for the proper use of these drugs and the institution of formulary restrictions are other methods by which inappropriate FQ use might be reduced [250–252].

Once resistance to a particular antimicrobial agent reaches a critical prevalence, the utility of that drug becomes severely compromised. Most often, alternative therapy will be prescribed. Much work has been done on the development of compounds that block multidrug efflux pumps (efflux pump inhibitors, or EPIs), many of which have FQs as substrates [253–255]. Increased efflux often is the first step along the pathway towards high-level target-based resistance and inhibition of this process may prevent such mutants from appearing. In addition, if efflux is the only mechanism of FQ resistance possessed by a particular pathogen, then the combination of a FQ with an EPI may result in the recovery of clinically useful activity of that drug. It has been shown in vitro that target-based resistance mutations occur much less frequently when an EPI is present in addition to the FQ [20, 21, 88, 256–258]. Currently, no EPI has been approved for clinical use. Preclinical trials testing a combination therapy of an EPI with a FQ against FQ-resistant organisms causing infections in patients with cystic fibrosis were discontinued due to tolerability issues [259]. However, EPI development remains a highly active area of research, with many recent advances reviewed in detail elsewhere [254, 256, 260].

References

1. Andriole VT. The quinolones: past, present, and future. Clin Infect Dis. 2005;41 Suppl 2:S113–9.
2. Lescher GY, Froelich ED, Gruet MD, Bailey JH, Brundage RP. 1,8-naphthyridine derivatives: a new class of chemotherapy agents. J Med Pharm Chem. 1962;5:1063–8.
3. O'Brien TP. Besifloxacin ophthalmic suspension, 0.6%: a novel topical fluoroquinolone for bacterial conjunctivitis. Adv Ther. 2012;29(6):473–90.
4. Keating GM. Sitafloxacin: in bacterial infections. Drugs. 2011; 71(6):731–44.
5. Blum MD, Graham DJ, McCloskey CA. Temafloxacin syndrome: review of 95 cases. Clin Infect Dis. 1994;18(6):946–50.
6. Stahlmann R. Clinical toxicological aspects of fluoroquinolones. Toxicol Lett. 2002;127(1–3):269–77.
7. Kocyigit I, Dortdudak S, Sipahioglu M, Unal A, Yucel HE, Tokgoz B, et al. Levofloxacin-induced delirium: is it a dangerous drug in patients with renal dysfunction? Ren Fail. 2012;34(5):634–6.
8. Mishra A, Dave N. Norfloxacin-induced hypoglycemia and urticaria. Indian J Pharmacol. 2012;44(3):415–6.
9. Ball P. Adverse drug reactions: implications for the development of fluoroquinolones. J Antimicrob Chemother. 2003;51 Suppl 1:21–7.
10. Martinez-Martinez L, Pascual A, Jacoby GA. Quinolone resistance from a transferable plasmid. Lancet. 1998;351(9105):797–9.
11. Hussein G, Perkins LT, Sternberg M, Bland C. Gatifloxacin-induced hypoglycemia: a case report and review of the literature. Clin Res Regul Aff. 2002;19(4):333–9.
12. Menzies DJ, Dorsainvil PA, Cunha BA, Johnson DH. Severe and persistent hypoglycemia due to gatifloxacin interaction with oral hypoglycemic agents. Am J Med. 2002;113(3):232–4.
13. Micheli L, Sbrilli M, Nencini C. Severe hypoglycemia associated with levofloxacin in Type 2 diabetic patients receiving polytherapy: two case reports. Int J Clin Pharmacol Ther. 2012;50(4): 302–6.
14. Licata A, Randazzo C, Morreale I, Butera G, D'Alessandro N, Craxi A. Fluoroquinolone-induced liver injury: three new cases and a review of the literature. Eur J Clin Pharmacol. 2012;68(5): 525–32.
15. Stahlmann R, Lode HM. Risks associated with the therapeutic use of fluoroquinolones. Expert Opin Drug Saf. 2013;12(4):497–505.
16. Etminan M, Forooghian F, Brophy JM, Bird ST, Maberley D. Oral fluoroquinolones and the risk of retinal detachment. JAMA. 2012;307(13):1414–9.
17. Matsumoto T, Uchino K, Yamaguchi H, Yoshida S, Takahashi M, Kodama H, et al. Study on the safety and efficacy of sitafloxacin—results of the use-results survey. Jpn J Antibiot. 2011;64(5): 319–37.
18. Drlica K. Mechanism of fluoroquinolone action. Curr Opin Microbiol. 1999;2(5):504–8.
19. Briales A, Rodriguez-Martinez JM, Velasco C, Diaz de Alba P, Dominguez-Herrera J, Pachon J, et al. In vitro effect of *qnrA1*, *qnrB1*, and *qnrS1* genes on fluoroquinolone activity against isogenic *Escherichia coli* isolates with mutations in *gyrA* and *parC*. Antimicrob Agents Chemother. 2011;55(3):1266–9.
20. Markham PN, Neyfakh AA. Inhibition of the multidrug transporter NorA prevents emergence of norfloxacin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1996; 40(11):2673–4.
21. Markham PN. Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine. Antimicrob Agents Chemother. 1999;43(4):988–9.
22. Lomovskaya O, Lee A, Hoshino K, Ishida H, Mistry A, Warren MS, et al. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 1999;43(6):1340–6.
23. Zhanel GG, Hoban DJ, Schurek K, Karlowsky JA. Role of efflux mechanisms on fluoroquinolone resistance in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. Int J Antimicrob Agents. 2004;24(6):529–35.
24. Hooper DC. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis. 2001;7(2):337–41.
25. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother. 2003;51(5):1109–17.
26. Woodford N, Livermore DM. Infections caused by Gram-positive bacteria: a review of the global challenge. J Infect. 2009;59 Suppl 1:S4–16.
27. Niedermann MS. Principles of appropriate antibiotic use. Int J Antimicrob Agents. 2005;26 Suppl 3:S170–5.
28. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis. 2005;41 Suppl 2:S120–6.
29. Schultsz C, Geerlings S. Plasmid-mediated resistance in *Enterobacteriaceae*: changing landscape and implications for therapy. Drugs. 2012;72(1):1–16.
30. Rice LB. Mechanisms of resistance and clinical relevance of resistance to beta-lactams, glycopeptides, and fluoroquinolones. Mayo Clin Proc. 2012;87(2):198–208.

31. Gellert M, Mizuuchi K, O'Dea MH, Nash HA. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc Natl Acad Sci U S A. 1976;73(11):3872–6.
32. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev. 1997;61(3):377–92.
33. Gellert M, Mizuuchi K, O'Dea MH, Itoh T, Tomizawa JI. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc Natl Acad Sci U S A. 1977;74(11):4772–6.
34. Kato J, Nishimura Y, Imamura R, Niki H, Hiraga S, Suzuki H. New topoisomerase essential for chromosome segregation in *E. coli*. Cell. 1990;63(2):393–404.
35. Ullsperger C, Cozzarelli NR. Contrasting enzymatic activities of topoisomerase IV and DNA gyrase from *Escherichia coli*. J Biol Chem. 1996;271(49):31549–55.
36. Peng H, Marians KJ. The interaction of *Escherichia coli* topoisomerase IV with DNA. J Biol Chem. 1995;270(42):25286–90.
37. Hoshino K, Kitamura A, Morrissey I, Sato K, Kato J, Ikeda H. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob Agents Chemother. 1994;38(11):2623–7.
38. Khodursky AB, Zechiedrich EL, Cozzarelli NR. Topoisomerase IV is a target of quinolones in *Escherichia coli*. Proc Natl Acad Sci U S A. 1995;92(25):11801–5.
39. Tse-Dinh YC. Exploring DNA, topoisomerases as targets of novel therapeutic agents in the treatment of infectious diseases. Infect Disord Drug Targets. 2007;7(1):3–9.
40. Drlica K, Hiasa H, Kerns R, Malik M, Mustaev A, Zhao X. Quinolones: action and resistance updated. Curr Top Med Chem. 2009;9(11):981–98.
41. Kampranis SC, Maxwell A. Conformational changes in DNA gyrase revealed by limited proteolysis. J Biol Chem. 1998;273(35):22606–14.
42. Khodursky AB, Cozzarelli NR. The mechanism of inhibition of topoisomerase IV by quinolone antibacterials. J Biol Chem. 1998;273(42):27668–77.
43. Willmott CJ, Critchlow SE, Eperon IC, Maxwell A. The complex of DNA gyrase and quinolone drugs with DNA forms a barrier to transcription by RNA polymerase. J Mol Biol. 1994;242(4):351–63.
44. Hiasa H, Yousef DO, Marians KJ. DNA strand cleavage is required for replication fork arrest by a frozen topoisomerase-quinolone-DNA ternary complex. J Biol Chem. 1996;271(42):26424–9.
45. Krueger S, Zaccai G, Wlodawer A, Langowski J, O'Dea M, Maxwell A, et al. Neutron and light-scattering studies of DNA gyrase and its complex with DNA. J Mol Biol. 1990;211(1):211–20.
46. Marians KJ, Hiasa H. Mechanism of quinolone action. A drug-induced structural perturbation of the DNA precedes strand cleavage by topoisomerase IV. J Biol Chem. 1997;272(14):9401–9.
47. Hawkey PM. Mechanisms of quinolone action and microbial response. J Antimicrob Chemother. 2003;51 Suppl 1:29–35.
48. Schmitz FJ, Higgins PG, Mayer S, Fluit AC, Dalhoff A. Activity of quinolones against gram-positive cocci: mechanisms of drug action and bacterial resistance. Eur J Clin Microbiol Infect Dis. 2002;21(9):647–59.
49. Yamagishi J, Kojima T, Oyamada Y, Fujimoto K, Hattori H, Nakamura S, et al. Alterations in the DNA topoisomerase IV *grlA* gene responsible for quinolone resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1996;40(5):1157–63.
50. Blanche F, Cameron B, Bernard FX, Maton L, Manse B, Ferrero L, et al. Differential behaviors of *Staphylococcus aureus* and *Escherichia coli* type II DNA topoisomerases. Antimicrob Agents Chemother. 1996;40(12):2714–20.
51. Yoshida H, Bogaki M, Nakamura M, Yamanaka LM, Nakamura S. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. Antimicrob Agents Chemother. 1991;35(8):1647–50.
52. Bruchmann S, Dotsch A, Nouri B, Chaberny IF, Haussler S. Quantitative contributions of target alteration and decreased drug accumulation to *Pseudomonas aeruginosa* fluoroquinolone resistance. Antimicrob Agents Chemother. 2013;57(3):1361–8.
53. Hooper DC. Mechanisms of quinolone resistance. In: Hooper DC, Rubenstein E, editors. Quinolone antimicrobial agents. Washington, DC: American Society for Microbiology; 2003. p. 41–67.
54. Horowitz DS, Wang JC. Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. J Biol Chem. 1987;262(11):5339–44.
55. Brown JC, Shanahan PM, Jesudason MV, Thomson CJ, Amyes SG. Mutations responsible for reduced susceptibility to 4-quinolones in clinical isolates of multi-resistant *Salmonella typhi* in India. J Antimicrob Chemother. 1996;37(5):891–900.
56. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ. 2008;86(4):260–8.
57. Lee CJ, Su LH, Huang YC, Chiu CH. First isolation of ciprofloxacin-resistant *Salmonella enterica* serovar Typhi in Taiwan. J Microbiol Immunol Infect. 2013;46(6):469–73.
58. Chitnis V, Chitnis D, Verma S, Hemvani N. Multidrug-resistant *Salmonella typhi* in India. Lancet. 1999;354(9177):514–5.
59. Gaind R, Paglietti B, Murgia M, Dawar R, Uzzau S, Cappuccinelli P, et al. Molecular characterization of ciprofloxacin-resistant *Salmonella enterica* serovar Typhi and Paratyphi A causing enteric fever in India. J Antimicrob Chemother. 2006;58(6):1139–44.
60. Morita M, Hirose K, Takai N, Terajima J, Watanabe H, Sagara H, et al. *Salmonella enterica* serovar Typhi in Japan, 2001−2006: emergence of high-level fluoroquinolone-resistant strains. Epidemiol Infect. 2010;138(3):318–21.
61. Chiu CH, Lee HY, Tseng LY, Chen CL, Chia JH, Su LH, et al. Mechanisms of resistance to ciprofloxacin, ampicillin/sulbactam and imipenem in *Acinetobacter baumannii* clinical isolates in Taiwan. Int J Antimicrob Agents. 2010;35(4):382–6.
62. Vila J, Ruiz J, Goni P, Marcos A, Jimenez de Anta T. Mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 1995;39(5):1201–3.
63. Vila J, Ruiz J, Goni P, Jimenez de Anta T. Quinolone-resistance mutations in the topoisomerase IV *parC* gene of *Acinetobacter baumannii*. J Antimicrob Chemother. 1997;39(6):757–62.
64. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrob Agents Chemother. 2001;45(12):3375–80.
65. Agnello M, Wong-Beringer A. Differentiation in quinolone resistance by virulence genotype in *Pseudomonas aeruginosa*. PLoS One. 2012;7(8):e42973.
66. Tessier PR, Nicolau DP. In vitro activity of novel gyrase inhibitors against a highly resistant population of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2013;57(6):2887–9.
67. Tari LW, Trzoss M, Bensen DC, Li X, Chen Z, Lam T, et al. Pyrrolopyrimidine inhibitors of DNA gyrase B (GyrB) and topoisomerase IV (ParE). Part I: Structure guided discovery and optimization of dual targeting agents with potent, broad-spectrum enzymatic activity. Bioorg Med Chem Lett. 2013;23(5):1529–36.
68. Trzoss M, Bensen DC, Li X, Chen Z, Lam T, Zhang J, et al. Pyrrolopyrimidine inhibitors of DNA gyrase B (GyrB) and topoisomerase IV (ParE), Part II: development of inhibitors with broad spectrum, Gram-negative antibacterial activity. Bioorg Med Chem Lett. 2013;23(5):1537–43.

69. Pillar C, Stapert L, Marchak M, Shinabarger D. Potent acivity of novel gyrase/topoisomerase inhibitors against gram-negative and gram-positive pathogens with important resistance phenotypes. In: ICAAC. San Francisco, CA: F-2019; 2012.
70. Delcour AH. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta. 2009;1794(5):808–16.
71. Chapman JS, Georgopapadakou NH. Routes of quinolone permeation in *Escherichia coli*. Antimicrob Agents Chemother. 1988; 32(4):438–42.
72. Hirai K, Aoyama H, Irikura T, Iyobe S, Mitsuhashi S. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. Antimicrob Agents Chemother. 1986;29(3):535–8.
73. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593–656.
74. Poole K. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother. 2005;56(1):20–51.
75. Cohen SP, Hooper DC, Wolfson JS, Souza KS, McMurry LM, Levy SB. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob Agents Chemother. 1988;32(8): 1187–91.
76. Thanassi DG, Suh GS, Nikaido H. Role of outer membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. J Bacteriol. 1995;177(4):998–1007.
77. Wiedemann B, Heisig P. Mechanisms of quinolone resistance. Infection. 1994;22 Suppl 2:S73–9.
78. Tavio MM, Aquili VD, Poveda JB, Antunes NT, Sanchez-Cespedes J, Vila J. Quorum-sensing regulator *sdiA* and *marA* overexpression is involved in in vitro-selected multidrug resistance of *Escherichia coli*. J Antimicrob Chemother. 2010; 65(6):1178–86.
79. Alekshun MN, Levy SB. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob Agents Chemother. 1997;41(10):2067–75.
80. Chou JH, Greenberg JT, Demple B. Posttranscriptional repression of *Escherichia coli* OmpF protein in response to redox stress: positive control of the *micF* antisense RNA by the *soxRS* locus. J Bacteriol. 1993;175(4):1026–31.
81. Dupont M, James CE, Chevalier J, Pages JM. An early response to environmental stress involves regulation of OmpX and OmpF, two enterobacterial outer membrane pore-forming proteins. Antimicrob Agents Chemother. 2007;51(9):3190–8.
82. Nikaido H. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob Agents Chemother. 1989;33(11): 1831–6.
83. Yoshimura F, Nikaido H. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J Bacteriol. 1982;152(2):636–42.
84. Nikaido H. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol. 2001;12(3):215–23.
85. Morita Y, Kataoka A, Shiota S, Mizushima T, Tsuchiya T. NorM of *Vibrio parahaemolyticus* is an Na(+)-driven multidrug efflux pump. J Bacteriol. 2000;182(23):6694–7.
86. Roberts MC. Update on acquired tetracycline resistance genes. FEMS Microbiol Lett. 2005;245(2):195–203.
87. Schindler BD, Patel D, Seo SM, Kaatz GW. Mutagenesis and modeling to predict structural and functional characteristics of the *Staphylococcus aureus* MepA Multidrug Efflux Pump. J Bacteriol. 2013;195(3):523–33.
88. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs. 2009;69(12):1555–623.
89. Nikaido H, Pages JM. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev. 2012;36(2):340–63.
90. Poole K. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob Agents Chemother. 2000; 44(9):2233–41.
91. Okusu H, Ma D, Nikaido H. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol. 1996;178(1):306–8.
92. Misra R, Bavro VN. Assembly and transport mechanism of tripartite drug efflux systems. Biochim Biophys Acta. 2009;1794(5): 817–25.
93. Nikaido H. Structure and mechanism of RND-type multidrug efflux pumps. Adv Enzymol Relat Areas Mol Biol. 2011;77:1–60.
94. Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V. The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc Natl Acad Sci U S A. 2009; 106(17):7173–8.
95. Zgurskaya HI, Nikaido H. Cross-linked complex between oligomeric periplasmic lipoprotein AcrA and the inner-membrane-associated multidrug efflux pump AcrB from *Escherichia coli*. J Bacteriol. 2000;182(15):4264–7.
96. Yu EW, Aires JR, Nikaido H. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J Bacteriol. 2003;185(19):5657–64.
97. Alekshun MN, Levy SB. The mar regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol. 1999;7(10):410–3.
98. Martin RG, Rosner JL. Genomics of the *marA*/*soxS*/*rob* regulon of *Escherichia coli*: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. Mol Microbiol. 2002;44(6):1611–24.
99. Zheng J, Cui S, Meng J. Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant *Salmonella Typhimurium*. J Antimicrob Chemother. 2009;63(1):95–102.
100. Ariza RR, Li Z, Ringstad N, Demple B. Activation of multiple antibiotic resistance and binding of stress-inducible promoters by *Escherichia coli* Rob protein. J Bacteriol. 1995;177(7):1655–61.
101. Hung LW, Kim HB, Murakami S, Gupta G, Kim CY, Terwilliger TC. Crystal structure of AcrB complexed with linezolid at 3.5 A resolution. J Struct Funct Genomics. 2013;14(2):71–5.
102. Eicher T, Cha HJ, Seeger MA, Brandstatter L, El-Delik J, Bohnert JA, et al. Transport of drugs by the multidrug transporter AcrB involves an access and a deep binding pocket that are separated by a switch-loop. Proc Natl Acad Sci U S A. 2012; 109(15):5687–92.
103. Murakami S, Nakashima R, Yamashita E, Matsumoto T, Yamaguchi A. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature. 2006;443(7108): 173–9.
104. Murakami S, Nakashima R, Yamashita E, Yamaguchi A. Crystal structure of bacterial multidrug efflux transporter AcrB. Nature. 2002;419(6907):587–93.
105. Das D, Xu QS, Lee JY, Ankoudinova I, Huang C, Lou Y, et al. Crystal structure of the multidrug efflux transporter AcrB at 3.1A resolution reveals the N-terminal region with conserved amino acids. J Struct Biol. 2007;158(3):494–502.
106. Elkins CA, Nikaido H. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. J Bacteriol. 2002;184(23):6490–8.
107. Seeger MA, Schiefner A, Eicher T, Verrey F, Diederichs K, Pos KM. Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. Science. 2006;313(5791):1295–8.

108. Sennhauser G, Amstutz P, Briand C, Storchenegger O, Grutter MG. Drug export pathway of multidrug exporter AcrB revealed by DARPin inhibitors. PLoS Biol. 2007;5(1):e7.
109. Bohnert JA, Schuster S, Seeger MA, Fahnrich E, Pos KM, Kern WV. Site-directed mutagenesis reveals putative substrate binding residues in the *Escherichia coli* RND efflux pump AcrB. J Bacteriol. 2008;190(24):8225–9.
110. Poole K. Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect. 2004;10(1):12–26.
111. Li Y, Mima T, Komori Y, Morita Y, Kuroda T, Mizushima T, et al. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. J Antimicrob Chemother. 2003;52(4):572–5.
112. Strateva T, Yordanov D. *Pseudomonas aeruginosa*—a phenomenon of bacterial resistance. J Med Microbiol. 2009;58(Pt 9): 1133–48.
113. Li XZ, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob Agents Chemother. 1994;38(8):1732–41.
114. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2000;44(12):3322–7.
115. Dunham SA, McPherson CJ, Miller AA. The relative contribution of efflux and target gene mutations to fluoroquinolone resistance in recent clinical isolates of *Pseudomonas aeruginosa*. Eur J Clin Microbiol Infect Dis. 2010;29(3):279–88.
116. Kiser TH, Obritsch MD, Jung R, MacLaren R, Fish DN. Efflux pump contribution to multidrug resistance in clinical isolates of *Pseudomonas aeruginosa*. Pharmacotherapy. 2010;30(7):632–8.
117. Colmer JA, Fralick JA, Hamood AN. Isolation and characterization of a putative multidrug resistance pump from *Vibrio cholerae*. Mol Microbiol. 1998;27(1):63–72.
118. Alonso A, Martinez JL. Cloning and characterization of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother. 2000;44(11):3079–86.
119. Morita Y, Kodama K, Shiota S, Mine T, Kataoka A, Mizushima T, et al. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob Agents Chemother. 1998;42(7):1778–82.
120. Miyamae S, Ueda O, Yoshimura F, Hwang J, Tanaka Y, Nikaido H. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides thetaiotaomicron*. Antimicrob Agents Chemother. 2001;45(12):3341–6.
121. Yoon EJ, Courvalin P, Grillot-Courvalin C. RND-type efflux pumps in multidrug-resistant clinical isolates of *Acinetobacter baumannii*: major role for AdeABC overexpression and AdeRS mutations. Antimicrob Agents Chemother. 2013;57(7):2989–95.
122. Coyne S, Rosenfeld N, Lambert T, Courvalin P, Perichon B. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2010;54(10):4389–93.
123. Damier-Piolle L, Magnet S, Bremont S, Lambert T, Courvalin P. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2008;52(2):557–62.
124. Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci U S A. 2002;99(8):5638–42.
125. Vetting MW, Hegde SS, Fajardo JE, Fiser A, Roderick SL, Takiff HE, et al. Pentapeptide repeat proteins. Biochemistry. 2006;45(1): 1–10.
126. Vetting MW, Hegde SS, Wang M, Jacoby GA, Hooper DC, Blanchard JS. Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor. J Biol Chem. 2011;286(28):25265–73.
127. Xiong X, Bromley EH, Oelschlaeger P, Woolfson DN, Spencer J. Structural insights into quinolone antibiotic resistance mediated by pentapeptide repeat proteins: conserved surface loops direct the activity of a Qnr protein from a gram-negative bacterium. Nucleic Acids Res. 2011;39(9):3917–27.
128. Ruiz J, Pons MJ, Gomes C. Transferable mechanisms of quinolone resistance. Int J Antimicrob Agents. 2012;40(3):196–203.
129. Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein QnrA with *Escherichia coli* topoisomerase IV. Antimicrob Agents Chemother. 2005;49(7): 3050–2.
130. Wang M, Tran JH, Jacoby GA, Zhang Y, Wang F, Hooper DC. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob Agents Chemother. 2003;47(7):2242–8.
131. Wang M, Sahm DF, Jacoby GA, Hooper DC. Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. Antimicrob Agents Chemother. 2004;48(4):1295–9.
132. Robicsek A, Sahm DF, Strahilevitz J, Jacoby GA, Hooper DC. Broader distribution of plasmid-mediated quinolone resistance in the United States. Antimicrob Agents Chemother. 2005;49(7): 3001–3.
133. Jacoby GA, Walsh KE, Mills DM, Walker VJ, Oh H, Robicsek A, et al. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob Agents Chemother. 2006;50(4):1178–82.
134. Ruiz J, Gomez J, Navia MM, Ribera A, Sierra JM, Marco F, et al. High prevalence of nalidixic acid resistant, ciprofloxacin susceptible phenotype among clinical isolates of *Escherichia coli* and other *Enterobacteriaceae*. Diagn Microbiol Infect Dis. 2002; 42(4):257–61.
135. Cesaro A, Bettoni RR, Lascols C, Merens A, Soussy CJ, Cambau E. Low selection of topoisomerase mutants from strains of *Escherichia coli* harbouring plasmid-borne qnr genes. J Antimicrob Chemother. 2008;61(5):1007–15.
136. Hansen LH, Jensen LB, Sorensen HI, Sorensen SJ. Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. J Antimicrob Chemother. 2007;60(1):145–7.
137. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, et al. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12(1):83–8.
138. Poirel L, Cattoir V, Nordmann P. Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. Front Microbiol. 2012;3:24.
139. Ahmed AM, Motoi Y, Sato M, Maruyama A, Watanabe H, Fukumoto Y, et al. Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. Appl Environ Microbiol. 2007;73(20):6686–90.
140. Liu BT, Wang XM, Liao XP, Sun J, Zhu HQ, Chen XY, et al. Plasmid-mediated quinolone resistance determinants *oqxAB* and *aac(6′)-Ib-cr* and extended-spectrum beta-lactamase gene *bla*CTX-M-24 co-located on the same plasmid in one *Escherichia coli* strain from China. J Antimicrob Chemother. 2011;66(7): 1638–9.
141. Soufi L, Saenz Y, Vinue L, Abbassi MS, Ruiz E, Zarazaga M, et al. *Escherichia coli* of poultry food origin as reservoir of sulphonamide resistance genes and integrons. Int J Food Microbiol. 2011;144(3):497–502.
142. Du XD, Li DX, Hu GZ, Wang Y, Shang YH, Wu CM, et al. Tn*1548*-associated *armA* is co-located with *qnrB2*, *aac(6′)-Ib-cr* and *bla*CTX-M-3 on an IncFII plasmid in a *Salmonella enterica* subsp. enterica serovar Paratyphi B strain isolated from chickens in China. J Antimicrob Chemother. 2012;67(1):246–8.

143. Gibson JS, Cobbold RN, Heisig P, Sidjabat HE, Kyaw-Tanner MT, Trott DJ. Identification of Qnr and AAC(6′)-1b-cr plasmid-mediated fluoroquinolone resistance determinants in multidrug-resistant *Enterobacter* spp. isolated from extraintestinal infections in companion animals. Vet Microbiol. 2010;143(2–4):329–36.
144. Martens R, Wetzstein HG, Zadrazil F, Capelari M, Hoffmann P, Schmeer N. Degradation of the fluoroquinolone enrofloxacin by wood-rotting fungi. Appl Environ Microbiol. 1996;62(11): 4206–9.
145. Wetzstein HG, Stadler M, Tichy HV, Dalhoff A, Karl W. Degradation of ciprofloxacin by basidiomycetes and identification of metabolites generated by the brown rot fungus *Gloeophyllum striatum*. Appl Environ Microbiol. 1999;65(4):1556–63.
146. Kim DW, Heinze TM, Kim BS, Schnackenberg LK, Woodling KA, Sutherland JB. Modification of norfloxacin by a *Microbacterium* sp. strain isolated from a wastewater treatment plant. Appl Environ Microbiol. 2011;77(17):6100–8.
147. Kaatz GW, Seo SM. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1995;39(12):2650–5.
148. Kaatz GW, Seo SM, Ruble CA. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1993;37(5):1086–94.
149. Kaatz GW, Seo SM, O'Brien L, Wahiduzzaman M, Foster TJ. Evidence for the existence of a multidrug efflux transporter distinct from NorA in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2000;44(5):1404–6.
150. Martinez-Garriga B, Vinuesa T, Hernandez-Borrell J, Vinas M. The contribution of efflux pumps to quinolone resistance in *Streptococcus pneumoniae* clinical isolates. Int J Med Microbiol. 2007;297(3):187–95.
151. Rodriguez-Martinez JM, Velasco C, Briales A, Garcia I, Conejo MC, Pascual A. Qnr-like pentapeptide repeat proteins in gram-positive bacteria. J Antimicrob Chemother. 2008;61(6):1240–3.
152. Montero C, Mateu G, Rodriguez R, Takiff H. Intrinsic resistance of *Mycobacterium smegmatis* to fluoroquinolones may be influenced by new pentapeptide protein MfpA. Antimicrob Agents Chemother. 2001;45(12):3387–92.
153. Arsene S, Leclercq R. Role of a *qnr*-like gene in the intrinsic resistance of *Enterococcus faecalis* to fluoroquinolones. Antimicrob Agents Chemother. 2007;51(9):3254–8.
154. Hegde SS, Vetting MW, Roderick SL, Mitchenall LA, Maxwell A, Takiff HE, et al. A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. Science. 2005; 308(5727):1480–3.
155. Jacoby GA, Hooper DC. Phylogenetic analysis of chromosomally determined *qnr* and related proteins. Antimicrob Agents Chemother. 2013;57(4):1930–4.
156. Ng EY, Trucksis M, Hooper DC. Quinolone resistance mutations in topoisomerase IV: relationship to the *flqA* locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in *Staphylococcus aureus*. Antimicrob Agents Chemother. 1996;40(8):1881–8.
157. Eliopoulos GM. Quinolone resistance mechanisms in pneumococci. Clin Infect Dis. 2004;38 Suppl 4:S350–6.
158. Neyfakh AA, Borsch CM, Kaatz GW. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrob Agents Chemother. 1993;37(1): 128–9.
159. Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J Bacteriol. 1990;172(12):6942–9.
160. Costa SS, Viveiros M, Amaral L, Couto I. Multidrug Efflux Pumps in *Staphylococcus aureus*: an update. Open Microbiol J. 2013; 7:59–71.
161. Yu JL, Grinius L, Hooper DC. NorA functions as a multidrug efflux protein in both cytoplasmic membrane vesicles and reconstituted proteoliposomes. J Bacteriol. 2002;184(5):1370–7.
162. Hsieh PC, Siegel SA, Rogers B, Davis D, Lewis K. Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. Proc Natl Acad Sci U S A. 1998;95(12):6602–6.
163. Kaatz GW, Thyagarajan RV, Seo SM. Effect of promoter region mutations and *mgrA* overexpression on transcription of *norA*, which encodes a *Staphylococcus aureus* multidrug efflux transporter. Antimicrob Agents Chemother. 2005;49(1):161–9.
164. Ingavale SS, Van Wamel W, Cheung AL. Characterization of RAT, an autolysis regulator in *Staphylococcus aureus*. Mol Microbiol. 2003;48(6):1451–66.
165. Ingavale S, van Wamel W, Luong TT, Lee CY, Cheung AL. Rat/MgrA, a regulator of autolysis, is a regulator of virulence genes in *Staphylococcus aureus*. Infect Immun. 2005;73(3):1423–31.
166. Luong TT, Newell SW, Lee CY. Mgr, a novel global regulator in *Staphylococcus aureus*. J Bacteriol. 2003;185(13):3703–10.
167. Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY. Transcription profiling of the *mgrA* regulon in *Staphylococcus aureus*. J Bacteriol. 2006;188(5):1899–910.
168. Gertz S, Engelmann S, Schmid R, Ziebandt AK, Tischer K, Scharf C, et al. Characterization of the sigma(B) regulon in *Staphylococcus aureus*. J Bacteriol. 2000;182(24):6983–91.
169. Wu S, de Lencastre H, Tomasz A. Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J Bacteriol. 1996;178(20):6036–42.
170. Hardwick SW, Pane-Farre J, Delumeau O, Marles-Wright J, Murray JW, Hecker M, et al. Structural and functional characterization of partner switching regulating the environmental stress response in *Bacillus subtilis*. J Biol Chem. 2007;282(15): 11562–72.
171. Truong-Bolduc QC, Dunman PM, Strahilevitz J, Projan SJ, Hooper DC. MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. J Bacteriol. 2005;187(7):2395–405.
172. Fournier B, Aras R, Hooper DC. Expression of the multidrug resistance transporter NorA from *Staphylococcus aureus* is modified by a two-component regulatory system. J Bacteriol. 2000; 182(3):664–71.
173. Truong-Bolduc QC, Zhang X, Hooper DC. Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. J Bacteriol. 2003;185(10):3127–38.
174. Truong-Bolduc QC, Ding Y, Hooper DC. Posttranslational modification influences the effects of MgrA on *norA* expression in *Staphylococcus aureus*. J Bacteriol. 2008;190(22):7375–81.
175. Truong-Bolduc QC, Hooper DC. Phosphorylation of MgrA and its effect on expression of the NorA and NorB efflux pumps of *Staphylococcus aureus*. J Bacteriol. 2010;192(10):2525–34.
176. Truong-Bolduc QC, Strahilevitz J, Hooper DC. NorC, a new efflux pump regulated by MgrA of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2006;50(3):1104–7.
177. Huang J, O'Toole PW, Shen W, Amrine-Madsen H, Jiang X, Lobo N, et al. Novel chromosomally encoded multidrug efflux transporter MdeA in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2004;48(3):909–17.
178. Yamada Y, Shiota S, Mizushima T, Kuroda T, Tsuchiya T. Functional gene cloning and characterization of MdeA, a multidrug efflux pump from *Staphylococcus aureus*. Biol Pharm Bull. 2006;29(4):801–4.
179. DeMarco CE, Cushing LA, Frempong-Manso E, Seo SM, Jaravaza TA, Kaatz GW. Efflux-related resistance to norfloxacin, dyes, and biocides in bloodstream isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother. 2007;51(9):3235–9.
180. Noguchi N, Nakaminami H, Nishijima S, Kurokawa I, So H, Sasatsu M. Antimicrobial agent of susceptibilities and antiseptic

resistance gene distribution among methicillin-resistant *Staphylococcus aureus* isolates from patients with impetigo and staphylococcal scalded skin syndrome. J Clin Microbiol. 2006; 44(6):2119–25.
181. Mayer S, Boos M, Beyer A, Fluit AC, Schmitz FJ. Distribution of the antiseptic resistance genes *qacA*, *qacB* and *qacC* in 497 methicillin-resistant and -susceptible European isolates of *Staphylococcus aureus*. J Antimicrob Chemother. 2001;47(6): 896–7.
182. Alam MM, Kobayashi N, Uehara N, Watanabe N. Analysis on distribution and genomic diversity of high-level antiseptic resistance genes *qacA* and *qacB* in human clinical isolates of *Staphylococcus aureus*. Microb Drug Resist. 2003;9(2):109–21.
183. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev. 1999;12(1):147–79.
184. Paulsen IT, Brown MH, Skurray RA. Proton-dependent multidrug efflux systems. Microbiol Rev. 1996;60(4):575–608.
185. Noguchi N, Hase M, Kitta M, Sasatsu M, Deguchi K, Kono M. Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett. 1999;172(2):247–53.
186. Noguchi N, Suwa J, Narui K, Sasatsu M, Ito T, Hiramatsu K, et al. Susceptibilities to antiseptic agents and distribution of antiseptic-resistance genes *qacA*/*B* and *smr* of methicillin-resistant *Staphylococcus aureus* isolated in Asia during 1998 and 1999. J Med Microbiol. 2005;54(Pt 6):557–65.
187. Littlejohn TG, Paulsen IT, Gillespie MT, Tennent JM, Midgley M, Jones IG, et al. Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. FEMS Microbiol Lett. 1992;74(2–3):259–65.
188. Nakaminami H, Noguchi N, Sasatsu M. Fluoroquinolone efflux by the plasmid-mediated multidrug efflux pump QacB variant QacBIII in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2010;54(10):4107–11.
189. Mitchell BA, Brown MH, Skurray RA. QacA multidrug efflux pump from *Staphylococcus aureus*: comparative analysis of resistance to diamidines, biguanidines, and guanylhydrazones. Antimicrob Agents Chemother. 1998;42(2):475–7.
190. Paulsen IT, Brown MH, Littlejohn TG, Mitchell BA, Skurray RA. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. Proc Natl Acad Sci U S A. 1996;93(8):3630–5.
191. Mitchell BA, Paulsen IT, Brown MH, Skurray RA. Bioenergetics of the staphylococcal multidrug export protein QacA. Identification of distinct binding sites for monovalent and divalent cations. J Biol Chem. 1999;274(6):3541–8.
192. Kuroda T, Tsuchiya T. Multidrug efflux transporters in the MATE family. Biochim Biophys Acta. 2009;1794(5):763–8.
193. He GX, Kuroda T, Mima T, Morita Y, Mizushima T, Tsuchiya T. An H(+)-coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. J Bacteriol. 2004;186(1):262–5.
194. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. Antimicrob Agents Chemother. 2005;49(10):4362–4.
195. Dridi L, Tankovic J, Petit JC. CdeA of *Clostridium difficile*, a new multidrug efflux transporter of the MATE family. Microb Drug Resist. 2004;10(3):191–6.
196. Borges-Walmsley MI, McKeegan KS, Walmsley AR. Structure and function of efflux pumps that confer resistance to drugs. Biochem J. 2003;376(Pt 2):313–38.
197. Kaatz GW, McAleese F, Seo SM. Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. Antimicrob Agents Chemother. 2005;49(5):1857–64.
198. McAleese F, Petersen P, Ruzin A, Dunman PM, Murphy E, Projan SJ, et al. A novel MATE family efflux pump contributes to the reduced susceptibility of laboratory-derived *Staphylococcus aureus* mutants to tigecycline. Antimicrob Agents Chemother. 2005;49(5):1865–71.
199. Kaatz GW, DeMarco CE, Seo SM. MepR, a repressor of the *Staphylococcus aureus* MATE family multidrug efflux pump MepA, is a substrate-responsive regulatory protein. Antimicrob Agents Chemother. 2006;50(4):1276–81.
200. Schindler BD, Seo SM, Jacinto PL, Kumaraswami M, Birukou I, Brennan RG, et al. Functional consequences of substitution mutations in MepR, a repressor of the *Staphylococcus aureus mepA* Multidrug Efflux Pump Gene. J Bacteriol. 2013;195(16): 3651–62.
201. Neyfakh AA, Bidnenko VE, Chen LB. Efflux-mediated multidrug resistance in *Bacillus subtilis*: similarities and dissimilarities with the mammalian system. Proc Natl Acad Sci U S A. 1991; 88(11):4781–5.
202. Neyfakh AA. The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the *Staphylococcus* NorA protein. Antimicrob Agents Chemother. 1992;36(2):484–5.
203. Ahmed M, Borsch CM, Taylor SS, Vazquez-Laslop N, Neyfakh AA. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. J Biol Chem. 1994;269(45):28506–13.
204. Kumaraswami M, Newberry KJ, Brennan RG. Conformational plasticity of the coiled-coil domain of BmrR is required for *bmr* operator binding: the structure of unliganded BmrR. J Mol Biol. 2010;398(2):264–75.
205. Zheleznova EE, Markham PN, Neyfakh AA, Brennan RG. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. Cell. 1999;96(3):353–62.
206. Heldwein EE, Brennan RG. Crystal structure of the transcription activator BmrR bound to DNA and a drug. Nature. 2001; 409(6818):378–82.
207. Newberry KJ, Huffman JL, Miller MC, Vazquez-Laslop N, Neyfakh AA, Brennan RG. Structures of BmrR-drug complexes reveal a rigid multidrug binding pocket and transcription activation through tyrosine expulsion. J Biol Chem. 2008;283(39): 26795–804.
208. Ahmed M, Lyass L, Markham PN, Taylor SS, Vazquez-Laslop N, Neyfakh AA. Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. J Bacteriol. 1995;177(14):3904–10.
209. Baranova NN, Danchin A, Neyfakh AA. Mta, a global MerR-type regulator of the *Bacillus subtilis* multidrug-efflux transporters. Mol Microbiol. 1999;31(5):1549–59.
210. Ohki R, Murata M. *bmr3*, a third multidrug transporter gene of *Bacillus subtilis*. J Bacteriol. 1997;179(4):1423–7.
211. Ohki R, Tateno K. Increased stability of *bmr3* mRNA results in a multidrug-resistant phenotype in *Bacillus subtilis*. J Bacteriol. 2004;186(21):7450–5.
212. Gill MJ, Brenwald NP, Wise R. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 1999;43(1):187–9.
213. Brenwald NP, Appelbaum P, Davies T, Gill MJ. Evidence for efflux pumps, other than PmrA, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. Clin Microbiol Infect. 2003;9(2):140–3.
214. Piddock LJ, Johnson MM, Simjee S, Pumbwe L. Expression of efflux pump gene *pmrA* in fluoroquinolone-resistant and -susceptible clinical isolates of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2002;46(3):808–12.

215. Montanari MP, Tili E, Cochetti I, Mingoia M, Manzin A, Varaldo PE. Molecular characterization of clinical *Streptococcus pneumoniae* isolates with reduced susceptibility to fluoroquinolones emerging in Italy. Microb Drug Resist. 2004;10(3):209–17.
216. Avrain L, Garvey M, Mesaros N, Glupczynski Y, Mingeot-Leclercq MP, Piddock LJ, et al. Selection of quinolone resistance in *Streptococcus pneumoniae* exposed in vitro to subinhibitory drug concentrations. J Antimicrob Chemother. 2007;60(5): 965–72.
217. Pestova E, Millichap JJ, Siddiqui F, Noskin GA, Peterson LR. Non-PmrA-mediated multidrug resistance in *Streptococcus pneumoniae*. J Antimicrob Chemother. 2002;49(3):553–6.
218. Robertson GT, Doyle TB, Lynch AS. Use of an efflux-deficient *Streptococcus pneumoniae* strain panel to identify ABC-class multidrug transporters involved in intrinsic resistance to antimicrobial agents. Antimicrob Agents Chemother. 2005;49(11): 4781–3.
219. El Garch F, Lismond A, Piddock LJ, Courvalin P, Tulkens PM, Van Bambeke F. Fluoroquinolones induce the expression of *patA* and *patB*, which encode ABC efflux pumps in *Streptococcus pneumoniae*. J Antimicrob Chemother. 2010;65(10):2076–82.
220. Marrer E, Schad K, Satoh AT, Page MG, Johnson MM, Piddock LJ. Involvement of the putative ATP-dependent efflux proteins PatA and PatB in fluoroquinolone resistance of a multidrug-resistant mutant of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2006;50(2):685–93.
221. Garvey MI, Baylay AJ, Wong RL, Piddock LJ. Overexpression of *patA* and *patB*, which encode ABC transporters, is associated with fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2011;55(1):190–6.
222. Boncoeur E, Durmort C, Bernay B, Ebel C, Di Guilmi AM, Croize J, et al. PatA and PatB form a functional heterodimeric ABC multidrug efflux transporter responsible for the resistance of *Streptococcus pneumoniae* to fluoroquinolones. Biochemistry. 2012;51(39):7755–65.
223. Hashimoto K, Ogawa W, Nishioka T, Tsuchiya T, Kuroda T. Functionally cloned *pdrM* from *Streptococcus pneumoniae* encodes a Na(+) coupled multidrug efflux pump. PLoS One. 2013;8(3):e59525.
224. Tocci N, Iannelli F, Bidossi A, Ciusa ML, Decorosi F, Viti C, et al. Functional analysis of pneumococcal drug efflux pumps associates the MATE DinF transporter with quinolone susceptibility. Antimicrob Agents Chemother. 2013;57(1):248–53.
225. Jonas BM, Murray BE, Weinstock GM. Characterization of *emeA*, a NorA homolog and multidrug resistance efflux pump, in *Enterococcus faecalis*. Antimicrob Agents Chemother. 2001; 45(12):3574–9.
226. van Veen HW, Callaghan R, Soceneantu L, Sardini A, Konings WN, Higgins CF. A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene. Nature. 1998;391(6664):291–5.
227. Jnawali HN, Hwang SC, Park YK, Kim H, Lee YS, Chung GT, et al. Characterization of mutations in multi- and extensive drug resistance among strains of *Mycobacterium tuberculosis* clinical isolates in Republic of Korea. Diagn Microbiol Infect Dis. 2013;76(2):187–96.
228. Takiff H, Guerrero E. Current prospects for the fluoroquinolones as first-line tuberculosis therapy. Antimicrob Agents Chemother. 2011;55(12):5421–9.
229. Wang JY, Hsueh PR, Jan IS, Lee LN, Liaw YS, Yang PC, et al. Empirical treatment with a fluoroquinolone delays the treatment for tuberculosis and is associated with a poor prognosis in endemic areas. Thorax. 2006;61(10):903–8.
230. Zhu C, Zhang Y, Shen Y, Siu GK, Wu W, Qian X, et al. Molecular characterization of fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates from Shanghai, China. Diagn Microbiol Infect Dis. 2012;73(3):260–3.
231. Jacobs MR. Fluoroquinolones as chemotherapeutic agents against mycobacterial infections. Curr Pharm Des. 2004;10:3213–20.
232. Poole K. Efflux-mediated resistance to fluoroquinolones in gram-positive bacteria and the mycobacteria. Antimicrob Agents Chemother. 2000;44(10):2595–9.
233. Von Groll A, Martin A, Jureen P, Hoffner S, Vandamme P, Portaels F, et al. Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in *gyrA* and *gyrB*. Antimicrob Agents Chemother. 2009;53(10):4498–500.
234. Ajbani K, Rodrigues C, Shenai S, Mehta A. Mutation detection and accurate diagnosis of extensively drug-resistant tuberculosis: report from a tertiary care center in India. J Clin Microbiol. 2011;49(4):1588–90.
235. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011;55(5):2032–41.
236. Sun Z, Zhang J, Zhang X, Wang S, Zhang Y, Li C. Comparison of *gyrA* gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. Int J Antimicrob Agents. 2008;31(2):115–21.
237. Feng Y, Liu S, Wang Q, Wang L, Tang S, Wang J, et al. Rapid diagnosis of drug resistance to fluoroquinolones, amikacin, capreomycin, kanamycin and ethambutol using genotype MTBDRsl assay: a meta-analysis. PLoS One. 2013;8(2):e55292.
238. Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplimer conformation analysis for rapid detection of *gyrA* mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrob Agents Chemother. 2004;48(2):596–601.
239. Pantel A, Petrella S, Veziris N, Brossier F, Bastian S, Jarlier V, et al. Extending the definition of the GyrB quinolone resistance-determining region in *Mycobacterium tuberculosis* DNA gyrase for assessing fluoroquinolone resistance in *M. tuberculosis*. Antimicrob Agents Chemother. 2012;56(4):1990–6.
240. Kim H, Nakajima C, Yokoyama K, Rahim Z, Kim YU, Oguri H, et al. Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. Antimicrob Agents Chemother. 2011;55(8):3661–7.
241. Veziris N, Martin C, Brossier F, Bonnaud F, Denis F, Aubry A. Treatment failure in a case of extensively drug-resistant tuberculosis associated with selection of a GyrB mutant causing fluoroquinolone resistance. Eur J Clin Microbiol Infect Dis. 2007;26(6):423–5.
242. Aubry A, Veziris N, Cambau E, Truffot-Pernot C, Jarlier V, Fisher LM. Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. Antimicrob Agents Chemother. 2006;50(1):104–12.
243. Malik S, Willby M, Sikes D, Tsodikov OV, Posey JE. New insights into fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional genetic analysis of *gyrA* and *gyrB* mutations. PLoS One. 2012;7(6):e39754.
244. Devasia R, Blackman A, Eden S, Li H, Maruri F, Shintani A, et al. High proportion of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates with novel gyrase polymorphisms and a *gyrA* region associated with fluoroquinolone susceptibility. J Clin Microbiol. 2012;50(4):1390–6.
245. Chen J, Chen Z, Li Y, Xia W, Chen X, Chen T, et al. Characterization of *gyrA* and *gyrB* mutations and fluoroquinolone resistance in *Mycobacterium tuberculosis* clinical isolates from Hubei Province, China. Braz J Infect Dis. 2012;16(2):136–41.

246. Khrapunov S, Cheng H, Hegde S, Blanchard J, Brenowitz M. Solution structure and refolding of the *Mycobacterium tuberculosis* pentapeptide repeat protein MfpA. J Biol Chem. 2008; 283(52):36290–9.
247. De Rossi E, Arrigo P, Bellinzoni M, Silva PA, Martin C, Ainsa JA, et al. The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*. Mol Med. 2002; 8(11):714–24.
248. Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*. Biochem J. 2002;367(Pt 1):279–85.
249. Pasca MR, Guglierame P, Arcesi F, Bellinzoni M, De Rossi E, Riccardi G. Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2004;48(8):3175–8.
250. Dowell D, Tian LH, Stover JA, Donnelly JA, Martins S, Erbelding EJ, et al. Changes in fluoroquinolone use for gonorrhea following publication of revised treatment guidelines. Am J Public Health. 2012;102(1):148–55.
251. Frei CR, Labreche MJ, Attridge RT. Fluoroquinolones in community-acquired pneumonia: guide to selection and appropriate use. Drugs. 2011;71(6):757–70.
252. Aspinall SL, Metlay JP, Maselli JH, Gonzales R. Impact of hospital formularies on fluoroquinolone prescribing in emergency departments. Am J Manag Care. 2007;13(5):241–8.
253. Zechini B, Versace I. Inhibitors of multidrug resistant efflux systems in bacteria. Recent Pat Antiinfect Drug Discov. 2009; 4(1):37–50.
254. Schindler BD, Jacinto P, Kaatz GW. Inhibition of drug efflux pumps in *Staphylococcus aureus*: current status of potentiating existing antibiotics. Future Microbiol. 2013;8(4):491–507.
255. Blair JM, Piddock LJ. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Curr Opin Microbiol. 2009;12(5):512–9.
256. Kourtesi C, Ball AR, Huang YY, Jachak SM, Vera DM, Khondkar P, et al. Microbial efflux systems and inhibitors: approaches to drug discovery and the challenge of clinical implementation. Open Microbiol J. 2013;7:34–52.
257. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, et al. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob Agents Chemother. 2001; 45(1):105–16.
258. Pletz MW, Michaylov N, Schumacher U, van der Linden M, Duesberg CB, Fuehner T, et al. Antihypertensives suppress the emergence of fluoroquinolone-resistant mutants in pneumococci: an in vitro study. Int J Med Microbiol. 2013;303(4):176–81.
259. Lomovskaya O, Zgurskaya HI, Totrov M, Watkins WJ. Waltzing transporters and 'the dance macabre' between humans and bacteria. Nat Rev Drug Discov. 2007;6(1):56–65.
260. Pages JM, Amaral L. Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. Biochim Biophys Acta. 2009;1794(5):826–33.
261. Bansal S, Tandon V. Contribution of mutations in DNA gyrase and topoisomerase IV genes to ciprofloxacin resistance in *Escherichia coli* clinical isolates. Int J Antimicrob Agents. 2011; 37(3):253–5.
262. Moon DC, Seol SY, Gurung M, Jin JS, Choi CH, Kim J, et al. Emergence of a new mutation and its accumulation in the topoisomerase IV gene confers high levels of resistance to fluoroquinolones in *Escherichia coli* isolates. Int J Antimicrob Agents. 2010;35(1):76–9.
263. Namboodiri SS, Opintan JA, Lijek RS, Newman MJ, Okeke IN. Quinolone resistance in *Escherichia coli* from Accra, Ghana. BMC Microbiol. 2011;11:44.
264. Wang S, Wang Y, Shen J, Wu Y, Wu C. Polymorphic mutation frequencies in clinical isolates of *Staphylococcus aureus*: the role of weak mutators in the development of fluoroquinolone resistance. FEMS Microbiol Lett. 2013;341(1):13–7.
265. Sanfilippo CM, Hesje CK, Haas W, Morris TW. Topoisomerase mutations that are associated with high-level resistance to earlier fluoroquinolones in *Staphylococcus aureus* have less effect on the antibacterial activity of besifloxacin. Chemotherapy. 2011;57(5): 363–71.

Plasmid-Mediated Quinolone Resistance

17

George A. Jacoby

1 Introduction

Mutations that reduce target affinity or decrease drug accumulation are responsible for most of the increased quinolone resistance in gram-negative pathogens but do not account for how rapidly resistance has developed or its frequent linkage to resistance to other antimicrobial agents. Three mechanisms for plasmid-mediated quinolone resistance (PMQR), long thought not to occur, have been discovered since 1998. Plasmid genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC* code for proteins of the pentapeptide repeat family that protect DNA gyrase and topoisomerase IV from quinolone inhibition. The *qnr* genes appear to have been acquired from aquatic bacteria, are usually associated on plasmids with mobilizing or transposable elements, and are often incorporated into sul1-type integrons. The second PMQR mechanism involves acetylation of certain quinolones by a variant of the common aminoglycoside acetyltransferase AAC(6′)-Ib. The third mechanism is enhanced efflux produced by plasmid genes for pumps QepAB and OqxAB. The plasmid-mediated mechanisms provide only low-level resistance that by itself does not exceed the clinical breakpoint for susceptibility but nonetheless facilitates selection of higher level resistance and makes pathogens containing PMQR genes harder to treat.

In the 1990s, the introduction of potent fluoroquinolones led to their increasing use and was followed by increasing quinolone resistance [1] due to the known resistance mechanisms of target modification and drug efflux but also with the unexpected emergence of novel plasmid-mediated quinolone resistance (PMQR). The first example of PMQR was discovered in a clinical strain of *Klebsiella pneumoniae* isolated in 1994 that could transfer low-level quinolone resistance along with resistance to several other antibiotics to *Escherichia coli* and other gram-negative organisms [2]. In *E. coli* the plasmid caused an 8- to 32-fold decrease in susceptibility to nalidixic acid and to all fluoroquinolones tested. Although the increased MIC did not exceed the susceptibility breakpoint as defined by the CLSI, the plasmid raised the mutant protective concentration [3] and facilitated the selection of truly quinolone resistant mutants [2, 4, 5].

The responsible gene was termed *qnrA* and coded for a 218 amino acid protein belonging to the pentapeptide repeat family that was shown with purified components to block inhibition of DNA gyrase and topoisomerase IV by ciprofloxacin [6, 7]. *qnrA* was followed by discovery of plasmid-mediated *qnrS* [8], *qnrB* [9], *qnrC* [10], and *qnrD* [11]. The *qnrVC* gene from *Vibrio cholerae* can also be located in a plasmid [12, 13] or in transmissible form as part of an integrating conjugative element [14]. These *qnr* genes generally differ in sequence by 35% or more from *qnrA* and each other. Allelic variants have also been described in each family differing by 10% or less: currently 8 alleles for *qnrA*, 9 for *qnrS*, and 85 for *qnrB* [15] (http://www.lahey.org/qnrstudies/). *qnr* genes are also found on the chromosome of both gram-negative and gram-positive bacteria from both clinical and environmental sources [16–19].

QnrA protein binds to both DNA gyrase and topoisomerase IV and to their subunits and decreases the binding of gyrase to DNA [7, 20]. The structures are known for three Qnr proteins and a related pentapeptide repeat protein from *Mycobacterium tuberculosis* [21]: chromosomally determined AhQnr [22] and plasmid-mediated QnrB1 [23] from gram-negative organisms and chromosomally encoded EfsQnr from *Enterococcus faecalis* [24]. All are rod-like C-terminal dimers and fold into a right-handed quadrilateral β helix with size, shape, and charge mimicking the β form of DNA. The monomers of QnrB1 and AhQnr have projecting loops of 8 and 12 amino acids that are important for their activity. Deletion of the smaller loop reduces protection while deletion of the larger or both loops destroys protective activity [22, 23]. MfpA and EfsQnr lack loops, but EfsQnr

G.A. Jacoby, M.D. (✉)
Part-Time, Harvard Medical School, Boston, MA, USA
e-mail: gajacoby50@gmail.com

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_17

differs from MfpA in having a 25-amino acid flexible extension required for full protective activity.

Qnr plasmids have been found around the world in a variety of Enterobacteriaceae, especially *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Salmonella enterica* but rarely in non-fermenting bacteria such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii* [25, 26]. Plasmids carrying *qnr* genes vary in size and incompatibility specificity, indicating that the spread of multiple plasmids has been responsible for their dissemination, and that plasmid acquisition has occurred multiple times. A mobile or transposable element is almost invariably associated with plasmid-mediated *qnr* genes, especially insertion sequences IS*CR1*, IS*Ecp1*, and IS*26*. The complex is often then inserted into a sul1-type integron. *qnrVC* is so far the only *qnr* gene located in a cassette with a linked *attC* site ready by itself for integron capture [27]. Because of such linkage *qnr* genes are often found on plasmids with genes for other resistance determinants such as extended-spectrum β-lactamases and carbapenemases. *qnr* prevalence seems to be increasing [28, 29] and has reached as high as 39 % in a sample of *Enterobacter cloacae* isolates at one hospital in China [30].

The likely origin of the *qnrA*, *qnrC*, and *qnrS* genes is the chromosome of an aquatic bacterium. QnrA1 is 98 % identical to the chromosomally determined Qnr of *Shewanella algae* [31], QnrS1 is 83 % identical to Qnr from *Vibrio splendidus* [32], and QnrC is 72 % identical to chromosomal Qnrs in *Vibrio orientalis* or *V. cholerae* [10]. QnrB homologs, on the other hand, are found on the chromosome of members of the *Citrobacter freundii* complex [33]. The wide distribution of *qnr* suggests an origin well before quinolones were discovered. Indeed, *qnrB* genes and pseudogenes have been discovered on the chromosome of *Citrobacter freundii* strains collected in the 1930s [34]. Their natural function is unknown, but possible hints come from study of how *qnr* genes are regulated. Expression of the chromosomal *qnrA* gene of *S. algae*, an organism adapted to growth at low temperature, is stimulated up to eightfold by cold shock [35]. Expression of *qnrB* alleles, on the other hand, is augmented up to ninefold by exposure to DNA damaging agents such as ciprofloxacin via an upstream LexA binding site and the classical SOS system [36, 37]. *qnrD* and the chromosomal *qnr* of *S. marcescens* are similarly regulated [38]. Expression of plasmid-mediated *qnrS1* or the related chromosomal *qnrVS1* of *V. splendidus* is also stimulated by ciprofloxacin up to 30-fold, but by a mechanism independent of the SOS system. No LexA binding site is found upstream from these *qnr* genes, but upstream sequence is required for quinolone stimulation to occur [39].

A second type of plasmid-mediated quinolone resistance involves enzymatic modification. AAC(6′)-Ib-cr is a bifunctional variant of a common aminoglycoside acetyltransferase that is also able to acetylate fluoroquinolones with an amino nitrogen on the piperazinyl ring, such as ciprofloxacin and norfloxacin [40]. Acetylation decreases the antibacterial potency raising the ciprofloxacin MIC and, as with Qnr, increasing the MPC as well. Compared to other AAC(6′)-Ib enzymes, the –cr variant has two unique amino acid substitutions: Trp102Arg and Asp179Tyr, both of which are required for quinolone activity. Models of enzyme action suggest that the Asp179Tyr replacement is particularly important in permitting π-stacking interactions with the quinolone ring to facilitate quinolone binding. The role of Trp102Arg is to position the Tyr face for optimal interaction [41] or to hydrogen bond to keto or carboxyl groups of the quinolone to fix it in place [42]. The *aac(6′)-Ib-cr* gene has been found worldwide in a variety of Enterobacteriaceae (especially *E. coli*) and is often more common than *qnr* alleles. It is usually found in a cassette as part of an integron in a multiresistance plasmid, which may contain other PMQR genes. Association with the worldwide ESBL leader CTX-M-15 is particularly common.

The third mechanism for plasmid-mediated quinolone resistance involves genes for efflux pumps that export quinolones out of the bacterial cytoplasm. Two are known: *qepAB* [43, 44] and *oqxAB* [45, 46]. Both seem less common than *qnr* or *aac(6′)-Ib-cr* but have not been so thoroughly studied.

In animal model infections the presence of a *qnr* gene makes an infecting agent harder to eliminate with quinolones. This detrimental effect has been shown in mice with pneumonia produced by *K. pneumoniae* or *E. coli* [47] [48] and in *E. coli* UTI models [49] [50]. Patients treated with levofloxacin for bloodstream infections caused by gram-negative organisms with elevated quinolone MICs that were still within the susceptible category have been shown to have worse outcomes than similar patients infected with more susceptible organisms [51]. A specific effect of PMQR carriage on outcome has been harder to document. In two studies with a relatively small number of *qnr*-positive enterobacter and klebsiella blood isolates no difference in mortality was evident between patients infected with strains with or without *qnr* genes [52, 53].

Thus although Qnr, AAC(6′)-Ib-cr, QepA, and OqxAB by themselves provide only modest losses of susceptibility (Table 17.1), they contribute to the rising prevalence of quinolone resistance, and their presence, at least in animal models, makes infections harder treat.

Table 17.1 Effect of different quinolone resistance mechanisms on quinolone susceptibility

	MIC μg/ml		
E. coli strain	Ciprofloxacin	Levofloxacin	Nalidixic acid
J53 R^-	0.008	0.015	4
J53 *gyrA* S83L	0.25	0.5	≥256
J53 pMG252 *qnrA1*	0.25	0.5	16
J53 pMG298 *qnrB1*	0.25	0.5	16
J53 pMG306 *qnrS1*	0.25	0.38	16
J53 pMG320 *aac(6′)-Ib-cr*	0.06	0.015[a]	4[a]
J53 pAT851 *qepA*	0.064	0.032	4
CLSI susceptibility breakpoint	≤1.0	≤2.0	≤16

[a]Susceptibility is not affected because only ciprofloxacin has the target N for acetylation

References

1. Neuhauser MM, Weinstein RA, Rydman R, Danziger LH, Karam G, Quinn JP. Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. JAMA. 2003;289:885–8.
2. Martínez-Martínez L, Pascual A, Jacoby GA. Quinolone resistance from a transferable plasmid. Lancet. 1998;351:797–9.
3. Drlica K. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother. 2003;52:11–7.
4. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis. 2005;41 Suppl 2:S120–6.
5. Rodríguez-Martínez JM, Velasco C, García I, Cano ME, Martínez-Martínez L, Pascual A. Mutant prevention concentrations of fluoroquinolones for *Enterobacteriaceae* expressing the plasmid-carried quinolone resistance determinant *qnrA1*. Antimicrob Agents Chemother. 2007;51:2236–9.
6. Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci U S A. 2002;99:5638–42.
7. Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein QnrA with *Escherichia coli* topoisomerase IV. Antimicrob Agents Chemother. 2005;49:3050–2.
8. Hata M, Suzuki M, Matsumoto M, Takahashi M, Sato K, Ibe S, Sakae K. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob Agents Chemother. 2005;49:801–3.
9. Jacoby GA, Walsh KE, Mills DM, Walker VJ, Oh H, Robicsek A, Hooper DC. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob Agents Chemother. 2006;50:1178–82.
10. Wang M, Guo Q, Xu X, Wang X, Ye X, Wu S, Hooper DC, Wang M. New plasmid-mediated quinolone resistance gene, *qnrC*, found in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother. 2009;53:1892–7.
11. Cavaco LM, Hasman H, Xia S, Aarestrup FM. *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. Antimicrob Agents Chemother. 2009;53:603–8.
12. Xia R, Guo X, Zhang Y, Xu H. *qnrVC*-like gene located in a novel complex class 1 integron harboring the IS*CR1* element in an *Aeromonas punctata* strain from an aquatic environment in Shandong Province, China. Antimicrob Agents Chemother. 2010;54:3471–4.
13. Singh R, Rajpara N, Tak J, Patel A, Mohanty P, Vinothkumar K, Chowdhury G, Ramamurthy T, Ghosh A, Bhardwaj AK. Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, the *qnr* gene and a mutation in gyrase A as mechanisms of multidrug resistance. J Med Microbiol. 2012;61:369–74.
14. Kim HB, Wang M, Ahmed S, Park CH, LaRocque RC, Faruque AS, Salam MA, Khan WA, Qadri F, Calderwood SB, Jacoby GA, Hooper DC. Transferable quinolone resistance in *Vibrio cholerae*. Antimicrob Agents Chemother. 2010;54:799–803.
15. Jacoby G, Cattoir V, Hooper D, Martínez-Martínez L, Nordmann P, Pascual A, Poirel L, Wang M. *qnr* gene nomenclature. Antimicrob Agents Chemother. 2008;52:2297–9.
16. Rodríguez-Martínez JM, Velasco C, Briales A, García I, Conejo MC, Pascual A. Qnr-like pentapeptide repeat proteins in gram-positive bacteria. J Antimicrob Chemother. 2008;61:1240–3.
17. Sánchez MB, Hernández A, Rodríguez-Martínez JM, Martínez-Martínez L, Martínez JL. Predictive analysis of transmissible quinolone resistance indicates *Stenotrophomonas maltophilia* as a potential source of a novel family of Qnr determinants. BMC Microbiol. 2008;8:148–61.
18. Boulund F, Johnning A, Pereira MB, Larsson DG, Kristiansson E. A novel method to discover fluoroquinolone antibiotic resistance (qnr) genes in fragmented nucleotide sequences. BMC Genomics. 2012;13:695.
19. Jacoby GA, Hooper DC. Phylogenetic analysis of chromosomally determined Qnr and related proteins. Antimicrob Agents Chemother. 2013;57:1930–4.
20. Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. Antimicrob Agents Chemother. 2005;49:118–25.
21. Hegde SS, Vetting MW, Roderick SL, Mitchenall LA, Maxwell A, Takiff HE, Blanchard JS. A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. Science. 2005;308:1480–3.
22. Xiong X, Bromley EH, Oelschlaeger P, Woolfson DN, Spencer J. Structural insights into quinolone antibiotic resistance mediated by pentapeptide repeat proteins: conserved surface loops direct the activity of a Qnr protein from a gram-negative bacterium. Nucleic Acids Res. 2011;39:3917–27.
23. Vetting MW, Hegde SS, Wang M, Jacoby GA, Hooper DC, Blanchard JS. Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor. J Biol Chem. 2011;286:25265–73.
24. Hegde SS, Vetting MW, Mitchenall LA, Maxwell A, Blanchard JS. Structural and biochemical analysis of the pentapeptide repeat protein *Efs*Qnr, a potent DNA gyrase inhibitor. Antimicrob Agents Chemother. 2011;55:110–7.
25. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis. 2006;6:629–40.

26. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev. 2009;22:664–89.
27. Fonseca EL, Dos Santos Freitas F, Vieira VV, Vicente AC. New *qnr* gene cassettes associated with superintegron repeats in *Vibrio cholerae* O1. Emerg Infect Dis. 2008;14:1129–31.
28. Strahilevitz J, Engelstein D, Adler A, Temper V, Moses AE, Block C, Robicsek A. Changes in *qnr* prevalence and fluoroquinolone resistance in clinical isolates of *Klebsiella pneumoniae* and *Enterobacter* spp. collected from 1990 to 2005. Antimicrob Agents Chemother. 2007;51:3001–3.
29. Kim HB, Park CH, Kim CJ, Kim EC, Jacoby GA, Hooper DC. Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. Antimicrob Agents Chemother. 2009; 53:639–45.
30. Zhao X, Xu X, Zhu D, Ye X, Wang M. Decreased quinolone susceptibility in high percentage of *Enterobacter cloacae* clinical isolates caused only by Qnr determinants. Diagn Microbiol Infect Dis. 2010;67:110–3.
31. Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob Agents Chemother. 2005;49:3523–5.
32. Cattoir V, Poirel L, Mazel D, Soussy CJ, Nordmann P. *Vibrio splendidus* as the source of plasmid-mediated QnrS-like quinolone resistance determinants. Antimicrob Agents Chemother. 2007;51:2650–1.
33. Jacoby GA, Griffin CM, Hooper DC. *Citrobacter* spp. as a source of *qnrB* alleles. Antimicrob Agents Chemother. 2011;55:4979–84.
34. Saga T, Sabtcheva S, Mitsutake K, Ishii Y, Tateda K, Yamaguchi K, Kaku M. Characterization of *qnrB*-like genes in *Citrobacter* species of the American Type Culture Collection. Antimicrob Agents Chemother. 2013;57:2863–6.
35. Kim HB, Park CH, Gavin M, Jacoby GA, Hooper DC. Cold shock induces *qnrA* expression in *Shewanella algae*. Antimicrob Agents Chemother. 2011;55:414–6.
36. Wang M, Jacoby GA, Mills DM, Hooper DC. SOS regulation of *qnrB* expression. Antimicrob Agents Chemother. 2009;53:821–3.
37. Da Re S, Garnier F, Guerin E, Campoy S, Denis F, Ploy MC. The SOS response promotes *qnrB* quinolone-resistance determinant expression. EMBO Rep. 2009;10:929–33.
38. Briales A, Rodriguez-Martinez JM, Velasco C, Machuca J, Diaz de Alba P, Blazquez J, Pascual A. Exposure to diverse antimicrobials induces the expression of *qnrB1, qnrD* and *smaqnr* genes by SOS-dependent regulation. J Antimicrob Chemother. 2012;67:2854–9.
39. Okumura R, Liao CH, Gavin M, Jacoby GA, Hooper DC. Quinolone Induction of *qnrVS1* in *Vibrio splendidus* and plasmid-carried *qnrS1* in *Escherichia coli*, a mechanism independent of the SOS system. Antimicrob Agents Chemother. 2011;55:5942–5.
40. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12:83–8.
41. Vetting MW, Park CH, Hegde SS, Jacoby GA, Hooper DC, Blanchard JS. Mechanistic and structural analysis of aminoglycoside *N*-acetyltransferase AAC(6′)-Ib and its bifunctional fluoroquinolone-active AAC(6′)-Ib-cr variant. Biochemistry. 2008; 47:9825–35.
42. Maurice F, Broutin I, Podglajen I, Benas P, Collatz E, Dardel F. Enzyme structural plasticity and the emergence of broad-spectrum antibiotic resistance. EMBO Rep. 2008;9:344–9.
43. Yamane K, Wachino J, Suzuki S, Kimura K, Shibata N, Kato H, Shibayama K, Konda T, Arakawa Y. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother. 2007;51:3354–60.
44. Périchon B, Courvalin P, Galimand M. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob Agents Chemother. 2007;51:2464–9.
45. Hansen LH, Johannesen E, Burmolle M, Sorensen AH, Sorensen SJ. Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in *Escherichia coli*. Antimicrob Agents Chemother. 2004;48:3332–7.
46. Hansen LH, Jensen LB, Sorensen HI, Sorensen SJ. Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. J Antimicrob Chemother. 2007;60: 145–7.
47. Rodríguez-Martínez JM, Pichardo C, García I, Pachón-Ibañez ME, Docobo-Pérez F, Pascual A, Pachón J, Martínez-Martínez L. Activity of ciprofloxacin and levofloxacin in experimental pneumonia caused by *Klebsiella pneumoniae* deficient in porins, expressing active efflux and producing QnrA1. Clin Microbiol Infect. 2008;14:691–7.
48. Domínguez-Herrera J, Velasco C, Docobo-Pérez F, Rodríguez-Martínez JM, López-Rojas R, Briales A, Pichardo C, Díaz-de-Alba P, Rodríguez-Baño J, Pascual A, Pachón J. Impact of *qnrA1, qnrB1* and *qnrS*1 on the efficacy of ciprofloxacin and levofloxacin in an experimental pneumonia model caused by *Escherichia col*i with or without the GyrA mutation Ser83Leu. J Antimicrob Chemother. 2013;68:1609–15.
49. Allou N, Cambau E, Massias L, Chau F, Fantin B. Impact of low-level resistance to fluoroquinolones due to *qnrA1* and *qnrS1* genes or a *gyrA* mutation on ciprofloxacin bactericidal activity in a murine model of *Escherichia coli* urinary tract infection. Antimicrob Agents Chemother. 2009;53:4292–7.
50. Jakobsen L, Cattoir V, Jensen KS, Hammerum AM, Nordmann P, Frimodt-Moller N. Impact of low-level fluoroquinolone resistance genes *qnrA1, qnrB19* and *qnrS1* on ciprofloxacin treatment of isogenic *Escherichia coli* strains in a murine urinary tract infection model. J Antimicrob Chemother. 2012;67:2438–44.
51. Defife R, Scheetz MH, Feinglass JM, Postelnick MJ, Scarsi KK. Effect of differences in MIC values on clinical outcomes in patients with bloodstream infections caused by gram-negative organisms treated with levofloxacin. Antimicrob Agents Chemother. 2009; 53:1074–9.
52. Chong YP, Choi SH, Kim ES, Song EH, Lee EJ, Park KH, Cho OH, Kim SH, Lee SO, Kim MN, Jeong JY, Woo JH, Kim YS. Bloodstream infections caused by *qnr*-positive Enterobacteriaceae: clinical and microbiologic characteristics and outcomes. Diagn Microbiol Infect Dis. 2010;67:70–7.
53. Liao CH, Hsueh P-R, Jacoby GA, Hooper DC. Risk factors and clinical characteristics of patients with *qnr*-positive *Klebsiella pneumoniae* bacteremia. J Antimicrob Chemother. 2013;68(12): 2907–14.

Resistance to Macrolides, Lincosamides, and Streptogramins

18

Vincent Cattoir and Roland Leclercq

1 Introduction

The structurally unrelated antimicrobials–macrolides, lincosamides, and streptogramins–are grouped into a single family, called the MLS family. This classification is based on a similar, although not identical, mechanism of action. Macrolides are composed of a minimum of two amino and/or neutral sugars attached to a lactone ring of variable size [1] (Fig. 18.1). Erythromycin, produced by a strain of the actinomycete *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*), is the first macrolide discovered in 1952. It actually corresponds to a mixture of antibiotics that includes erythromycin A, which is the active compound and has a 14-membered lactone ring with two sugars, cladinose and an amino sugar (e.g., desosamine). Other commercially available macrolides derived from erythromycin A include clarithromycin, dirithromycin, roxithromycin, as well as azithromycin that has an enlarged 15-membered ring resulting from a nitrogen insertion. Structural modifications of erythromycin A resulted in improved pharmacokinetic profiles and better tolerance, but cross-resistance between members of this class of antimicrobials was still observed. Some 16-membered ring macrolides are also available in a few countries (spiramycin, josamycin, midecamycin, and miocamycin) or for veterinary use (tylosin). The most recent class of ketolides comprises telithromycin and cethromycin (ABT-773), which are derived from clarithromycin and have two major modifications, replacement of cladinose by a keto-function and an 11-12-carbamate extension with an alkyl-aryl modification in telithromycin. The first fluoroketolide solithromycin (CEM-101), exhibiting a different side chain and a fluorine atom linked to C-2 of the lactone, shows higher in vitro activity and enhanced accumulation in macrophages as compared to telithromycin [2].

Lincosamides form a small group of antibiotics of naturally occurring compounds or semisynthetic derivatives that contain an amino acid, a proline residue, attached by a peptide bond to a galactoside ring [3] (Fig. 18.1). Lincomycin is produced by the actinomycete *Streptomyces lincolnensis*. Clindamycin (7-chloro-7-deoxy lincomycin), a semisynthetic derivative of lincomycin in which a hydroxyl group has been replaced by chlorine, is the most important in clinical use. This minor difference in the structure of the molecules results in a noteworthy increase of the molecule affinity for its target [3].

The streptogramin antibiotics are composed of two chemically distinct compounds, namely type A and type B streptogramins [4]. The type A streptogramins are polyunsaturated cyclic macrolactones whereas type B streptogramins are cyclic hepta- or hexadepsipeptides (Fig. 18.1) [4–6]. Originally, streptogramins are natural mixtures produced by different members of *Streptomyces* or related genera [6, 7]. Every antibiotic producer synthesizes a mixture of various A and B components with a predominant member within each group. For instance, *Streptomyces pristinaespiralis* produces a mixture of group B compounds called pristinamycins I (pristinamycin I_A, pristinamycin I_B, and pristinamycin I_C with a ratio of 80–90 %, 3–5 %, and 2–5 %, respectively) and a mixture of group A compounds called pristinamycins II (pristinamycin II_A and pristinamycin II_B) [6]. Note that pristinamycin II_A is predominant in the pristinamycin II mixture. Actually, pristinamycin, an oral streptogramin produced by *S. pristinaespiralis* is essentially a mixture of pristinamycin I_A and pristinamycin II_A in a 30:70 ratio by weight [5, 6]. This drug is not commercially available except in some countries such as France and some African countries. Virginiamycin is another oral streptogramin used in livestock in certain countries for growth promotion and prevention of infection. Quinupristin and dalfopristin (hemisynthetic derivatives from pristinamycin IA and pristinamycin II_A, respectively)

V. Cattoir, Pharm.D., Ph.D. (✉) • R. Leclercq, M.D., Ph.D.
Department of Clinical Microbiology, School of Medicine, Caen University Hospital, University of Caen Normandie, Caen, France
e-mail: vincent.cattoir@chu-rennes.fr

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_18

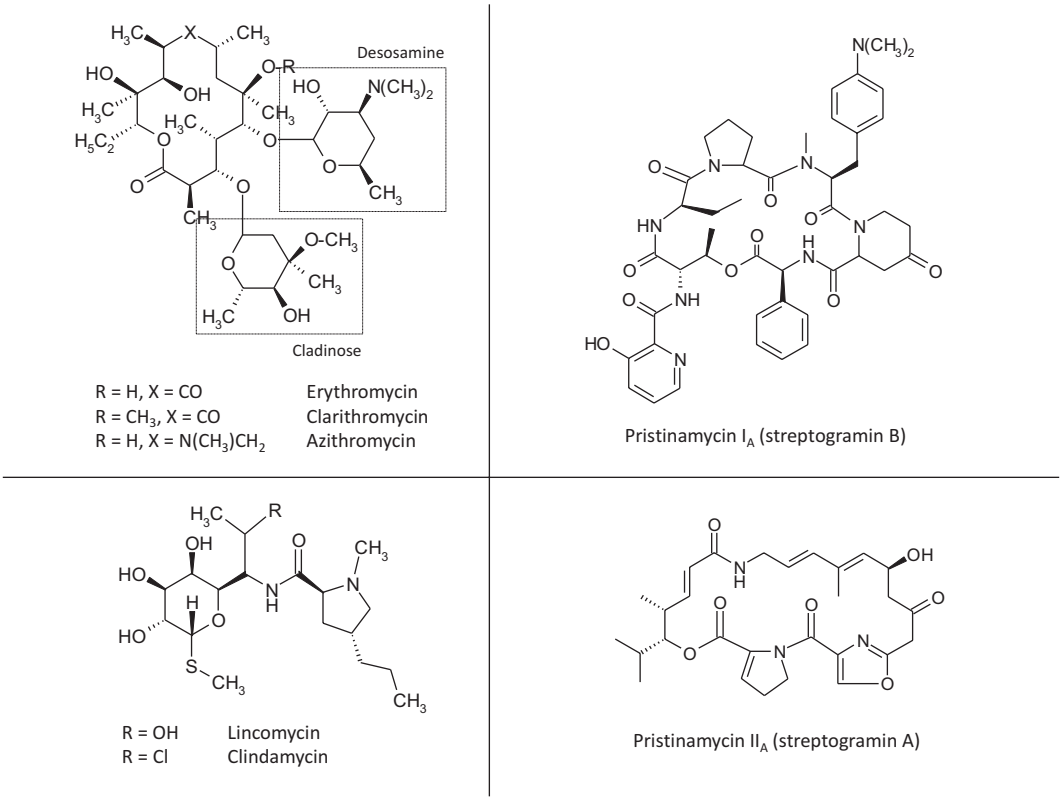


Fig. 18.1 Structure of selected macrolides (erythromycin, clarithromycin, and azithromycin), lincosamides (lincomycin, clindamycin), and streptogramins A (pristinamycin II_A) and B (pristinamycin I_A)

are combined in an injectable formulation with a 30:70 ratio (w/w) of methane sulfonate salts [5, 6]. More recently, an orally bioavailable combination (NXL 103) composed of linopristin (type B) and flopristin (type A) has been developed by Novexel SA and recently acquired by AstraZeneca [8].

2 Mode of Action

MLS antibiotics are bacteriostatic antibiotics that inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and ultimately inhibit microbial growth [1–3, 9]. The ribosome is composed of two subunits 30S and 50S built with RNAs and proteins, which assemble to produce a structure functional for protein synthesis. Each part undertakes a specific function. The small subunit 30S decodes mRNA, whereas in the large 50S part, the protein is formed by the polymerization of amino acids according to the genetic code. tRNA molecules carry the amino acids. Ribosomes possess three tRNA-binding sites A, P, and E, hosting the aminoacyl-tRNA, the peptidyl-tRNA, and the exiting tRNA, respectively. Each elongation cycle involves the advancement of the mRNA together with A → P → E site passage of the tRNA molecule driven by GTPase activity [10]. The 50S subunit is formed in part by 23S rRNA, which is organized into six domains. The domain V loop, called peptidyl transferase center (PTC), contains the active site of the peptide bond formation [11, 12]. This PTC loop is positioned at the bottom of a cavity located at the interface of the two subunits, adjacent to the entrance of the peptide tunnel. This tunnel crosses the 50S subunit and emerges on the back of the ribosome. Three-dimensional molecular structure of the ribosome was revealed by electron-cryomicroscopic studies and at atomic level by RX crystallography at high resolution [13]. From three bacterial species (*Thermus thermophilus*, *Haloarcula marismortui*, and *Deinococcus radiodurans*) chosen as a model for the high stability of their ribosomes, much has been learned about the antibiotics that inhibit ribosome function. Although some differences may occur in the ribosomal binding of macrolides and lincosamides according to bacterial species, common features have been found [14, 15].

The binding sites for the MLS antibiotics are located in the PTC or in the near vicinity of PTC at the beginning of the peptide tunnel, before it is constricted by the ribosomal proteins L4 and L22 [16]. The common nucleotide moieties

involved in hydrogen bond interactions of the 23S rRNA with macrolides and clindamycin are the nitrogen bases of the nucleotide residues A2058, a crucial MLS-binding site, and A2059 [16]. However, each class of drugs forms its own unique set of interactions with specific additional nucleotides. According to its position, the antibiotic inhibits peptide bond formation or peptide nascent chain progression. All the macrolides attach their lactone ring inside the peptide tunnel at the upper portion, and can protrude their appendage into the PTC cavity [17]. The mechanism of action depends on their size and sugar components [18]. Important contacts are formed between the C5 monosaccharide (desosamine) or disaccharide side chain of 14-15-16-membered macrolides and rRNA [19]. The shape of desosamine sugar of the macrolactone ring in erythromycin fits exactly with that of cavity formed by several nucleotides including A2058, and this interaction is considered to be required for ribosome binding [20].

The telithromycin macrolactone ring had additional hydrogen bond and hydrophobic interactions involving the three keto groups and two nucleotides residues of PTC. Several telithromycin- and erythromycin-binding sites within the 23S RNA overlap exactly. Telithromycin binds 10 times more strongly to ribosomes than the parent macrolide erythromycin, largely because of the alkyl-aryl substituent extending from the macrolactone ring position 11 and 12 that generates a hydrogen bond with nucleotide U2609 [14]. Both macrolides and ketolides act by producing a steric blockage of the ribosome exit tunnel, hence hampering the progression of nascent peptide [16].

Clindamycin binds in an elongated conformation oriented with its long axis roughly parallel to the axis of the exit tunnel. The proline residue occupies the same cleft as the site A substrate puromycin and blocks PTC activity by hampering the binding of transfer RNA to the A site. Clindamycin interacts directly with the A and P sites and blocks the formation of peptide bond by disturbing the positioning of tRNA in A and P sites [14]. The overlapping of some binding sites may explain why macrolides and clindamycin bind competitively to ribosome and why modification of binding sites confers cross-resistance [9].

Type A streptogramins block substrate attachment to both A and P sites of the PTC, competing with the binding of tRNAs to either the A- or P-site, and thus preventing the two early steps of elongation [7, 21]. Type B streptogramins share overlapping binding sites with macrolides and lincosamides (domains II and V), and act similarly by inhibiting translocation, preventing polypeptide extension, and triggering the premature release of incomplete protein chains [7, 21]. In addition, binding of type A streptogramins induces a conformational change in the ribosome near the PTC that subsequently unmasks a high-affinity binding site for streptogramins B leading to an increasing of their activity by ca. 100-fold [4, 7, 21].

3 Spectrum of Activity

MICs of MLS for important pathogenic bacteria are shown in Table 18.1. Macrolides have a spectrum of activity limited to Gram-positive cocci and bacilli, notably staphylococci, β-hemolytic streptococci, and pneumococci, as well as Gram-negative cocci. Gram-negative bacilli are generally resistant with the exception of some clinically important species, such as *Bordetella pertussis*, *Moraxella catarrhalis*, *Campylobacter* spp., and *Helicobacter pylori*. Macrolides also exhibit in vitro activity against intracellular bacteria, such as chlamydiae, mycoplasmas, and *Legionella pneumophila*. Note that clarithromycin has a good in vitro and in vivo activity against nontuberculous mycobacteria, especially *Mycobacterium avium* complex.

Lincosamides have a spectrum of activity closely related to that of macrolides, despite their different structure. Noteworthy, *Enterococcus faecalis* has an intrinsic resistance to lincosamides and streptogramins A (LS_A phenotype) that is shared with other species of enterococci, such as *Enterococcus avium*, *Enterococcus gallinarum*, and *Enterococcus casseliflavus*. By contrast, *Enterococcus faecium*, *Enterococcus hirae*, and *Enterococcus durans* are intrinsically susceptible to lincosamides. A particular feature of clindamycin is its activity against anaerobic bacteria, in particular, *Clostridium* spp., *Peptostreptococcus* spp., and Gram-negative rods. However, incidence of acquired resistance is now relatively high in the *Bacteroides fragilis* group. Also, *Clostridium sporogenes*, *Clostridium tertium*, and *Clostridium difficile* are frequently resistant to clindamycin. Finally, clindamycin has some activity against *Toxoplasma gondii* and *Pneumocystis jirovecii*.

Like macrolides and lincosamides, the spectrum of activity of streptogramins includes a broad range of aerobic and anaerobic Gram-positive bacteria, with MIC_{50} generally ≤1 μg/mL (Table 18.1). Noteworthy, *E. faecalis* is a gap in the antimicrobial spectrum since this Gram-positive species is intrinsically resistant due to a LS_A phenotype (see below).

4 Mechanisms of Resistance and Clinical Implications

Resistance to MLS can be mediated by multiple mechanisms including target modification, enzymatic drug inactivation, and active efflux. Target modification usually encompasses methylation of A2058, which is, as previously mentioned, a key residue with which macrolides, lincosamides, and streptogramins B interact. It also can be due to mutations in 23S rRNA or in conserved regions of ribosomal proteins L4 and L22. In pathogenic microorganisms, the impact of these mechanisms is unequal in terms of incidence and of clinical implications. Modification of the ribosomal target confers

Table 18.1 MICs of MLS antibiotics for susceptible pathogenic bacteria

	MIC_{50} (μg/mL)[a]								
Bacterial species	Ery	Cla	Azi	Tel	Lin	Cli	Pri	Q-D	F-L
Aerobes									
Gram-positive bacteria									
Staphylococcus aureus	0.25	0.25	1	0.03	0.5	0.12	0.25	0.25	0.12
Staphylococcus epidermidis	0.25	0.12	0.5	0.03	0.5	0.25	0.12	0.12	0.06
Streptococcus pyogenes	0.06	0.01	0.06	0.06	0.06	0.03	0.12	0.25	0.06
Streptococcus pneumoniae	0.06	0.01	0.06	0.03	0.25	0.06	0.25	0.5	0.25
Streptococcus viridans	0.06	0.01	0.06	0.03	0.25	0.12	0.5	1	0.12
Corynebacterium diphtheriae	<0.01	<0.01	0.01	<0.01	0.5	0.25	0.12	0.25	0.03
Gram-negative bacteria									
Bordetella pertussis	0.01	<0.01	<0.01	0.03	–	–	0.06	0.12	0.03
Moraxella catarrhalis	0.12	0.06	0.03	0.06	–	–	0.25	1	0.06
Haemophilus influenzae	4	4	1	1	32	8	1	2	0.25
Campylobacter jejuni	1	1	0.12	1	>8	>8	–	–	–
Helicobacter pylori	0.25	0.01	0.25	0.25	4	0.5	–	–	–
Intracellular bacteria									
Legionella pneumophila	0.25	0.03	0.12	0.03	16	4	0.06	0.5	0.03
Chlamydophila pneumoniae	0.12	0.06	0.12	0.06	–	–	0.5	2	0.25
Mycoplasma pneumoniae	<0.01	<0.01	<0.01	<0.01	–	–	0.25	0.12	0.12
Chlamydia trachomatis	0.25	0.06	0.12	0.06	–	–	0.12	0.5	0.12
Mycoplasma hominis	>16	>16	4	2	–	–	0.5	1	0.25
Mycoplasma genitalium	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–
Ureaplasma urealyticum	0.25	0.03	0.25	0.03	–	–	0.5	1	0.25
Anaerobes									
Bacteroides fragilis group	16	2	8	16	1	0.1	2	2	–
Prevotella spp.	0.5	0.06	0.12	0.12	0.25	0.01	–	–	–
Fusobacterium spp.	64	16	8	16	0.5	<0.1	0.06	0.06	–
Actinomyces spp.	0.03	0.03	<0.01	<0.01	0.25	0.06	0.12	0.25	0.06
Propionibacterium spp.	0.01	<0.01	0.03	<0.01	0.5	0.03	0.03	0.12	0.03
Clostridium perfringens	1	0.5	0.5	0.12	0.5	0.12	0.12	0.25	0.06
Peptostreptococcus spp.	4	2	4	0.06	0.5	0.05	0.12	0.25	0.12

[a]*Azi* azithromycin, *Cla* clarithromycin, *Cli* clindamycin, *Ery* erythromycin, *F-L* flopristin-linopristin, *Lin* lincomycin, *Pri* pristinamycin, *Q-D* quinupristin-dalfopristin, *Tel* telithromycin, – not available

broad-spectrum resistance to MLS, whereas enzymatic modification affects only structurally related antibiotics. These mechanisms have been found in antibiotic producers, which often combine several self-protective mechanisms against the antimicrobial that they produce.

4.1 Ribosomal Methylation

4.1.1 *erm* Genes

Ribosomal modification by methylation was the first mechanism of resistance to macrolides elucidated. This mechanism results from the acquisition of an *erm* gene (erythromycin ribosome methylase) usually carried by plasmids or transposons in pathogenic bacteria. Biochemical studies indicated that *erm* genes encode methylases that add one or two methyl groups to a single position (A2058) in bacterial 23S rRNA [22]. As a consequence of methylation, the activity of antibiotics that have the A2058 nucleotide as a key nucleotide for their binding to the ribosome is impaired. The overlapping binding sites in the peptidyl transferase region of 23S ribosomal RNA of macrolides, lincosamides, and streptogramins B account for cross-resistance (the so-called MLS_B resistance phenotype).

A wide range of microorganisms that are targets for macrolides and lincosamides express Erm methylases. More than 40 different *erm* genes have been reported so far (http://faculty.washington.edu/marilynr/), of which six major classes are detected in pathogenic microorganisms: *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), and *erm*(X). Both *erm*(A) and *erm*(C) typically are staphylococcal gene classes. Genes belonging to the *erm*(B) class and to a subclass of the *erm*(A)

gene class, previously called *erm*(TR), are widespread in β-hemolytic streptococci and enterococci. The *erm*(F) and *erm*(G) class genes are detected in *Bacteroides* spp. and other anaerobic bacteria whereas the *erm*(X) class genes are identified in Gram-positive rods. Although each class is relatively confined to a bacterial genus, it is not strictly genus specific. For instance, *erm*(B) genes may be found in staphylococci and anaerobes. Although all members of the *erm* family methylate the adenine of 23S rRNA located at position 2058, they differ by their capacity to monomethylate or dimethylate this nucleotide position. The major Erm methylases detected in pathogens, Erm(A), Erm(B), and Erm(C), generally function as dimethylases that confer a high-level cross-resistance to MLS_B drugs (including telithromycin). However, Erm(B) and Erm(A) (formerly *ermTR*) may function as monomethylases in *Streptococcus pneumoniae* and *Streptococcus pyogenes*, respectively [23, 24]. In fact, this makes a difference for ketolides, which are weakly affected by monomethylation, but not for erythromycin and clindamycin that are poorly active whether the ribosome is mono- or dimethylated.

MLS_B resistance may be constitutively or inducibly expressed [25, 26]. In inducible resistance, the bacteria produce inactive mRNA that is unable to encode methylase. In the model of the staphylococcal gene *erm*(C), the inactivity of the mRNA is due to the structure of its 5′ untranslated region (UTR) which has a set of inverted repeats that sequester the initiation sequences (ribosome-binding site and initiation codon) for the methylase by base-pairing in the absence of erythromycin [26]. Thus, the methylase cannot be produced since the initiation motifs for translation of the enzyme are not accessible to the ribosomes. Induction is related to the presence of an open-reading frame encoding a short 14-amino acid peptide upstream of the *erm*(C) structural gene. In the presence of low concentrations of erythromycin, binding of the antibiotic to a ribosome translating the leader peptide causes the ribosome to stall. Ribosome stalling likely induces destabilization of the pairing and conformational rearrangements in the mRNA that would then unmask the initiation sequences for the methylase, allowing synthesis to proceed by available ribosomes.

The *erm*(C) regulation model designated as posttranscriptional (or translational) attenuation would also account for the regulation of the *erm*(A) and *erm*(B) determinants [26]. For a given attenuator, the inducing capacity of the macrolides depends on the antibiotic structure. Whereas 14-membered macrolides (erythromycin, roxithromycin, and clarithromycin) and 15-membered macrolides (azithromycin) are inducers for the production of most Erm methylases, ketolides and lincosamides are generally not. Mutations in the attenuator may modify the induction pattern. In particular, lincosamides may become inducers in the case of mutation of the attenuator. This feature has been reported in laboratory mutants [27] and rarely for clinical isolates of *S. aureus* [28]. In staphylococci that typically contain *erm*(A) or *erm*(C) genes, inducible resistance leads to dissociated phenotypes of resistance between inducers (erythromycin) that are not active and noninducers (clindamycin) that remain active. The phenotype of MLS_B-inducible resistance expressed by staphylococci is characteristic, provided that the strains are tested by the disk-diffusion technique. A blunting of the clindamycin inhibition zone, similar to the shape of the letter D and referred as to a D-shaped zone, can be observed, provided that a disk of erythromycin is placed nearby (Fig. 18.2b). Which holds true for staphylococci is not for streptococci that usually harbor *erm*(B) genes. Indeed, the inducible *erm*(B) gene generally confers a cross-resistance to erythromycin and clindamycin, which differs from the dissociated resistance conferred by the staphylococcal *erm*(A) and *erm*(C) genes. The particular expression of *erm*(B) might be related to methylation of various proportions of ribosomes even in the absence of erythromycin [24]. This paradox could be explained by a nonstringent control of the expression of the methylase by the *erm*(B) attenuator. Fusion of the mutated *erm*(B) attenuator with a *lacZ* reporter gene has confirmed that the expression of the methylase can be partly derepressed in some strains [29]. By contrast, the control of methylase expression by the staphylococcal *erm*(A) and *erm*(C) methylases appears more strict. Other additional features, such as differences in the promoter strength or in the copy number of the *erm*(B) gene, may also account for the various levels of ribosomal methylation. The presence of basal levels of methylase appears sufficient to confer resistance to lincosamides, explaining the cross-resistance between macrolides and lincosamides in streptococci containing inducible *erm*(B) genes [28]. The expression in streptococci of the *erm*(A) gene (formerly *ermTR*) resembles that of the staphylococcal *erm*(A) gene [25].

In constitutive expression, active methylase mRNA is produced in the absence of an inducer, and the strains express cross-resistance to MLS_B antibiotics, regardless of the nature of the *erm* gene (Fig. 18.2c). In the laboratory, mutants derived from inducible strains of staphylococci and expressing constitutive MLS_B resistance can be selected on agar plates containing inhibitory concentrations of clindamycin at frequencies varying between 10^{-6} and 10^{-8}, depending on the strain [25, 30]. In addition, clinical isolates constitutively resistant to erythromycin are widespread, especially among methicillin-resistant staphylococci. It has been shown both in laboratory mutants and in clinical isolates that constitutive expression is due to deletions, duplications, or point mutations in the attenuator sequence leading to derepressed production of the methylase [26]. Similarly, in vitro selection by clindamycin of constitutive resistance at a frequency of 10^{-7} has been reported in a clinical isolate of *S. pyogenes* inducibly resistant to erythromycin and harboring *erm*(TR), a subclass of *erm*(A) genes [31].

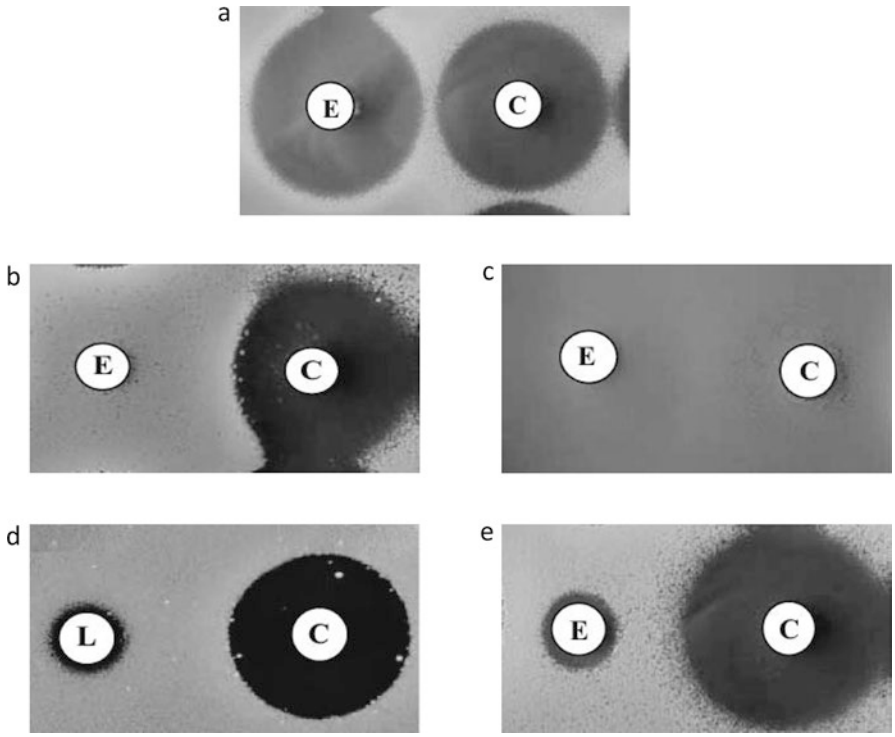


Fig. 18.2 Phenotypes of resistance to macrolides and clindamycin in *S. aureus*. (**a**) *S. aureus* susceptible to erythromycin and clindamycin; (**b**) *S. aureus* containing an *erm*(C) gene inducibly expressed (a D-shaped zone can be observed for the clindamycin zone of inhibition on the edge closest to the erythromycin zone of inhibition); (**c**) *S. aureus* containing an *erm*(C) gene constitutively expressed; (**d**) *S. aureus* containing an *lnu*(A) gene responsible for inactivation of lincosamides; (**e**) *S. aureus* resistant to erythromycin by *msr*(A)-mediated efflux (note the absence of D-shaped zone). *C* clindamycin, *E* erythromycin, *L* lincomycin

The use of clindamycin for the treatment of an infection due to an inducibly resistant strain of *S. aureus* is not devoid of risk. As previously mentioned, constitutive mutants can be selected in vitro in the presence of clindamycin at a relatively high frequency. Bacterial inocula exceeding 10^7 cfu can be found in mediastinitis and in certain lower respiratory tract infections. The risk to patients is illustrated by reports of selection of constitutive mutants during the course of clindamycin therapy administered to patients with severe infections due to inducibly erythromycin-resistant *S. aureus* [30, 32–37]. However, clinical evidence regarding the risk of emergence of clindamycin resistance is based only on a few case reports which are summarized in Table 18.2, and there are also reports of successful use of clindamycin in treating patients with D-test-positive isolates. Although it seems reasonable to discourage the use of clindamycin in deep-seated infections or in infections with heavy bacterial inoculum that increases the risk for selection of constitutive mutants, there are no criteria to confidently predict the success or the failure of clindamycin therapy in infections due to MLS_B-inducible staphylococci. Nonetheless, it is worth noting that isolates containing the inducible *erm*(C) present significantly higher frequencies of mutational resistance than those harboring the *erm*(A) gene [38]. More prospective studies of cases of staphylococcal or streptococcal infections treated with clindamycin are needed to better define the role of this antimicrobial in infections due to microorganisms with various macrolide resistance phenotypes. Noteworthy, the bactericidal activity of streptogramins against staphylococci expressing (like numerous MRSA isolates) a constitutive MLS_B phenotype is generally altered [39].

4.1.2 *cfr* Gene

Ribosomal methylation, occurring at a different site than the A2058 previously mentioned, may confer resistance to lincosamides but not to macrolides. Initially identified in staphylococcal isolates from animal sources, it has been recently detected in human *S. aureus* and *E. faecalis* clinical isolates [40–43]. Interestingly, in a linezolid-resistant MRSA clinical isolate, the *cfr* gene was located downstream of an *erm*(B) gene, both genes being co-transcribed [41]. The resistance is due to the production of the Cfr (chloramphenicol florfenicol resistance) protein that specifically methylates the 23S rRNA at the A2503 residue [44]. This still rare mechanism causes cross-resistance to five different antibiotic families: phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramins A (the so-called $PhLOPS_A$ phenotype) [45]. Although almost exclusively found on plasmids, chromosomal location has also been reported [40, 41].

4.2 Ribosomal Mutations

Studies with mutants obtained in the laboratory and reports of clinical isolates have revealed that several structures participating in the binding of macrolides, particularly domains

Table 18.2 Failures of clindamycin therapy in infections due to *S. aureus* with inducible MLS_B phenotype resistance [26–31]

No. of patients treated with clindamycin	No. of failures	No. of MLS_B constitutive isolates	Reference
3	2	1	[20]
2	2	2	[21]
3	1	1	[22]
2	2	1	[23]
1	1	1	[24]
1	1	1	[25]
All cases (n = 12)	9	7	

V and II of 23S rRNA and proteins L4 and L22, can display mutations responsible for macrolide/lincosamide resistance. The resistance phenotype conferred by alterations in the ribosomal target varies according to the nature of the mutated structure, but there is generally cross-resistance to MLS. In addition, since bacteria generally have several copies of the *rrl* gene coding for the 23S rRNA, susceptibility to macrolides and lincosamides varies according to the number of mutated copies and decreases as the number of the mutated copies increases [46]. Ribosomal mutations are rare in clinical isolates of staphylococci and streptococci [25], but are the main mechanism of resistance to macrolides in some bacterial species, such as *Campylobacter* spp., *H. pylori*, *P. acnes*, and *M. avium* complex [47].

4.3 Enzymatic Inactivation

Unlike target modification, inactivation of MLS antibiotics only confers resistance to structurally related antibiotics. Different esterases and phosphorylases have been identified in strains resistant to macrolides, almost exclusively in Gram-negative bacteria. Indeed, members of the family *Enterobacteriaceae* highly resistant to erythromycin due to the presence of these resistance determinants have been reported. Most of the strains were isolated from stool or blood cultures during selective digestive tract decontamination in neutropenic patients [48]. The isolates inactivate the lactone ring of 14-membered ring macrolides by production of erythromycin esterases or macrolide 2′-phosphotransferases that add phosphate to the 2′-hydroxyl group of an amino sugar [49–51]. Two types (I and II) of esterases, encoded by *ere*(A) and *ere*(B) (erythromycin esterase) genes, respectively, have been identified so far. Note that the G+C content of *ere*(B) (36 %), unlike that of *ere*(A) (50 %), is significantly different from the base composition of the *Escherichia coli* chromosome (50 %), suggesting that *ere*(B) is of exogenous origin, possibly a Gram-positive coccus. The *ere*(B) gene was detected in only 5 of 851 isolates (0.6 %) of erythromycin-resistant MRSA strains collected from 24 European hospitals while no *ere*(A) gene could be detected [52]. There are two groups of phosphotransferases, MPH(2′)-I (encoded by *mph*(A) and *mph*(D) genes) that inactivates 14- and 15-membered ring macrolides more efficiently than 16-membered ones, and MPH(2′)-II (encoded by *mph*(B) and *mph*(C) genes) that inactivates both groups of macrolides [53]. *mph*(A) and *mph*(B) are the most prevalent genes among Gram-negative bacteria. Notably, the plasmid-borne *mph*(A) gene conferring resistance to azithromycin has emerged in *Shigella sonnei* isolates responsible for an outbreak in Paris area while *E. coli* could constitute a major reservoir for this gene [54, 55]. An *mph*(C) gene, distinct from *mph*(A) and *mph*(B), has been described in a few strains of *S. aureus* [56].

Specific resistance to lincosamides is due to enzymatic inactivation of those antibiotics. Phosphorylation and nucleotidylation of the hydroxyl group at position 3 or 4 of lincosamides have been detected in several species of *Streptomyces*. In both animal and human isolates, lincosamide nucleotidyltransferases encoded by *lnu* genes (formerly *lin*) were reported. In clinical isolates, five *lnu* class genes have been described: *lnu*(A), *lnu*(B), *lnu*(C), *lnu*(D), and *lin*(F) [57–62]. The *O*-nucleotidyltransferases encoded by these genes inactivate lincosamides by adenylylation [58]. The *lnu*(A) genes have been reported in staphylococci and *Bacteroides* spp. [57, 60]. Initially described in *E. faecium*, *lnu*(B) is the most prevalent *lnu* gene among streptococci of human and animal origin [58]. The *lnu*(F) gene has been rarely described in *E. coli* and *Salmonella* spp. [59]. The *lnu*(C) gene was first characterized in a *Streptococcus agalactiae* clinical isolate, being located on a small mobilizable transposon [61, 63]. A second report of *lnu*(C) was recently published in a *Streptococcus anginosus* clinical isolate [64]. The *lnu*(D) gene was first described in a clinical isolate of *Streptococcus uberis* responsible for a case of bovine mastitis, and was then detected in two other *S. uberis* veterinary isolates [62, 65, 66]. Mechanistically, LnuA nucleotidyltransferase modifies a hydroxyl group of clindamycin and lincomycin at positions 3 and 4, respectively, whereas LnuB modifies a hydroxyl at position 3 in both clindamycin and lincomycin [58].

Although Lnu(A), Lnu(B), Lnu(C), and Lnu(D) nucleotidyltransferases inactivate in vitro more efficiently clindamycin than lincomycin, the corresponding genes confer resistance to lincomycin (MICs from 16 to 32 μg/mL) but not to clindamycin (MICs from 0.06 to 0.12 μg/mL), the so-called L phenotype [57, 58, 62, 63] (Fig. 18.2d). By contrast, when the *lnu*(A), *lnu*(B), *lnu*(C), and *lnu*(D) genes were cloned into *E. coli*, they conferred cross-resistance to lincomycin and clindamycin [57, 58, 62, 63]. A similar phenotype was observed for the *lin*(F) gene in *E. coli* [59]. The reason for the difference in phenotypic expression of the resistance determinant in the two backgrounds remains unexplained. Hypothetically, the difference between the two lincosamides might be related to differences in relative affinities of clindamycin and lincomycin for the ribosomes

of Gram-positive and Gram-negative organisms and for the Lnu enzymes: clindamycin might have better affinity for the Gram-positive ribosomes than for Lnu(C), explaining why its activity is maintained. Although the activity of clindamycin against the Gram-positive hosts of the *lnu* gene was only weakly affected by the mechanism of resistance, a 100-fold increase in the bacterial inoculum led to a three-dilution increase in the MIC of clindamycin for *S. agalactiae* UCN36 containing *lnu*(C) [61] and the bactericidal activity of clindamycin (already weak against susceptible strains) was totally abolished against a staphylococcal strain with *lnu*(A) [57].

Inactivation of type A streptogramins is due to *O*-acetylation by acetyltransferases encoded by *vat* genes [5, 7, 67]. These enzymes transfer an acetyl group from acetyl-CoA to the secondary hydroxyl of type A streptogramins. Type B streptogramins can be inactivated by enzymes called lyases or lactonases, which are encoded by *vgb* genes [5, 7, 67]. They cause a cleavage of the ester linkage leading to a linearization of the molecule.

4.4 Active Efflux

Efflux was reported as responsible for the intrinsic resistance to macrolides and lincosamides of *E. coli* and other Gram-negative bacteria, and as putatively responsible for the intrinsic resistance of *E. faecalis* to lincosamides and streptogramins A. In *E. coli*, inactivation of the tripartite pump AcrAB-TolC renders this organism susceptible to erythromycin and clindamycin [68]. In *E. faecalis* OG1RF, cross-resistance to lincosamides and streptogramins A (the so-called LS_A phenotype) was related to the expression of a species-specific chromosomal *lsa* gene, renamed *lsa*(A), coding for an ABC protein [69]. Inactivation of the *lsa*(A) gene resulted in entire susceptibility to clindamycin, dalfopristin, and quinupristin–dalfopristin, whereas trans-complementation with a recombinant plasmid bearing an intact *lsa* gene restored resistance to these antibiotics. In *Staphylococcus sciuri*, a LS_A phenotype was demonstrated to be related to the expression of the plasmid-mediated *lsa*(B) gene coding for a Lsa(A) homolog [70]. A similar LS_A phenotype was observed from *S. agalactiae* clinical isolates from New Zealand, and was due to a Lsa(A)-like protein encoded by the chromosomal *lsa*(C) gene [71, 72]. The last *lsa*-like gene, called *lsa*(E), has been recently identified in MRSA isolates of swine origin [73]. As opposed to *E. faecalis*, *E. faecium* is intrinsically susceptible to all macrolides and related compounds, but the LS_A phenotype may be selected in vitro and in vivo [74]. The resistance is due to a unique mutation within a gene coding for an ABC homologue showing 66 % amino acid identity with Lsa(A), leading to an amino acid substitution. The wild-type allele was named *eat*(A) (for Enterococcus ABC transporter) and its mutated resistant variant, *eat*(A)v [75]. Interestingly, the phenotype conferred by Lsa-like proteins actually comprises lincosamides, streptogramins A, and pleuromutilins (e.g., tiamulin), and is known as LS_AP phenotype [72, 75].

Acquired efflux of lincosamides (as a LS_A phenotype) has also been detected in staphylococcal isolates. This phenotype, similar to that mediated by Lsa-like proteins, is due to the acquisition of plasmid genes *vga*(A), *vga*(A)v, or $vga(A)_{LC}$, which also code for ABC proteins responsible for a low-level resistance to lincosamides and streptogramins A [76–78].

Active efflux has been reported as an acquired mechanism of resistance to macrolides in clinical isolates of Gram-positive organisms. In particular, the efflux pump *msr*(A) responsible for the MS_B phenotype (resistance to erythromycin and streptogramins B) in staphylococci and the dual efflux pump *mef*(A)/*mel* responsible for the M phenotype (resistance to erythromycin) in streptococci [79]. *msr*(A) and *mel* belong to the ABC transporter family whereas *mef*(A) is part of the Major Facilitator Superfamily [80]. Note that these mechanisms that are widely spread do not affect the activity of lincosamides, and that the activity of ketolides is affected by *mef*(A) only at a very low level, being likely not clinically significant. *mef*(A)/*mel* genes are borne by a transposon [81, 82] and have been described in a variety of species, mostly *S. pneumoniae* and *S. pyogenes*. The *msr*(A) gene is usually found in staphylococci but has also been detected in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* [83]. Different *msr*(A) homologs have also been described, such as *msr*(C) in *Enterococcus*, *msr*(D) in many genera and linked to *mef*(A), and *msr*(E) in some Gram-negative bacteria.

As opposed to Mef(A) that is undoubtedly an efflux pump, the biochemical basis of resistance remains unclear for aforementioned Lsa-, Vga-, and Msr-like proteins. They all belong to the family of ABC systems, of which most of them are involved in import and export, and then called ABC transporters [84]. These "classical" transporters share a common organization with two hydrophobic transmembrane domains (TMDs) and two intracytoplasmic nucleotide-binding domains (NBDs) implicated in ATP hydrolysis. Actually, Lsa-, Vga-, and Msr-like proteins belong to a third group of ABC proteins (named class 2) that lack TMDs consisting of two NBDs fused into a single protein [84]. Even though these class 2 ABC proteins are presumed to function as efflux pumps, the biochemical mechanism of resistance has been poorly elucidated. Only two studies that showed about Msr(A) suggest that Msr(A) and $vga(A)_{LC}$ might be able to hijack the TMDs of ABC transporters to mediate efflux [77, 85], but no membrane partners have been identified so far [86]. A ribosomal-related mechanism of resistance, such as ribosomal protection, might also be hypothesized.

5 Reports of Susceptibility Tests by the Laboratory

5.1 Staphylococci

Both clindamycin and erythromycin have to be tested. As noted above, resistance to both erythromycin and clindamycin relates to constitutive MLS_B resistance and is easily recognized. Dissociated susceptibility results for erythromycin and clindamycin require the attention of the clinical microbiology laboratory. The following cases can be discussed.

5.1.1 Strains Resistant to Erythromycin but Susceptible to Clindamycin

When clindamycin is active, the identification of the phenotype is required. The inducible MLS_B resistance can be detected only by methods showing induction of clindamycin resistance. As previously mentioned, the disk-diffusion method is an easy method to detect this phenotype by placing an erythromycin disk near a clindamycin disk on an agar growth medium, using a standard disk dispenser [87]. The presence of a D-shape zone is the signature of the MLS_B-inducible phenotype (Fig. 18.2b). This approach is recommended by the CLSI susceptibility testing standards. When staphylococci are tested using a broth-based method (including automated instruments), the CLSI recommends placing erythromycin (15 µg) and clindamycin (2 µg) disks nearly 15–26 mm apart (center to center) on the blood agar plate that is used to control the purity of the bacterial inoculum [88, 89]. Isolates displaying a D-shaped zone, therefore inducibly resistant to MLSB antibiotics, should be reported as clindamycin resistant by the laboratory [88]. However, the clinical laboratory may add the following comment: "This isolate is presumed to be resistant based on detection of inducible clindamycin resistance; clindamycin may still be effective in some patients." Note that certain automated systems also propose a liquid-based induction test. The final decision to treat or not the patient with clindamycin should be based on the analysis of each specific case, and if a clindamycin therapy is started, it requires close follow-up of the patient for failure. In the absence of D-shaped zone, the staphylococcal isolate is presumably resistant to erythromycin by active efflux through acquisition of the *msr*(A) gene (Fig. 18.2e). Since clindamycin is neither an inducer nor a substrate for this pump, the isolate can safely be reported as susceptible to clindamycin. Strains of *S. aureus* ATCC strain BAA-977 containing *erm*(A) and *S. aureus* ATCC BAA-976 harboring the efflux pump encoded by *msr*(A) are recommended as positive and negative control organisms, respectively [90].

5.1.2 Strains Susceptible to Erythromycin but Resistant to Lincosamides

This dissociated phenotype of resistance is rare in *S. aureus*, found in less than 1 % of the strains, but is more frequent in coagulase-negative staphylococci, with frequencies ranging from 1 to 7 % of strains depending on the staphylococcal species [57]. Two phenotypes of resistance should be distinguished: the LS_A type of resistance that is detected as a resistance or an intermediate susceptibility to both clindamycin and lincomycin, and the L phenotype resistance that can be identified only if lincomycin is tested since MIC of clindamycin or zone size diameter for the disk of clindamycin remain within the range of those for a susceptible isolate. This phenotype can be easily identified by testing both lincomycin and clindamycin, which display an unusual dissociated susceptibility to clindamycin and resistance to lincomycin. By the disk-diffusion technique, lincosamide inactivation can be easily predicted by observing the appearance of the clindamycin inhibition zone edge. A sharply demarcated edge correlates with the production of lincosamide nucleotidyltransferases (Fig. 18.2d). There is no recommendation for the interpretation of the result for clindamycin and the clinical relevance is unknown.

5.2 Other Organisms

For streptococci, concerns about the activity of clindamycin against isolates susceptible to this antibiotic but with an inducible MLS_B phenotype could also be raised. However, routine testing for inducible resistance for pneumococci is not recommended since isolates containing an inducible *erm*(B) gene usually display cross-resistance between erythromycin and clindamycin, as mentioned above. Only rare isolates with an inducible MLS_B phenotype are susceptible to clindamycin and clinical significance has not been established. The same observation can be made for β-hemolytic streptococci containing an inducible *erm*(B) gene. However, β-hemolytic streptococci might contain an inducible *erm*(A) gene (formerly *ermTR*) with a positive D-shaped zone test. In this case, although no clinical failure has been reported, the use of clindamycin does not seem safe. By contrast, isolates of *S. pneumoniae* or *S. pyogenes* expressing the efflux pumps MefA/Mel remain fully susceptible to clindamycin. Resistance to clindamycin in *Bacteroides fragilis* is frequent (generally more than 30 % of isolates) and is mostly due to ribosomal methylation (MLS_B phenotype) mostly by *erm*(F), *erm*(G), and *erm*(B) genes. The resistance is often expressed at a high level. *C. perfringens* is rarely resistant to clindamycin. Again, resistant isolates expressing an MLS_B phenotype which, in some cases of inducible expression, can be detected only after 48 h of incubation. For some fastidious organisms (e.g., *H. pylori*, *M. avium* complex), molecular detection of 23S rRNA mutations is a good option since the number and the position of mutations conferring macrolide resistance are limited. Many different approaches, particularly real-time PCR assays, have been developed. For instance, it is possible to detect most of mutations conferring clarithromycin resistance in *H. pylori*, even directly from gastric biopsies [91].

6 Conclusion

Favorable properties of macrolides and clindamycin, in terms of tissue distribution, convenient oral or intravenous dosing, and low cost explain why these antibiotics, available for more than 40 years, remain widely used. However, a multiplicity of mechanisms has emerged in staphylococci, streptococci, enterococci, and anaerobes that confer resistance to this group of antimicrobials and lead to complex resistance phenotypes. Identification of the corresponding resistance mechanisms has a clinical importance as regards to the use of macrolides and clindamycin. The clinical relevance of the inducible MLS_B type of resistance for activity of clindamycin still remains to be fully evaluated. Epidemiological aspects of resistance to macrolides and lincosamides have not been discussed in this chapter, since it is highly variable according to the country and even within a single country. The frequencies of resistance to clindamycin cannot be deduced from those to erythromycin since cross-resistance is unpredictable. In particular, efflux mechanisms affect the activity of erythromycin but not that of clindamycin, both in streptococci and staphylococci. The reverse is also true for other mechanisms of resistance. Therefore, specific surveys of macrolide and lincosamide resistance in pathogens are required. Both surveillance of the incidence of resistance and of the respective prevalence of the various resistance mechanisms is justified by the rapid variations in resistance observed in several countries.

References

1. Takashima H. Structural consideration of macrolide antibiotics in relation to the ribosomal interaction and drug design. Curr Top Med Chem. 2003;3:991–9.
2. Llano-Sotelo B, Dunkle J, Klepacki D, Zhang W, Fernandes P, Cate JH, Mankin AS. Binding and action of CEM-101, a new fluoroketolide antibiotic that inhibits protein synthesis. Antimicrob Agents Chemother. 2010;54:4961–70.
3. Spizek J, Novotna J, Rezanka T. Lincosamides: chemical structure, biosynthesis, mechanism of action, resistance, and applications. Adv Appl Microbiol. 2004;56:121–54.
4. Mukhtar TA, Wright GD. Streptogramins, oxazolidinones, and other inhibitors of bacterial protein synthesis. Chem Rev. 2005;105:529–42.
5. Canu A, Leclercq R. Overcoming bacterial resistance by dual target inhibition: the case of streptogramins. Curr Drug Targets Infect Disord. 2001;1:215–25.
6. Barriere JC, Berthaud N, Beyer D, Dutka-Malen S, Paris JM, Desnottes JF. Recent developments in streptogramin research. Curr Pharm Des. 1998;4:155–80.
7. Johnston NJ, Mukhtar TA, Wright GD. Streptogramin antibiotics: mode of action and resistance. Curr Drug Targets. 2002;3:335–44.
8. Politano AD, Sawyer RG. NXL-103, a combination of flopristin and linopristin, for the potential treatment of bacterial infections including community-acquired pneumonia and MRSA. Curr Opin Investig Drugs. 2010;11:225–36.
9. Verdier L, Bertho G, Gharbi-Benarous J, Girault JP. Lincomycin and clindamycin conformations. A fragment shared by macrolides, ketolides and lincosamides determined from TRNOE ribosome-bound conformations. Bioorg Med Chem. 2000;8:1225–43.
10. Agmon I, Amit M, Auerbach T, Bashan A, Baram D, Bartels H, Berisio R, Greenberg I, Harms J, Hansen HA, Kessler M, Pyetan E, Schluenzen F, Sittner A, Yonath A, Zarivach R. Ribosomal crystallography: a flexible nucleotide anchoring tRNA translocation, facilitates peptide-bond formation, chirality discrimination and antibiotics synergism. FEBS Lett. 2004;567:20–6.
11. Ban N, Nissen P, Hansen J, Moore PB, Steitz TA. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. Science. 2000;289:905–20.
12. Yonath A. Ribosomal crystallography: peptide bond formation, chaperone assistance and antibiotics activity. Mol Cells. 2005;20:1–16.
13. Harms J, Schluenzen F, Zarivach R, Bashan A, Gat S, Agmon I, Bartels H, Franceschi F, Yonath A. High resolution structure of the large ribosomal subunit from a mesophilic *Eubacterium*. Cell. 2001;107:679–88.
14. Tu D, Blaha G, Moore PB, Steitz TA. Structures of MLSBK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell. 2005;121:257–70.
15. Wilson DN, Harms JM, Nierhaus KH, Schlünzen F, Fucini P. Species-specific antibiotic-ribosome interactions: implications for drug development. Biol Chem. 2005;386:1239–52.
16. Schlünzen F, Zarivach R, Harms J, Bashan A, Tocilj A, Albrecht R, Yonath A, Franceschi F. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. Nature. 2001;413:814–21.
17. Polacek N, Mankin AS. The ribosomal peptidyl transferase center: structure, function, evolution, inhibition. Crit Rev Biochem Mol Biol. 2005;40:285–311.
18. Poehlsgaard J, Douthwaite S. The bacterial ribosome as a target for antibiotics. Nat Rev Microbiol. 2005;13:870–81.
19. Gaynor M, Mankin AS. Macrolide antibiotics: binding site, mechanism of action, resistance. Curr Top Med Chem. 2003;3:949–60.
20. Yonath A, Bashan A. Ribosomal crystallography: initiation, peptide bond formation, and amino acid polymerization are hampered by antibiotics. Annu Rev Microbiol. 2004;58:233–51.
21. Cocito C, Di Giambattista M, Nyssen E, Vannuffel P. Inhibition of protein synthesis by streptogramins and related antibiotics. J Antimicrob Chemother. 1997;39(Suppl A):7–13.
22. Weisblum B. Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother. 1995;39:577–85.
23. Liu M, Douthwaite S. Activity of the ketolide telithromycin is refractory to Erm monomethylation of bacterial rRNA. Antimicrob Agents Chemother. 2002;46:1629–33.
24. Douthwaite S, Jalava J, Jakobsen L. Ketolide resistance in *Streptococcus pyogenes* correlates with the degree of rRNA dimethylation by Erm. Mol Microbiol. 2005;58:613–22.
25. Leclercq R, Courvalin P. Resistance to macrolides and related antibiotics in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2002;46:2727–34.
26. Weisblum B. Insights into erythromycin action from studies of its activity as inducer of resistance. Antimicrob Agents Chemother. 1995;39:797–805.
27. Tanaka T, Weisblum B. Mutant of *Staphylococcus aureus* with lincomycin- and carbomycin-inducible resistance to erythromycin. Antimicrob Agents Chemother. 1974;5:538–40.
28. Clarebout G, Nativelle E, Leclercq R. Unusual inducible cross resistance to macrolides, lincosamides, and streptogramins B by methylase production in clinical isolates of *Staphylococcus aureus*. Microb Drug Resist. 2001;7:317–22.

29. Rosato A, Vicarini H, Bonnefoy A, Chantot JF, Leclercq R. A new ketolide, HMR 3004, active against streptococci inducibly resistant to erythromycin. Antimicrob Agents Chemother. 1998;42:1392–6.
30. Lewis II JS, Jorgensen JH. Inducible clindamycin resistance in staphylococci: should clinicians and microbiologists be concerned? Clin Infect Dis. 2005;40:280–5.
31. Fines M, Gueudin M, Ramon A, Leclercq R. In vitro selection of resistance to clindamycin related to alterations in the attenuator of the *erm*(TR) gene of *Streptococcus pyogenes* UCN1 inducibly resistant to erythromycin. J Antimicrob Chemother. 2001;48:411–6.
32. Rao GG. Should clindamycin be used in treatment of patients with infections caused by erythromycin-resistant staphylococci? J Antimicrob Chemother. 2000;45:715–6.
33. McGehee RF, Barrett FF, Finland F. Resistance of *Staphylococcus aureus* to lincomycin, clindamycin, and erythromycin. Antimicrob Agents Chemother. 1968;13:392–7.
34. Drinkovic D, Fuller ER, Shore KP, Holland DJ, Ellis-Pegler R. Clindamycin treatment of *Staphylococcus aureus* expressing inducible clindamycin resistance. J Antimicrob Chemother. 2001;48:315–6.
35. Frank AI, Marcinak JF, Mangat PD, Tjhio JT, Kelkar S, Schreckenberger PC, Quinn JP. Clindamycin treatment of methicillin-resistant *Staphylococcus aureus* infections in children. Pediatr Infect Dis J. 2002;21:530–4.
36. Siberry GK, Tekle T, Carroll K, Dick J. Failure of clindamycin treatment of methicillin-resistant *Staphylococcus aureus* expressing inducible clindamycin resistance in vitro. Clin Infect Dis. 2003;37:1257–60.
37. Levin TP, Suh B, Axelrod P, Truant AL, Fekete T. Potential clindamycin resistance in clindamycin-susceptible, erythromycin-resistant *Staphylococcus aureus*: report of a clinical failure. Antimicrob Agents Chemother. 2005;49:1222–4.
38. Daurel C, Huet C, Dhalluin A, Bes M, Etienne J, Leclercq R. Differences in potential for selection of clindamycin-resistant mutants between inducible *erm*(A) and *erm*(C) *Staphylococcus aureus* genes. J Clin Microbiol. 2008;46:546–50.
39. Clarebout G, Nativelle E, Bozdogan B, Villers C, Leclercq R. Bactericidal activity of quinupristin-dalfopristin against strains of *Staphylococcus aureus* with the MLS(B) phenotype of resistance according to the *erm* gene type. Int J Antimicrob Agents. 2004; 24:444–9.
40. Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. Antimicrob Agents Chemother. 2000;44:2530–3.
41. Toh SM, Xiong L, Arias CA, Villegas MV, Lolans K, Quinn J, Mankin AS. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol. 2007;64:1506–14.
42. Witte W, Cuny C. Emergence and spread of *cfr*-mediated multiresistance in staphylococci: an interdisciplinary challenge. Future Microbiol. 2011;6:925–31.
43. Diaz L, Kiratisin P, Mendes RE, Panesso D, Singh KV, Arias CA. Transferable plasmid-mediated resistance to linezolid due to *cfr* in a human clinical isolate of *Enterococcus faecalis*. Antimicrob Agents Chemother. 2012;56:3917–22.
44. Kehrenberg C, Schwarz S, Jacobsen L, Hansen LH, Vester B. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Mol Microbiol. 2005;57:1064–73.
45. Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. Antimicrob Agents Chemother. 2006;50:2500–5.
46. Tait-Kamradt A, Davies T, Cronan M, Jacobs MR, Appelbaum PC, Sutcliffe J. Mutations in 23S rRNA and ribosomal protein L4 account for resistance in pneumococcal strains selected in vitro by macrolide passage. Antimicrob Agents Chemother. 2000;44: 2118–25.
47. Roberts MC. Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. FEMS Microbiol Lett. 2008;282:147–59.
48. Arthur M, Andremont A, Courvalin P. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob Agents Chemother. 1987;3:404–9.
49. Barthélémy P, Autissier D, Gerbaud G, Courvalin P. Enzymic hydrolysis of erythromycin by a strain of *Escherichia coli.* A new mechanism of resistance. J Antibiot. 1984;37:1692–6.
50. Noguchi N, Emura A, Matsuyama H, O'Hara K, Sasatsu M, Kono M. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2′-phosphotransferase I in *Escherichia coli*. Antimicrob Agents Chemother. 1995;39: 2359–63.
51. Noguchi N, Katayama J, O'Hara K. Cloning and nucleotide sequence of the mphB gene for macrolide 2′-phosphotransferase II in *Escherichia coli*. FEMS Microbiol Lett. 1996;144:197–202.
52. Schmitz FJ, Sadurski R, Kray A, Boos M, Geisel R, Kohrer K, Verhoef J, Fluit AC. Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. J Antimicrob Chemother. 2000;45: 891–4.
53. Chesneau O, Tsvetkova K, Courvalin P. Resistance phenotypes conferred by macrolide phosphotransferases. FEMS Microbiol Lett. 2007;269:317–22.
54. Boumghar-Bourtchai L, Mariani-Kurkdjian P, Bingen E, Filliol I, Dhalluin A, Ifrane SA, Weill FX, Leclercq R. Macrolide-resistant *Shigella sonnei*. Emerg Infect Dis. 2008;14:1297–9.
55. Phuc Nguyen MC, Woerther PL, Bouvet M, Andremont A, Leclercq R, Canu A. *Escherichia coli* as reservoir for macrolide resistance genes. Emerg Infect Dis. 2009;15:1648–50.
56. Matsuoka M, Endou K, Kobayashi H, Inoue M, Nakajima Y. A plasmid that encodes three genes for resistance to macrolide antibiotics in *Staphylococcus aureus*. FEMS Microbiol Lett. 1998;167: 221–7.
57. Leclercq R, Brisson-Noel A, Duval J, Courvalin P. Phenotypic expression and genetic heterogeneity of lincosamides inactivation in *Staphylococcus* spp. Antimicrob Agents Chemother. 1987;31: 1887–91.
58. Bozdogan B, Berrezouga L, Kuo M, Yurek D, Farley K, Stockman B, Leclercq R. A new resistance gene, *linB*, conferring resistance to lincosamides by nucleotidylation in *Enterococcus faecium* HM1025. Antimicrob Agents Chemother. 1999;43:925–9.
59. Heir E, Lindstedt BA, Leegaard TM, Gjernes E, Kapperud G. Prevalence and characterisation of integrons in blood culture Enterobacteriaceae and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class I integron-located lincosamide resistance gene. Ann Clin Microbiol Antimicrob. 2004;3:12.
60. Wang J, Shoemaker N, Wang GR, Salyers A. Characterization of a *Bacteroides* mobilizable transposon of a functional lincomycin resistance gene. J Bacteriol. 2000;182:3559–71.
61. Achard A, Villers C, Pichereau V, Leclercq R. New *lnu*(C) gene conferring resistance to lincomycin by nucleotidylation in *Streptococcus agalactiae* UCN36. Antimicrob Agents Chemother. 2005;49:2716–9.
62. Petinaki E, Guérin-Faublée V, Pichereau V, Villers C, Achard A, Malbruny B, Leclercq R. Lincomycin resistance gene *lnu*(D) in *Streptococcus uberis*. Antimicrob Agents Chemother. 2008;52: 626–30.
63. Achard A, Leclercq R. Characterization of a small mobilizable transposon, MTnSag1, in *Streptococcus agalactiae*. J Bacteriol. 2007;189:4328–31.

64. Gravey F, Galopin S, Grall N, Auzou M, Andremont A, Leclercq R, Cattoir V. Lincosamide resistance mediated by *lnu*(C) (L phenotype) in a *Streptococcus anginosus* clinical isolate. J Antimicrob Chemother. 2013;68(11):2464–7.
65. Haenni M, Saras E, Bertin S, Leblond P, Madec JY, Payot S. Diversity and mobility of integrative and conjugative elements in bovine isolates of *Streptococcus agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. uberis*. Appl Environ Microbiol. 2010;76: 7957–65.
66. Haenni M, Saras E, Chaussière S, Treilles M, Madec JY. *ermB*-mediated erythromycin resistance in *Streptococcus uberis* from bovine mastitis. Vet J. 2011;189:356–8.
67. Hershberger E, Donabedian S, Konstantinou K, Zervos MJ. Quinupristin-dalfopristin resistance in gram-positive bacteria: mechanism of resistance and epidemiology. Clin Infect Dis. 2004;38:92–8.
68. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria. Drugs. 2004;64:159–204.
69. Singh KV, Weinstock GM, Murray BE. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob Agents Chemother. 2002;46:1845–50.
70. Kehrenberg C, Ojo KK, Schwarz S. Nucleotide sequence and organization of the multiresistance plasmid pSCFS1 from *Staphylococcus sciuri*. J Antimicrob Chemother. 2004;54:936–9.
71. Malbruny B, Werno AM, Anderson TP, Murdoch DR, Leclercq R. A new phenotype of resistance to lincosamides and streptogramin A-type antibiotics in *Streptococcus agalactiae* in New Zealand. J Antimicrob Chemother. 2004;54:1040–4.
72. Malbruny B, Werno AM, Murdoch DR, Leclercq R, Cattoir V. Cross-resistance to lincosamides, streptogramins A, and pleuromutilins due to the *lsa*(C) gene in *Streptococcus agalactiae* UCN70. Antimicrob Agents Chemother. 2011;55:1470–4.
73. Li B, Wendlandt S, Yao J, Liu Y, Zhang Q, Shi Z, Wei J, Shao D, Schwarz S, Wang S, Ma Z. Detection and new genetic environment of the pleuromutilin-lincosamide-streptogramin A resistance gene *lsa*(E) in methicillin-resistant *Staphylococcus aureus* of swine origin. J Antimicrob Chemother. 2013;68:1251–5.
74. Dowzicky M, Talbot GH, Feger C, Prokocimer P, Etienne J, Leclercq R. Characterization of isolates associated with emerging resistance to quinupristin/dalfopristin (Synercid) during a worldwide clinical program. Diagn Microbiol Infect Dis. 2000;37: 57–62.
75. Isnard C, Malbruny B, Leclercq R, Cattoir V. Genetic basis for in vitro and in vivo resistance to lincosamides, streptogramins A and pleuromutilins (LS_AP phenotype) in *Enterococcus faecium*. Antimicrob Agents Chemother. 2013;57(9):4463–9.
76. Haroche J, Morvan A, Davi M, Allignet J, Bimet F, El Solh N. Clonal diversity among streptogramin A-resistant *Staphylococcus aureus* isolates collected in French hospitals. J Clin Microbiol. 2003;41:586–91.
77. Novotna G, Janata J. A new evolutionary variant of the streptogramin A resistance protein, $Vga(A)_{LC}$, from *Staphylococcus haemolyticus* with shifted substrate specificity towards lincosamides. Antimicrob Agents Chemother. 2006;50:4070–6.
78. Tessé S, Trueba F, Berthet N, Hot C, Chesneau O. Resistance genes underlying the LS_A phenotype of French staphylococcal isolates. Antimicrob Agents Chemother. 2013;57(9):4543–6.
79. Ambrose KD, Nisbet R, Stephens DS. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. Antimicrob Agents Chemother. 2005;48:4203–9.
80. Leclercq R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin Infect Dis. 2002;34:482–92.
81. Santagati M, Iannelli F, Oggioni MR, Stefani S, Pozzi G. Characterization of a genetic element carrying the macrolide efflux gene *mef*(A) in *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2000;44:2585–7.
82. Gay K, Stephens DS. Structure and dissemination of a chromosomal insertion element encoding macrolide efflux in *Streptococcus pneumoniae*. J Infect Dis. 2001;184:56–65.
83. Ojo KK, Striplin MJ, Ulep CC, Close NS, Zittle J, Luis H, Bernardo M, Leitao J, Roberts MC. *Staphylococcus* efflux *msr*(A) gene characterized in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* isolates. Antimicrob Agents Chemother. 2006;50:1089–91.
84. Davidson AL, Dassa E, Orelle C, Chen J. Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol Mol Biol Rev. 2008;72:317–64.
85. Ross JI, Eady EA, Cove JH, Cunliffe WJ, Baumberg S, Wootton JC. Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. Mol Microbiol. 1990;4:1207–14.
86. Reynolds E, Ross JI, Cove JH. Msr(A) and related macrolide/streptogramin resistance determinants: incomplete transporters? Int J Antimicrob Agents. 2003;22:228–36.
87. Fiebelkorn KR, Crawford SA, McElmeel ML, Jorgensen JH. Practical disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci. J Clin Microbiol. 2003;41:4740–4.
88. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 21th informational supplement. M100-S11. Wayne, PA: CLSI; 2011.
89. Jorgensen JH, Crawford SA, McElmeel ML, Fiebelkorn KR. Detection of inducible clindamycin resistance of staphylococci in conjunction with performance of automated broth susceptibility testing. J Clin Microbiol. 2004;42:1800–2.
90. Zelazny AM, Ferraro MJ, Glennen A, Hindler JF, Mann LM, Munro S, Murray PR, Reller LB, Tenover FC, Jorgensen JH. Selection of strains for quality assessment of the disk induction method for detection of inducible clindamycin resistance in staphylococci: a CLSI collaborative study. J Clin Microbiol. 2005;43:2613–5.
91. Burucoa C, Garnier M, Silvain C, Fauchère JL. Quadruplex real-time PCR assay using allele-specific scorpion primers for detection of mutations conferring clarithromycin resistance to *Helicobacter pylori*. J Clin Microbiol. 2008;46:2320–6.

Mechanisms of Resistance in Metronidazole

19

Shira I. Doron, Kirthana R. Beaulac, Abhay Dhand, and David R. Snydman

1 Introduction

Metronidazole is the drug of choice for human infections caused by various anaerobic and micro-aerophilic bacteria. Therapeutic failures are being noted in the treatment of *Helicobacter pylori*, *Bacteroides* spp., *Trichomonas vaginalis, Giardia*, and *Clostridium difficile*, and some of these have been attributed to the emerging resistance to metronidazole. With improvements in molecular detection, reports of resistance to metronidazole are slowly increasing.

Because of limited alternative treatment options, this emerging resistance poses various diagnostic and therapeutic dilemmas. Mechanisms of resistance are being defined and a better understanding is the key for prevention of resistance and improved management of these infections.

Metronidazole [1-(2 hydroxyethyl)-2-methyl-5-nitroimidazole] was introduced in 1960s.

Since then it has been drug of choice for human infections caused by various anaerobic and micro-aerophilic bacteria (*Bacteroides, Clostridia, Helicobacter*) and parasites (*Trichomonas, Giardia, Entamoeba*). Other gram positive anaerobes (e.g., lactobacilli, *Propionibacterium acnes*, majority of the periodontal pathogens, peptostreptococci) are known to be inherently resistant to metronidazole. Virtually all the anaerobic gram-negative rods are known to be susceptible to metronidazole.

Sensitivity testing for anaerobes is not performed routinely. Therefore, resistance to metronidazole is underreported. With improvements in molecular detection, increasing resistance rates are being noted. This emerging resistance to metronidazole poses various diagnostic and therapeutic dilemmas. Mechanisms of resistance are being defined and a better understanding is the key for prevention of resistance and improved management of these infections.

2 Antimicrobial Mechanism of Action

5-Nitroimidazole is administered as a pro-drug. It enters the cell by passive diffusion and is activated in either the cytoplasm in bacteria, Entamoeba, and Giardia, or in a specialized organelle called hydrogenosome in Trichomonas. Activation to its cytotoxic form occurs via transfer of an electron from various donors to the nitro group, which converts it to a nitroso free radical form. This toxic metabolite interacts primarily with DNA, RNA, or intracellular proteins leading to DNA strand breakage, inhibited repair, and disrupted transcription. If the disruption of DNA is faster than its repair, it ultimately leads to cell death.

The selective toxicity and effectiveness of metronidazole depends on the cytoplasmic environment in the anaerobic and microaerophilic organisms, which provide a sufficiently low redox potential environment required for the activation of the drug. Metronidazole has very low reduction potential E_{17} -486 mV and will be activated only in conditions where low redox status in maintained. Oxygen has higher affinity for an electron than metronidazole E_{17} –150 mV. Therefore, oxygen can either successfully compete with 5-nitroimidazole for the electron from the electron carrier or be able to remove the electron from the activated nitroso group, thereby re-forming the parent drug—the phenomenon known as futile cycling. Similarly downregulation of various intracellular

S.I. Doron, M.D., M.S. (✉)
Tufts University School of Medicine,
800 Washington Street, Box # 041, Boston, MA 02111, USA

Division of Geographic Medicine and Infectious Diseases,
Tufts Medical Center, Boston, MA, USA
e-mail: sdoron@tuftsmedicalcenter.org

K.R. Beaulac, Pharm.D., B.C.P.S.
Tufts Medical Center, 800 Washington Street, Box # 041, Boston, MA 02111, USA

A. Dhand, M.D.
Division of Geographic Medicine and Infectious Diseases,
Tufts Medical Center, Boston, MA, USA

D.R. Snydman, M.D., F.A.C.P.
Tufts University School of Medicine and Division of Geographic Medicine and Infectious Diseases,
Tufts Medical Center, Boston, MA, USA

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_19

electron donors may prevent activation of the pro-drug and therefore lack of efficacy.

3 Mechanism of Resistance

Proposed Mechanisms of Resistance

1. Decreased drug uptake or increased efflux.
2. Decreased drug activation/change in the biological target.
3. Increased oxygen scavenging capabilities (SOD/catalase/peroxidase).
4. Enhanced activity of DNA repair enzymes.

3.1 Bacteroides

Metronidazole-resistant (MTZ-R) *Bacteroides fragilis* was first reported in a patient with Crohn's disease after long-term therapy with metronidazole [1]. Metronidazole resistance in Bacteroides spp. is quite rare but has been reported in several countries [2–9]. Advances in technology have improved susceptibility assessment, resulting in an increased reported rate of resistance more recently [10]. Time kill curves have suggested that MTZ may remain bactericidal against MTZ-R isolates (MIC $\geq$ 256) if a C_{max} of 16 is achieved [11]. Metronidazole resistance among Bacteroides spp. is of concern as these species can also be resistant to a wide variety of antimicrobial agents including β-lactams, tetracycline, clindamycin, cefoxitin, and imipenem [12].

Breuil et al. (1989) and Reysset et al. (1993) showed that all Bacteroides strains that were resistant to 5-nitroimidazole harbored a genetic determinant, which was either plasmid borne or on the chromosome [13, 14]. This resistance was shown to be transferable by a conjugation-like process to susceptible strains with frequency ranging from 10^{-3} to 10^{-7} per donor. These genetic determinants have been shown to be specific nitroimidazole-resistant genes (*nim*), presumably encoding a nitroimidazole reductase that converted nitroimidazole to aminoimidazole, thus avoiding the formation of toxic nitroso radicals that are essential for antimicrobial activity. So far eight *nim* genes (*nim* A,B,C,D,E,F,G,J) have been described [15]. These genes are commonly transcribed from promoters located within different insertion elements. Gal et al. (2004) studied 50 resistant isolates and found the nim A gene was the most common, followed by nim B and nim E [16]. Although the presence of a nim gene does not always equate to therapeutic resistance, it is often associated with higher MICs and potential for development of resistance [9]. Prolonged exposure of nim-gene carrying Bacteroides spp. to metronidazole can select therapeutic resistance. MTZ resistance in the absence of detected nim genes suggests that either the current PCR primers are incompletely detecting the gene or that alternative genetic determinants are contributing to MTZ resistance [17, 18]. DNA repair proteins, such as RecA, may putatively repair oxidative damage caused by MTZ and confer MTZ resistance [18]. Diniz et al. (2004) used combination of proteomics for identification of differentially expressed proteins and other genes involved in the adaptive response to metronidazole [19]. Protein profile of resistant strains showed upregulation of lactate dehydrogenase and downregulation of flavodoxin and impaired enzymatic activity of pyruvate-ferrodoxin oxidase reductase. They also suggested that multiple enzymes involved in oxidation/reduction and electron transfer reactions may be important in activation of MTZ and possible mechanisms of inducing resistance. This supports the idea that there is no one specific gene for MTZ resistance and multiple possible pathways for resistance to exist.

3.2 Helicobacter Pylori

High rates of metronidazole resistance in *H. pylori* have also been reported worldwide [20]. Twenty to forty-five percent of isolates of *H. pylori* in Western Europe have been reported as MTZ-R. This rate is higher in developing countries, within immigrant populations and in young women who may have received this agent in the past for parasitic infections or gynecologic infections [21–23]. Thompson et al. (1995) showed that inactivation of recA (a gene needed for generalized DNA repair and recombination) severely impaired the ability of *H. pylori* mutants to survive treatment with UV light, ciprofloxacin, and metronidazole. Expression of a cloned recA gene obtained from a resistant strain of *H. pylori* in *E. coli* raised its level of resistance [24]. Smith et al. (1997) showed that a relationship existed between the intracellular oxygen scavenging ability of *H. pylori* and sensitivity of the bacterium to metronidazole. MTZ-R strains of *H. pylori* possessed considerably lower soluble cytosolic NADH oxidase activity than MTZ-S strains [25]. Goodwin et al. (1998) first demonstrated that a major mechanism of MTZ resistance in *H. pylori* is due to null mutations in the rdxA gene, which encodes an oxygen-insensitive NAD(P)H nitroreductase. Using a cosmid cloning approach in MTZ-R strains, they identified an open reading frame (ORF) that had protein level homology to classical oxygen-insensitive NAD(P)H nitroreductases. An *H. pylori* gene corresponding to this ORF was designated rdxA. In a series of elegant experiments Goodwin et al. also showed that *E. coli* (normally MTZ-R) was rendered MTZ-S by a functional rdxA gene, introduction of rdxA on a shuttle vector plasmid into formerly MTZ-R *H. pylori* rendered it MTZ-S, and replacement of rdxA in MTZ-S *H. pylori* with a rdxA::camR null insertion allele resulted in MTZ-R phenotype [26]. Kwon et al. (2000) reported on the role of an additional gene frxA,

which encodes NAD(P)H flavin oxidoreductase, in MTZ resistance in *H. pylori* [27]. Using a lambda phage genomic library, they identified a MTZ nitroreductase encoding gene, NAD(P)H flavinoxidoreductase (frxA). Frameshift mutations leading to premature termination of frxA protein were associated with metronidazole resistance in *H. pylori*. This was further confirmed by insertion activation of frxA and/or rdxA genes. In addition, cloned frxA gene expressed in *E. coli* showed nitroreductase activity and rendered normally Metronidazole-resistant *E. coli* sensitive. Strains carrying frxA null alleles enhanced MTZ resistance in rdxA-deficient cells. Also, inactivation of genes that encode ferredoxin-like protein (fdxB) along with previously described frxA and rdxA gene increased the MIC of MTZ-S strains [28]. This suggested multiple possible factors might be involved in high-level resistance to MTZ. Jeong et al. (2001) suggested two types of MTZ-S strains by genetic (mutational) and molecular tests on the basis of need of inactivation of rdxA alone or along with frxA gene to render *H. pylori* resistant [29]. Subsequent work suggested that rdxA gene might play a major role in the high-level resistance to metronidazole [30]. The cagA mutation, which is typically associated with more inflammation and severe disease, has been shown to be protective of metronidazole resistance. It is theorized that the cagA gene results in higher bacterial burden and rapid cell turnover, optimizing opportunities for metronidazole to be effective [31, 32]. There are various mutations within the Fur regulatory protein that result in metronidazole decreased-susceptibility or resistance. These mutations may alter iron-binding, dimerization and stability, binding to specific DNA sites, altered binding to promoters (sodB) allowing superoxide dismutase production, and other poorly defined alterations [33].

3.3 Trichomonas

The first report of resistance appeared in *Trichomonas vaginalis* about 2 years after introduction of metronidazole [34]. Early attempts to correlate treatment failure with resistance were hampered by the necessity to perform the investigation in the presence of oxygen [35] but Muller et al. subsequently fine-tuned the resistance testing technique using a multiwall assay and demonstrated a correlation between susceptibility and clinical outcome, while at the same time concluding that most patients who fail treatment do not have metronidazole-resistant infection [36]. More recently, concerns have been raised about a troubling rise in the frequency of metronidazole-resistant trichomoniasis associated with therapeutic failures [37].

The prevalence of metronidazole-resistant Trichomonas strains varies in different parts of the world, with a rate of 2–5 % in the USA and as high as 17 % in Papua New Guinea [38].

Figure 19.1 below from the CDC's STD surveillance network demonstrates susceptibility to metronidazole and tinidazole amongst 538 Trochomonas isolates from 6 US cities.

In trichomonads, activation of MTZ occurs within specialized organelles, hydrogenosomes, which contain pyruvate:ferrodoxin oxidoreductase (PFOR) and ferrodoxin (Fd). PFOR catalyzes the decarboxylation of pyruvate to acetyl CoA, transferring the electron to ferrodoxin. MTZ replaces protons as the acceptor of electrons donated by ferrodoxin. In the absence of the drug, these protons would normally be reduced to molecular hydrogen by hydrogenase. Yarlett et al. (1986) provided evidence that the reductive activation of metronidazole is diminished in resistant strains relative to drug-sensitive strains [40, 41]. Quon et al. (1992) examined the intracellular levels of Fd and its mRNA in four

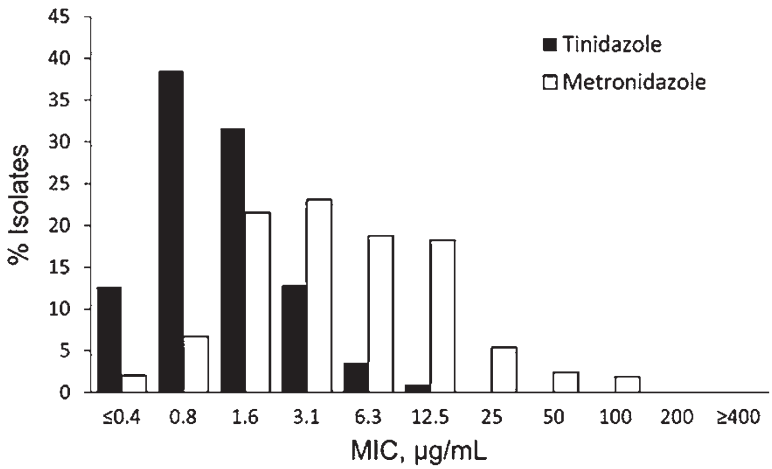


Fig. 19.1 Distribution of minimum lethal concentrations (MLCs) of tinidazole and metronidazole, STD Surveillance Network, 2009–2010 ($n = 538$). Susceptibility to metronidazole and tinidazole are defined as MLC ≤ 25 µg/mL, low-level resistance as MLC 50–100 µg/mL, moderate-level resistance as MLC 200 µg/mL, and high-level resistance as MLC ≥ 400 µg/mL [39]

clinically resistant strains and demonstrated decreased levels of ferrodoxin and its mRNA. This was attributed to reduced transcription of the ferrodoxin gene as determined by nuclear run-on assays [42]. Cerkasovova et al. (1988) noted that strains of *Tritrichomonas foetus*, a bovine reproductive system parasite in the order Trichomonadida, that are highly resistant to MTZ lack detectable enzymatic activity for pyruvate:ferrodoxin oxidoredutase and hydrogenase [43]. The molecular basis for these altered enzyme activities has not been established.

In 2006, Xio et al. [44] proposed that infection of Trichomonads by *Mycoplasma hominis*, a bacterial pathogen commonly found in the lower genital tract, may be the cause of T. *vaginalis* resistance. They found that Trichomonads infected with *M. hominis* demonstrated a tenfold higher minimal lethal concentration (MLC) of metronidazole. A 2010 study by the Centers for Disease Control and Prevention failed to confirm any association between *M. hominis*-infected strains and the presence of metronidazole resistance [45].

In 2012, Leitsch et al. [46] reported downregulated or absent flavin reductase activity and downregulated alcohol dehydrogenase 1 activity in metronidazole-resistant strains of Trichomonas. The former could affect oxygen scavenging and the latter detoxification of intracellular acetaldehyde. No conclusions can be made from these data regarding cause and effect.

3.4 Clostridium spp.

Clostridium species are usually sensitive to metronidazole but *Clostridium ramnosum* may require higher concentrations for inhibition [47, 48].

Though susceptibility testing is not routinely done, it is generally accepted that *Clostridium difficile* is predictably susceptible to metronidazole. However, Palaez et al. reported on the first series of resistant strains in 1994 [49] and subsequently described the resistance trends of the isolates from their institution in Madrid from 1993 to 2000 [50]. The overall rate of resistance was 6.3 %. The rate was highest in patients with HIV. Molecular typing revealed the absence of clonality among the isolates. In 2013, Lynch et al. sequenced the genome and examined the phenotype of a stably resistant isolate of *C. difficile*. Compared to metronidazole-susceptible strains, genomic analysis of the resistant isolate revealed single nucleotide polymorphism (SNP) level variation in genes related to electron transport, iron utilization, and energy production. Four phenotypic characteristics were apparent: (1) aberrant growth in liquid media; (2) attenuated cell wall separation; (3) lack of spore production by 48 h; and (4) heteroresistance [51].

Santengelo et al. (1991) developed E. coli F19 recA, a nitrate reductase-deficient mutant that was rendered MTZ-S by isolating and expressing *Clostridium acetobutylicum* genes on recombinant plasmids. Further tests on these isolates revealed that flavodoxin and hydrogenase genes were responsible for the electron transfer system, suggesting its possible role in metronidazole resistance [52]. Church et al. (1990, 1988) provided biochemical evidence that hydrogenase 1 of *Clostridium pasteuranicum* plays a critical enzymatic role in the reduction of metronidazole via a ferrodoxin-linked mechanism [53, 54].

3.5 Entamoeba and Giardia

In vivo and in vitro assays have demonstrated the existence of metronidazole-resistant Giardia isolates, and metronidazole treatment failures occur with a frequency of 10–20 % [55–59].

There is no reported clinical resistance in Entamoeba, but resistant strains have been generated in vitro in various laboratories. Purified PFOR and ferrodoxin have been shown to activate MTZ in vitro. Upcroft et al. characterized biochemical markers in a clinically resistant isolate and showed that PFOR is downregulated up to fivefold. Ferrodoxin 1, which is the next electron acceptor in the transport chain, is also downregulated about seven times [60]. Increased efflux of the drug also might be responsible for protecting the parasite.

Entamoeba produces SOD, catalase, and peroxidase for detoxification of oxygen and its breakdown products. Only one 2-oxoacid oxidoreductase, PFOR, has been detected in Entamoeba and it is predominantly membrane bound. Upcroft et al. showed marked increase in superoxide dismutase activity in MTZ-resistant E. histolytica while PFOR activity remained constant [60]. Wassmann et al. (1999) confirmed lack of change in PFOR activity in resistant strains. They also showed increased expression of iron containing FE-SOD and peroxiredoxin while expression of flavin reductase and ferrodoxin1 was decreased [61].

4 Cross-Resistance

There is documented cross-resistance between all the currently used 5-nitroimidazole drugs [60, 62].

5 Mechanism of Spread of Resistance

Although both plasmid-mediated and chromosomally mediated resistance has been described, the transfer to metronidazole-sensitive Bacteroides species does not yet appear to be a problem. Also, a combination of several mechanisms may be required for emergence of high-level resistance in various organisms that might lead to therapeutic failures.

6 Resistance Testing

There are many barriers that hinder local laboratories performing routine susceptibility testing on all clinical isolates of anaerobic bacteria. A significant amount of equipment and labor must be dedicated to the task, yielding what some consider relatively predictable results [63]. Recent consensus guidelines recommend against anaerobic culture of intra-abdominal pathogens, incorporating assumptions about anaerobic susceptibility [64]. The 2012 Clinical and Laboratory Standards Institute (CLSI) guidelines suggested anaerobic susceptibility testing only for specific settings, like critical illness, known resistance, persistent infection despite appropriate antibiotics, or to confirm antimicrobial activity when long courses of antibiotics are indicated [65]. For those instances in which resistance testing is warranted, not all hospital laboratories are equipped to perform susceptibility testing, with 15 % of surveyed hospitals sending specimens to a reference lab for testing [66]. Although susceptibility testing may be limited, 89 % of hospital laboratories and 100 % of reference laboratories report testing susceptibility to metronidazole.

There are various laboratory methods that can be employed for testing metronidazole susceptibility in anaerobic bacteria [67]. Agar dilution is considered the gold standard; however, it requires significant labor and expertise in interpretation [65]. It is very time consuming and is not efficient for testing single or small numbers of organisms. Broth microdilution may be performed more simply using commercially available panels, testing many antimicrobials at the same time; however, it is ineffective for poorly growing oxygen-sensitive organisms and CLSI only has suggested breakpoints for *B. fragilis* [68]. MIC gradient diffusion tests are also commercially available, yielding a precise MIC value at a relatively low cost. However, this test can overestimate metronidazole resistance if strict anaerobic conditions are not upheld throughout the testing process [69]. Disk diffusion tests are not considered accurate, as the results do not correlate well with agar dilution methods [68]. Anaerobic resistance testing in the clinical environment can be challenging, and epidemiologic data may be the primary resource in assessing resistance patterns and empiric therapy choices.

7 Alternative Agents

7.1 Helicobacter Pylori

Virtually all *H. pylori* isolates are susceptible in vitro to a variety of antimicrobial agents, including bismuth salts, amoxicillin, macrolides, nitrofurans, tetracyclines, and aminoglycosides. Combination therapy with a bismuth salt and two antibiotics has been widely used. After treatment failure, a second course of triple therapy may still be effective; alternatively, a regimen not including imidazoles may be used.

7.2 Trichomonas Vaginalis

If infection persists in a patient treated with a 7-day regimen and reinfection can be ruled out, other options include treating with 2 g of metronidazole orally daily for 3–5 days, 1–2 g of metronidazole daily for 14 days along with 500 mg intravaginally daily, high-dose intravenous metronidazole, [70], intravaginal paromomycin [71, 72] and tinidazole, which has recently been approved by the FDA. Tinidazole has been shown to be effective in some cases of metronidazole-resistant *T. vaginalis* infection [73]. Cromwell et al. found that although in vitro activities of metronidazole and tinidazole against the parasite are highly correlated, the tinidazole does have lower minimum inhibitory concentrations (MICs) than metronidazole [62].

7.3 Giardia

Alternatives to metronidazole for treatment of Giardia include paramomycin, nitazoxanide, and the anti-helminthic benzmidazoles (albendazole and mebendazole) [74].

7.4 Clostridium spp.

Two agents, vancomycin and fidaxomicin, are FDA approved for the treatment of *Clostridium difficile*. Despite successful cure in the majority of cases, recurrences are extremely common.

Other Clostridium species are typically susceptible to clindamycin. Other active agents include penicillin, tetracycline, erythromycin, chloramphenicol, rifampin, and some cephalosporins. *C. tertium* is susceptible to vancomycin.

References

1. Ingham HR, Eaton S, Venables CW, Adams PC. Bacteroides fragilis resistant to metronidazole after long-term therapy. Lancet. 1978;1(8057):214.
2. Elsaghier AA, Brazier JS, James EA. Bacteraemia due to Bacteroides fragilis with reduced susceptibility to metronidazole. J Antimicrob Chemother. 2003;51(6):1436–7.
3. Brazier JS, Stubbs SL, Duerden BI. Metronidazole resistance among clinical isolates belonging to the Bacteroides fragilis group: time to be concerned? J Antimicrob Chemother. 1999;44(4):580–1.

4. Marina M, Ivanova M, Kantardjiev T. Antimicrobial susceptibility of anaerobic bacteria in Bulgaria. Anaerobe. 2009;15:127–32.
5. Jamal W, Shain M, Rotimi VO. Surveillance trends of antimicrobial resistance among clinical isolates of anaerobes in Kuwait hospitals from 2002 to 2007. Anaerobe. 2010;16:1–5.
6. Toprak NU, Uzunkaya OD, Soki J, Soyletir G. Susceptibility profiles and resistance genes for carbapeems (*cfiA*) and metronidazole (*nim*) among *Bacteroides* species in a Turkish University Hospital. Anaerobe. 2012;18:169–71.
7. Takesue Y, Wanaabe A, Hanaki H, Kusachi S, Matsumoto T, Iwamoto A, et al. Nationwide surveillance of antimicrobial susceptibility patterns of pathogens isolated from surgical site infections in Japan. J Infect Chemother. 2012;18:816–26.
8. Karlowsky JA, Walkty AJ, Adam HJ, Baxter MR, Hoban DJ, Zhanel GG. Prevalence of antimicrobial resistance among clinical isolates of Bacteroides fragilis group in Canada in 2010–2011: CANWARD Surveillance Study. Antimicrob Agents Chemother. 2012;56(3):1247–52.
9. Nanako V, Silva AN, Merino VRC, Wexler HM, Avila-Campos MJ. Antimicrobial resistance and prevalence of resistance genes in intestinal *Bacteroidales* strains. Clinics. 2011;66(4):543–7.
10. Trevino M, Areses P, Penalver MD, Cortizo S, Pardo F, del Molino MLP, et al. Susceptibility trends of *Bacteroides fragilis* group and characterization of carbapenemase-producing strains by automated REP-PCR and MALDI-TOF. Anaerobe. 2012;18:34–43.
11. Schaumann R, Funke M, Janssen E, Rodloff AC. *In vitro* activities of clindamycin, imipenem, metronidazole, and piperacillin-tazobactam against susceptible and resistant isolates of *Bacteroides fragilis* evaluated by kill kinetics. Antimicrob Agents Chemother. 2012;56(6):3414–6.
12. Falagas ME, Siakavellas E. Bacteroides, Prevotella, and Porphyromonas species: a review of antibiotic resistance and therapeutic options. Int J Antimicrob Agents. 2000;15(1):1–9.
13. Breuil J, Dublanchet A, Truffaut N, Sebald M. Transferable 5-nitroimidazole resistance in the Bacteroides fragilis group. Plasmid. 1989;21(2):151–4.
14. Reysset G, Haggoud A, Sebald M. Genetics of resistance of Bacteroides species to 5-nitroimidazole. Clin Infect Dis. 1993;16 Suppl 4:S401–3.
15. Husain F, Veerangouda Y, His J, Meggersee R, Abratt V, Wexler HM. Two multidrug-resistant clinical isolates of *Bacteroides fragilis* carry a novel metronidazole resistance *nim* gene (*nimJ*). Antimicrob Agents Chemother. 2013;57(8):3767–74.
16. Gal M, Brazier JS. Metronidazole resistance in Bacteroides spp. carrying nim genes and the selection of slow-growing metronidazole-resistant mutants. J Antimicrob Chemother. 2004; 54(1):109–16.
17. Dubreuil L, Odou MF. Anaerobic bacteria and antibiotics: what kind of unexpected resistance could I find in my laboratory tomorrow? Anaerobe. 2010;16:555–9.
18. Steffens LS, Nicholson S, Paul LV, Nord CE, Patrick S, Abratt VB. *Bacteroides fragilis RecA* protein overexpression causes resistance to metronidazole. Res Microbiol. 2010;161:346–54.
19. Diniz CG, Farias LM, Carvalho MA, Rocha ER, Smith CJ. Differential gene expression in a Bacteroides fragilis metronidazole-resistant mutant. J Antimicrob Chemother. 2004;54(1):100–8.
20. Boyanova L, Mitov I. Geographic map and evolution of primary *Helicobacter pylori* resistance to antibacterial agents. Expert Rev Anti Infect Ther. 2010;8(1):59.
21. Xia HX, Daw MA, Beattie S, Keane CT, O'Morain CA. Prevalence of metronidazole-resistant Helicobacter pylori in dyspeptic patients. Ir J Med Sci. 1993;162(3):91–4.
22. Megraud F. Resistance of Helicobacter pylori to antibiotics. Aliment Pharmacol Ther. 1997;11 Suppl 1:43–53.
23. European Study Group on Antibiotic Susceptibility of Helicobacter pylori. Results of a multicentre European survey in 1991 of metronidazole resistance in Helicobacter pylori. Eur J Clin Microbiol Infect Dis. 1992;11(9):777–81.
24. Thompson SA, Blaser MJ. Isolation of the Helicobacter pylori recA gene and involvement of the recA region in resistance to low pH. Infect Immun. 1995;63(6):2185–93.
25. Smith MA, Edwards DI. Oxygen scavenging, NADH oxidase and metronidazole resistance in Helicobacter pylori. J Antimicrob Chemother. 1997;39(3):347–53.
26. Goodwin A, Kersulyte D, Sisson G, Veldhuyzen van Zanten SJ, Berg DE, Hoffman PS. Metronidazole resistance in Helicobacter pylori is due to null mutations in a gene (rdxA) that encodes an oxygen-insensitive NADPH nitroreductase. Mol Microbiol. 1998; 28(2):383–93.
27. Kwon DH, Kato M, El-Zaatari FA, Osato MS, Graham DY. Frame-shift mutations in NAD(P)H flavin oxidoreductase encoding gene (frxA) from metronidazole resistant Helicobacter pylori ATCC43504 and its involvement in metronidazole resistance. FEMS Microbiol Lett. 2000;188(2):197–202.
28. Kwon DH, El-Zaatari FA, Kato M, et al. Analysis of rdxA and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of Helicobacter pylori. Antimicrob Agents Chemother. 2000;44(8):2133–42.
29. Jeong JY, Mukhopadhyay AK, Akada JK, Dailidiene D, Hoffman PS, Berg DE. Roles of FrxA and RdxA nitroreductases of Helicobacter pylori in susceptibility and resistance to metronidazole. J Bacteriol. 2001;183(17):5155–62.
30. Yang YJ, Wu JJ, Sheu BS, Kao AW, Huang AH. The rdxA gene plays a more major role than frxA gene mutation in high-level metronidazole resistance of Helicobacter pylori in Taiwan. Helicobacter. 2004;9(5):400–7.
31. Khan A, Farooqui A, Manzoor H, Akhtar SS, Quraishy MS, Kazmi SU. Antibiotic resistance and *cagA* gene correlation: a looming crisis of *Helicobacter pylori*. World J Gastroenterol. 2012;18(18): 2245–52.
32. Vilaichone RK, Mahachai V, Tumwasorn S, Kachintorn U. J Gastroenterol Hepatol. 2011;16 Suppl 3:46–8.
33. Choi SS, Chivers PT, Berg DE. Point mutations in *Helicobacter pylori*'s *fur* regulatory gene that alter resistance to metronidazole, a prodrug activated by chemical reduction. PLoS One. 2011;6(3): e18236.
34. Robinson S. Trichomonal vaginitis resistant to metronidazole. Can Med Assoc J. 1962;86:665.
35. Watt L, Jennison RF. Clinical evaluation of metronidazole. A new systemic trichomonacide. Br Med J. 1960;2(5203):902–5.
36. Müller M, Lossick JG, Gorrell TE. In vitro susceptibility of trichomonas vaginalis to metronidazole and treatment outcome in vaginal trichomoniasis. Sex Transm Dis. 1988;15(1):17–24.
37. Sobel JD, Nagappan V, Nyirjesy P. Metronidazole-resistant vaginal trichomoniasis—an emerging problem. N Engl J Med. 1999;341(4): 292–3.
38. Schwebke JR, Barrientes FJ. Prevalence of trichomonas vaginalis isolates with resistance to metronidazole and tinidazole. Antimicrob Agents Chemother. 2006;50(12):4209–10.
39. Kirkcaldy RD, Augostini P, Asbel LE, Bernstein KT, Kerani RP, Mettenbrink CJ, Pathela P, Schwebke JR, Secor WE, Workowski KA. Trichomonas vaginalis antimicrobial drug resistance in 6 US cities, STD surveillance network, 2009–2010. Emerg Infect Dis. 2012;18(6):939–43.
40. Yarlett N, Yarlett NC, Lloyd D. Metronidazole-resistant clinical isolates of Trichomonas vaginalis have lowered oxygen affinities. Mol Biochem Parasitol. 1986;19(2):111–6.
41. Yarlett N, Yarlett NC, Lloyd D. Ferredoxin-dependent reduction of nitroimidazole derivatives in drug-resistant and susceptible strains of Trichomonas vaginalis. Biochem Pharmacol. 1986;35(10): 1703–8.

42. Quon DV, d'Oliveira CE, Johnson PJ. Reduced transcription of the ferredoxin gene in metronidazole-resistant Trichomonas vaginalis. Proc Natl Acad Sci U S A. 1992;89(10):4402–6.
43. Cerkasovova A, Cerkasov J, Kulda J. Metabolic differences between metronidazole resistant and susceptible strains of Tritrichomonas foetus. Mol Biochem Parasitol. 1984;11:105–18.
44. Xiao J, Xie L, Fang S, Gao M, Zhu Y, Song L, Zhong H, Lun Z. Symbiosis of mycoplasma hominis in trichomonas vaginalis may link metronidazole resistance in vitro. Parasitol Res. 2006;100(1): 123–30.
45. Butler SE, Augostini P, Secor WE. Mycoplasma hominis infection of trichomonas vaginalis is not associated with metronidazole-resistant trichomoniasis in clinical isolates from the united states. Parasitol Res. 2010;107(4):1023–7.
46. Leitsch D, Drinić M, Kolarich D, Duchêne M. Down-regulation of flavin reductase and alcohol dehydrogenase-1 (ADH1) in metronidazole-resistant isolates of trichomonas vaginalis. Mol Biochem Parasitol. 2012;183(2):177–83.
47. Alexander CJ, Citron DM, Brazier JS, Goldstein EJ. Identification and antimicrobial resistance patterns of clinical isolates of Clostridium clostridioforme, Clostridium innocuum, and Clostridium ramosum compared with those of clinical isolates of Clostridium perfringens. J Clin Microbiol. 1995;33(12):3209–15.
48. Brazier JS, Levett PN, Stannard AJ, Phillips KD, Willis AT. Antibiotic susceptibility of clinical isolates of clostridia. J Antimicrob Chemother. 1985;15(2):181–5.
49. Peláez T, Sánchez R, Blázquez R, Catalán P, Muñoz P, Bouza E. 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, abstr. E-34. 1994; p. 50.
50. Peláez T, Alcala L, Alonso R, Rodriguez-Creixems M, Garcia-Lechuz JM, Bouza E. Reassessment of clostridium difficile susceptibility to metronidazole and vancomycin. Antimicrob Agents Chemother. 2002;46(6):1647–50.
51. Lynch T, Chong P, Zhang J, Hizon R, Du T, Graham MR, Beniac DR, Booth TF, Kibsey P, Miller M, Gravel D, Mulvey MR, Canadian Nosocomial Infection Surveillance Program (CNISP). Characterization of a stable, metronidazole-resistant clostridium difficile clinical isolate. PLoS One. 2013;8(1):e53757.
52. Santangelo JD, Jones DT, Woods DR. Metronidazole activation and isolation of Clostridium acetobutylicum electron transport genes. J Bacteriol. 1991;173(3):1088–95.
53. Church DL, Rabin HR, Laishley EJ. Reduction of 2-, 4- and 5-nitroimidazole drugs by hydrogenase 1 in Clostridium pasteurianum. J Antimicrob Chemother. 1990;25(1):15–23.
54. Church DL, Rabin HR, Laishley EJ. Role of hydrogenase 1 of Clostridium pasteurianum in the reduction of metronidazole. Biochem Pharmacol. 1988;37(8):1525–34.
55. Upcroft J, Upcroft P. Drug resistance and giardia. Parasitol Today. 1993;9(5):187–90.
56. Müller J, Ley S, Felger I, Hemphill A, Muller N. Identification of differentially expressed genes in a giardia lamblia WB C6 clone resistant to nitazoxanide and metronidazole. J Antimicrob Chemother. 2008;62(1):72–82.
57. Lemee V, Zaharia I, Nevez G, Rabodonirina M, Brasseur P, Ballet JJ, Favennec L. Metronidazole and albendazole susceptibility of 11 clinical isolates of giardia duodenalis from france. J Antimicrob Chemother. 2000;46(5):819–21.
58. Upcroft JA, Upcroft P. Drug susceptibility testing of anaerobic protozoa. Antimicrob Agents Chemother. 2001;45(6):1810–4.
59. Tejman-Yarden N, Millman M, Lauwaet T, Davids BJ, Gillin FD, Dunn L, Upcroft JA, Miyamoto Y, Eckmann L. Impaired parasite attachment as fitness cost of metronidazole resistance in giardia lamblia. Antimicrob Agents Chemother. 2011;55(10):4643–51.
60. Upcroft P, Upcroft JA. Drug targets and mechanisms of resistance in the anaerobic protozoa. Clin Microbiol Rev. 2001;14(1): 150–64.
61. Wassmann C, Hellberg A, Tannich E, Bruchhaus I. Metronidazole resistance in the protozoan parasite Entamoeba histolytica is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. J Biol Chem. 1999;274(37): 26051–6.
62. Crowell AL, Sanders-Lewis KA, Secor WE. In vitro metronidazole and tinidazole activities against metronidazole-resistant strains of Trichomonas vaginalis. Antimicrob Agents Chemother. 2003;47(4): 1407–9.
63. Strobel JH. Basic laboratory culture methods for anaerobic bacteria. Methods Mol Biol. 2009;581:247–61.
64. Somolkin JS, Mazuski JE, Bradley JS, Rodvold KA, Goldstein EJC, Baron EJ, et al. Diagnosis and management of intra-abdominal infection in adults and children: guidelines by the Surgical Infection Society and the Infectious Diseases Society of America. Clin Infect Dis. 2010;50:133–64.
65. CLSI. Methods for antimicrobial susceptibility testing of anaerobic bacteria; Approved standard—8th edn. CLSI document M11-A8. Wayne: Clinical and Laboratory Standards Institute; 2012.
66. Goldstein EJC, Citron DM, Goldman PJ, Goldman RJ. National hospital survey of anaerobic culture and susceptibility methods: III. Anaerobe. 2008;14(2):68–72.
67. Schuetz AN. Antimicrobial resistance and susceptibility testing of anaerobic bacteria. Clin Infect Dis. 2014;59(5):698–705.
68. CLSI. Performance standards for antimicrobial susceptibility testing; 23rd informational supplement. CLSI document M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
69. Cormican MG, Erwin ME, Jones RN. False resistance to metronidazole by E-test among anaerobic bacteria investigations of contributing test conditions and medium quality. Diagn Microbiol Infect Dis. 1996;24:117–9.
70. Lossick JG, Kent HL. Trichomoniasis: trends in diagnosis and management. Am J Obstet Gynecol. 1991;165(4 Pt 2):1217–22.
71. Nyirjesy P, Sobel JD, Weitz MV, Leaman DJ, Gelone SP. Difficult-to-treat trichomoniasis: results with paromomycin cream. Clin Infect Dis. 1998;26(4):986–8.
72. Coelho DD. Metronidazole resistant trichomoniasis successfully treated with paromomycin. Genitourin Med. 1997;73(5):397–8.
73. Sobel JD, Nyirjesy P, Brown W. Tinidazole therapy for metronidazole-resistant vaginal trichomoniasis. Clin Infect Dis. 2001;33(8):1341–6.
74. Stover KR, Riche DM, Gandy CL, Henderson H. What would we do without metronidazole? Am J Med Sci. 2012;343(4):316–9.

Glycopeptide-Resistance in Enterococci

20

Florence Depardieu and Patrice M. Courvalin

1 Introduction

Glycopeptides, such as vancomycin and teicoplanin, act by blocking cell wall formation. Resistance to this class of antibiotics, detected first in 1986, is due to synthesis of altered peptidoglycan precursor ending in D-alanine-D-lactate or D-alanine-D-serine in place of D-alanine-D-alanine and by the removal of precursors terminating in D-alanine. Resistance can be acquired or intrinsic and strains may be resistant to vancomycin and teicoplanin, or to vancomycin only. Nine types of glycopeptide resistance, forming the *van* alphabet, and their biochemical mechanisms have been described. Furthermore, strains that are dependent on vancomycin for growth have been isolated from clinical samples. Data suggest that resistance could originate in glycopeptide-producing organisms or in soil organisms for VanA type and in anaerobes for VanB type. Since the years 2000, resistance to glycopeptide has disseminated from enterococci to *Staphylococcus aureus* clinical isolates.

2 Enterococci

Enterococci are part of the normal intestinal flora of humans and various animals. They are found in the feces of a high proportion of healthy adults. Enterococci are able to grow in variable environmental conditions at temperatures from 10 to 45 °C, in hypotonic, hypertonic, acidic, or alkaline media, under anaerobic or aerobic conditions. *Enterococcus faecalis* and *Enterococcus faecium* are the two major species of enterococci and represent more than 95 % of clinical isolates [1–4]. *E. faecalis* was more prevalent than *E. faecium* and accounted for 57–77 % of clinical isolates [5]. Currently, *E. faecium* is much more frequently resistant to vancomycin and ampicillin and becomes almost as common as a cause of nosocomial infections as *E. faecalis* [1, 6]. Enterococci are opportunistic pathogens and can be responsible for endocarditis and urinary tract infections, as well as intra-abdominal and pelvic sepsis and surgical wound infections [1]. They present intrinsic resistance to low concentrations of aminoglycosides, clindamycin, and trimethoprim–sulfamethoxazole [3] and to β-lactams due to the low affinity of several penicillin-binding proteins (PBP) for the penicillins [7–9]. Furthermore, they easily become resistant to other antibiotics by mutation or acquisition of foreign genetic material carried by conjugative transposons and pheromone-response or broad-host-range plasmids. Resistance to higher levels of penicillins, by overproduction or alteration of PBP5 [7, 10] or rarely by synthesis of a β-lactamase [11], and to aminoglycosides, chloramphenicol, macrolides-lincosamides-streptogramins, tetracyclines, fluoroquinolones, rifampin, and to the glycopeptides, has been described [12, 13]. Thus, treatment of enterococcal infections is often difficult.

3 Glycopeptides

Glycopeptide antibiotics, such as vancomycin and teicoplanin, are active against many important Gram-positive pathogens. Vancomycin, produced by *Amycolatopsis orientalis*, was the first glycopeptide used in the treatment of serious infections due to Gram-positive bacteria. The structure of glycopeptides is based on a heptapeptide domain in which five amino acid residues are common to all glycopeptides [14, 15]. The biologically active part of the molecule is located in the structure containing seven amino acid residues [15]. Glycopeptides act by inhibiting cell wall formation (Fig. 20.1). They bind with high affinity by five hydrogen bonds to the D-alanyl-D-alanine (D-Ala-D-Ala) C-terminal of precursors containing the pentapeptide moiety (Fig. 20.2a) synthesized by the D-Ala:D-Ala ligase (Ddl). They thus inhibit addition by transglycosylation of the pentapeptide

F. Depardieu, Ph.D. • P.M. Courvalin, M.D., F.R.C.P. (✉)
Unité des Agents Antibactériens, Institut Pasteur,
25, rue du Docteur Roux, Paris 75724 Cedex 15, France
e-mail: pcourval@pasteur.fr

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_20

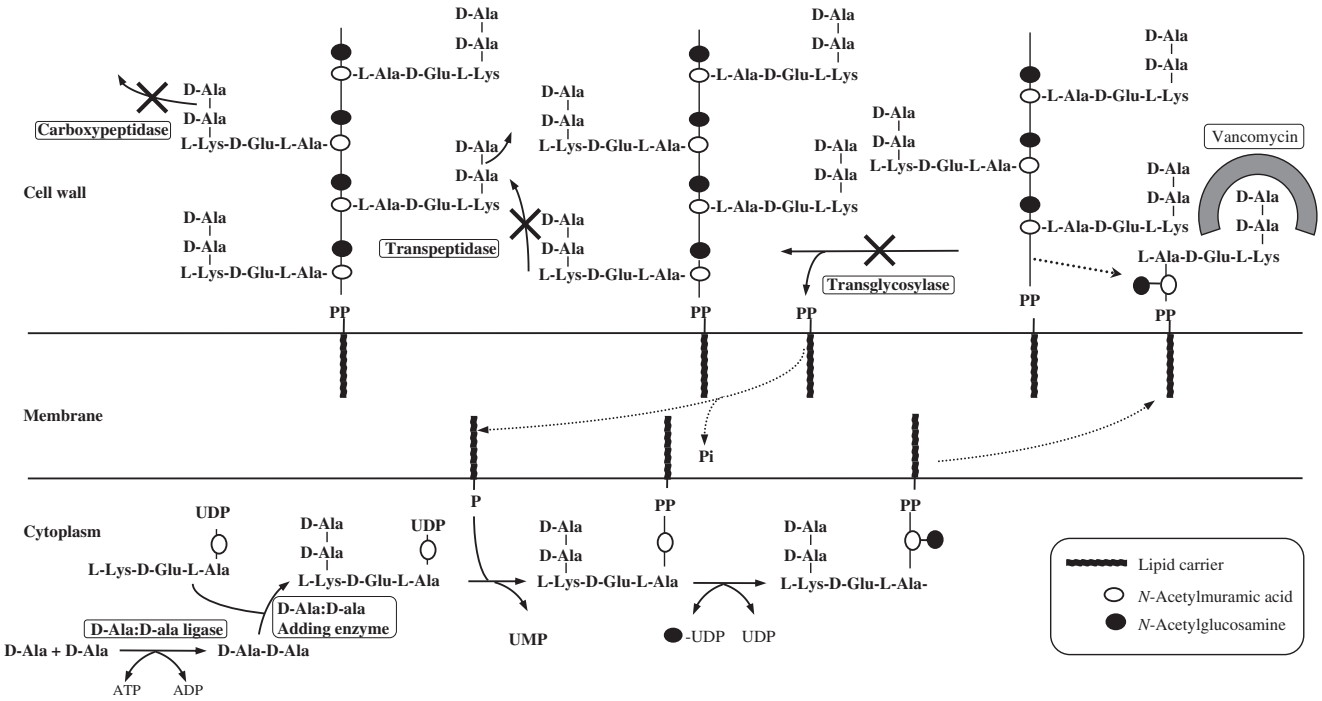


Fig. 20.1 Schematic representation of the mode of action of vancomycin on peptidoglycan biosynthesis (from [37]). Binding of vancomycin on the C-terminal D-Ala-D-Ala prevents transglycosylation, transpeptidation, and carboxypeptidation steps

precursors to the nascent peptidoglycan chain and prevent subsequent cross-linking catalyzed by the D,D-transpeptidases [14]. These reactions occur outside the cytoplasmic membrane [16]. The drugs do not penetrate into the cytoplasm and interaction with the target can only take place after translocation of the precursors to the outer surface of the membrane [14, 17]. Gram-negative bacteria are insensitive to this group of antibiotics due to the presence of the outer membrane which is impermeable to glycopeptides.

4 Glycopeptide Resistance in Enterococci

The first enterococcal isolates resistant to high levels of vancomycin and teicoplanin were reported in 1988 [18, 19]. The number of infections with vancomycin-resistant enterococci in US hospitals increased from 9820 in 2000 to 21,352 in 2006 [20]. In the United States, the percentage of *E. faecium* isolates that were resistant to vancomycin rose from 0 % before the mid-1980s to more than 80 % by 2007 [21]; in contrast, only 5 % of *E. faecalis* are vancomycin resistant [6]. In 2007, a significant increase in rates of bacteraemia due to vancomycin-resistant enterococci was observed in Canada with 63 % of clinical isolates identified as *E. faecium* [22]. Although a decrease in the prevalence of vancomycin-resistant enterococci in animals in Europe was initially observed, after the ban of avoparcin, a glycopeptide used as a food additive which displays cross-resistance with vancomycin, there has been an increase in nosocomial ampicillin and/or vancomycin-resistant enterococcal infections over the past decade [23–27].

Glycopeptide resistance is due to the replacement of the normal peptidoglycan precursor by modified precursors ending in D-Ala-D-lactate (D-Ala-D-Lac) or D-Ala-D-serine (D-Ala-D-Ser) in place of D-Ala-D-Ala. This alteration is responsible for diminished binding affinity of glycopeptides for their target. In the case of precursors ending in D-Ala-D-Lac, the affinity is 1000-fold lower because the substitution eliminates a critical central hydrogen bond (Fig. 20.2b) [28]. The replacement of D-Ala by D-Ser should not affect the number of hydrogen bonds that can be formed between vancomycin and the altered precursors but the binding affinity is altered (sevenfold lower) [29], probably due to conformational changes (Fig. 20.2c). In addition to production of modified peptidoglycan precursors, resistant strains are also able to eliminate the precursors normally synthesized by the host. Combination of these two pathways, synthesis of modified precursors and elimination of classical precursors, leads to resistance. Therefore, resistance to glycopeptides is a complex system involving several genes.

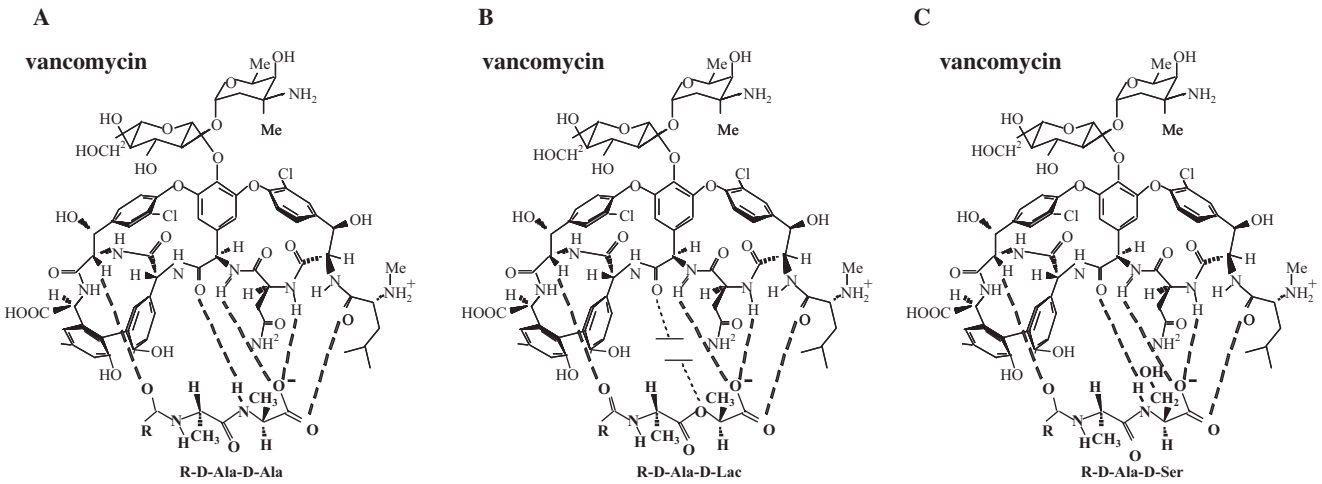


Fig. 20.2 Interactions between vancomycin and (**A**) N-Acetyl-D-Ala-D-Ala, (**B**) N-Acetyl D-Ala-D-Lac, (**C**) N-Acetyl-D-Ala-D-Ser. Hydrogen bonds are indicated by dotted lines. With the D-Ala-D-Lac depsipeptide, a central hydrogen bond is missing due to substitution of a NH group by an oxygen and repulsion between the two oxygens and 3-D alteration of the target; with the D-Ala-D-Ser pentapeptide, replacement of a CH_3 group by a CH_2OH group is responsible for conformational changes

Table 20.1 Glycopeptide resistance in enterococci

Resistance	Acquired					Intrinsic
Type	VanA	VanB	VanD	VanE	VanG	VanC
MIC (mg/L)						
Vancomycin	64–1000	4–1000	64–128	8–32	8–16	2–32
Teicoplanin	16–512	0.5–1	4–64	0.5	0.5	0.5–1
Expression	Inducible		Constitutive	Inducible	Inducible	Constitutive
						Inducible
Location	Plasmid		Chromosome	Chromosome	Chromosome	Chromosome
	Chromosome					
Modified target	D-Ala-D-Lac			D-Ala-D-Ser		

5 The *van* Alphabet

Nine types of glycopeptide resistance have been described to date in enterococci: eight are acquired (VanA, B, D, E, G, L, M, and N) [30–34] and one, VanC, is an intrinsic property of *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens* [35, 36]. The MIC ranges of vancomycin and teicoplanin against the various types overlap (Table 20.1) and classification of glycopeptide resistance is based on the primary sequence of the structural gene for the resistance ligase.

5.1 Glycopeptide Resistance due to Synthesis of Modified Peptidoglycan Precursors Ending in D-Ala-D-Lac

5.1.1 VanA

This is the most frequently encountered type of glycopeptide resistance in enterococci. VanA-type strains are characterized by a high level of resistance to both vancomycin and teicoplanin due to synthesis of modified peptidoglycan precursors ending in D-Ala-D-Lac (Table 20.1). The *vanA* gene cluster, generally located on transposon Tn*1546* [37] or related elements [38], can be found on, both, transferable

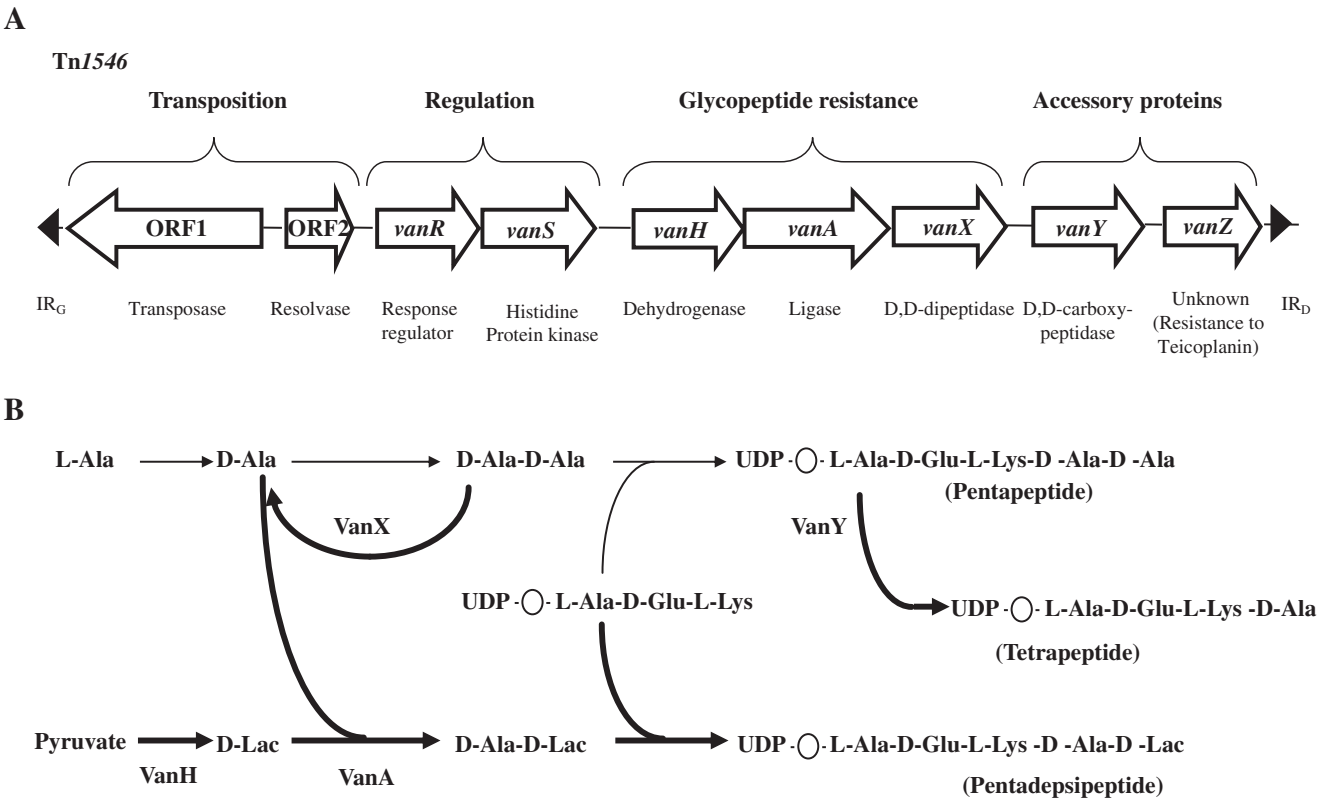


Fig. 20.3 (**A**) Organization of Tn*1546*. IR_G and IR_D indicate inverted repeat sequences at the ends of the transposon. (**B**) Schematic representation of the synthesis of peptidoglycan precursors in a VanA-type-resistant strain after induction with glycopeptides. Ddl, D-Ala:D-Ala ligase. ○, *N*-acetylmuramic acid

and nontransferable plasmids as well as part of the bacterial chromosome (Table 20.1). It has been reported mainly in *E. faecium* and *E. faecalis* but also in *E. avium* [39], *E. durans* [40–42], *E. gallinarum*, and *E. casseliflavus* [43], and in *Bacillus circulans* [44]. Since the years 2000, the *vanA* gene cluster was found in 11 isolates of *Staphylococcus aureus* [45–48].

Tn*1546*, originally detected on plasmid pIP816 from *E. faecium* BM4147, is composed of nine genes that can be assigned to different functional groups: two encode a transposase and a resolvase (ORF1 and ORF2) responsible for the movements of the element and the remaining seven genes are involved in regulation (*vanRS*) and expression of glycopeptide resistance (*vanHAXYZ*) (Fig. 20.3a). The *vanH*, *vanA*, and *vanX* genes code for proteins that are necessary for expression of resistance (Fig. 20.3b). VanH is a dehydrogenase that converts pyruvate to D-Lac [49], VanA a ligase that uses D-Lac and a D-Ala residue to synthesize the depsipeptide D-Ala-D-Lac which is incorporated into the peptidoglycan precursors in place of D-Ala-D-Ala [28], and VanX is a D,D-dipeptidase that hydrolyses the dipeptide D-Ala-D-Ala formed by the endogenous chromosomal D-Ala:D-Ala ligase (Ddl) [50, 51], thus reducing the level of normal peptidoglycan precursors ending in D-Ala-D-Ala. The penicillin-insensitive D,D-carboxypeptidase VanY, not essential for resistance, cleaves the D-Ala C-terminal residue of the pentapeptide precursors synthesized from the D-Ala-D-Ala dipeptide that has escaped VanX hydrolysis [52, 53]. Vancomycin has no affinity for the resulting tetrapeptide precursors. VanZ alone confers low-level resistance to teicoplanin by an unknown mechanism [54] that does not involve incorporation of a substituent of D-Ala-D-Ala into peptidoglycan precursors.

Expression of glycopeptide resistance is regulated by two genes, *vanR* and *vanS*, for a two-component regulatory system located upstream from *vanH* [32, 55]. VanS is a membrane-associated sensor that contains, in the C-terminal cytoplasmic domain, a histidine residue which is phosphorylated in response to the presence of glycopeptides in the medium [56]. VanR acts as a transcriptional activator that can be phosphorylated on an aspartate residue by acquisition of the phosphoryl group of the activated VanS [56]. In summary, in the presence of glycopeptides in the culture medium, a signal leads to autophosphorylation of VanS on a specific histidine residue and the phosphoryl group is then transferred to a specific aspartate residue of VanR (Fig. 20.4). In the absence of glycopeptides, VanS stimulates dephosphorylation of VanR leading to a negative regulation of resistance genes and thus preventing accumulation of VanR

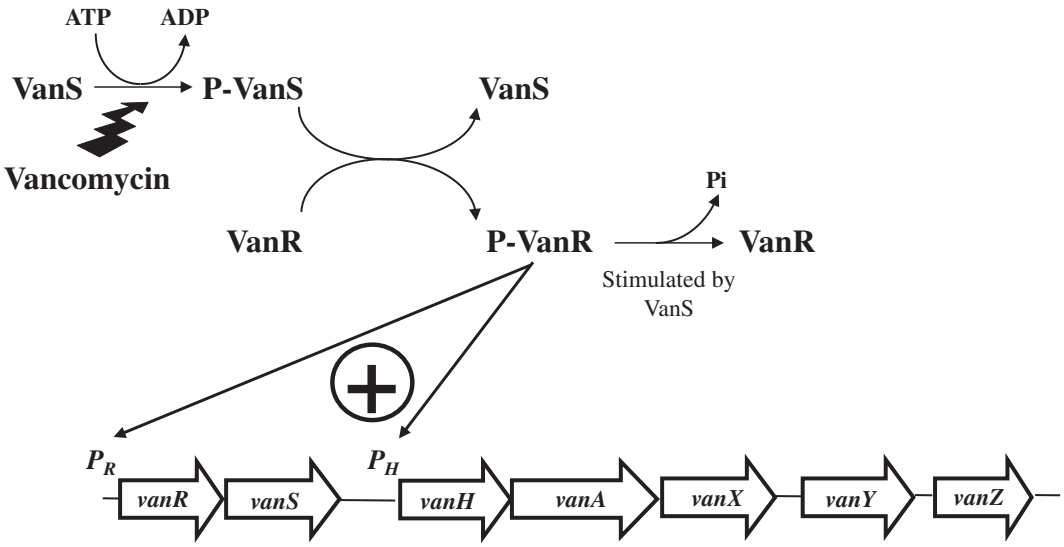


Fig. 20.4 Schematic representation of activation of the *PR* and *PH* promoters of the *vanA* operon by phospho-VanR after induction with vancomycin

phosphorylated by acetyl phosphate or by a kinase encoded by the host chromosome [56–58]. The resistance and regulatory genes are transcribed from two distinct promoters, P_H and P_R, respectively, that are coordinately regulated [57, 58]. Phospho-VanR binds to P_H and P_R and activates transcription of the two sets of genes [59].

VanA-type resistance in clinical isolates of enterococci is mediated by genetic elements identical or closely related to Tn*1546* that are generally carried by self-transferable plasmids [18, 60–63] and, occasionally, by the host chromosome as part of larger conjugative elements [38]. Tn*1546*-like elements are highly conserved except for the presence of insertion sequences in intergenic regions not essential for expression of glycopeptide resistance. The high degree of sequence conservation in the *vanRSHAX* cluster from isolates that are geographically and epidemiologically unrelated suggests that diversification of VanA elements occurred following the transfer of a progenitor Tn*1546* to enterococci. Only very few point mutations have been identified in this gene cluster with a single mutation in *orf1*, *vanS*, *vanA*, *vanX*, and *vanY* genes [63–67]. Much greater diversity is found upstream from the *vanR* gene or downstream from *vanX* and results from the presence of deletions, rearrangements, and insertion sequences (IS) in genes not essential for glycopeptide resistance (*orf1*, *orf2*, *vanY*, and *vanZ*) and in the intergenic regions [66–69]. IS*1251* has been found between *vanS* and *vanH*, in particular in VanA elements from strains collected in the United States [67, 70, 71] but also occasional isolates from Ireland, Norway [72], and Germany [73]. Less frequently, IS*1542* has been detected in the *orf2*-*vanR* intergenic region [74–77] and IS*1476* in the *vanY* gene from strains isolated in Canada [78]. In contrast, IS*1216V* appears to be ubiquitous since insertions have been found in the *vanX*-*vanY* intergenic region [66, 67, 75, 79], upstream from *vanR* [67], in *orf2* [66], in *vanS* [80, 81], and complexed with an IS*3*-like element at the left terminus [38, 66, 67, 76, 82] or with IS*1542* in the *orf2*-*vanR* intergenic region [76, 79, 83]. The multiple insertion sites suggest that this element is actively mobile and indicate that the movement of IS is likely to be crucial in the evolution of VanA elements. Conjugal transfer of plasmids that have acquired Tn*1546*-like elements by transposition appears to be responsible for the spread of glycopeptide resistance in enterococci.

5.1.2 VanB

The VanB type is characterized by a variable level of resistance to vancomycin only (Table 20.1) [84]. The *vanB* operon also confers resistance by production of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac and its organization is similar to that of *vanA* (Fig. 20.5a) but differs in its regulation since vancomycin, but not teicoplanin, is an inducer (Table 20.1) [85]. The *vanB* cluster contains the $vanH_BBX_B$ resistance genes encoding, respectively, a dehydrogenase, a ligase, and a dipeptidase that have high levels of sequence identity (67–76 %) with VanHAX of the *vanA* operon, the accessory $vanY_B$ gene for a D,D-carboxypeptidase, and the $vanR_BS_B$ regulatory genes encoding a two-component regulatory system only distantly related to VanRS (34 and 24 % identity) [86]. The function of *vanW* is unknown.

The $vanR_BS_B$ two-component regulatory system and $vanY_BWH_BBX_B$ resistance genes are inducibly co-transcribed from the P_{RB} and P_{YB} promoters, respectively [86, 87]. In common with VanS, purified VanSB also acts as both

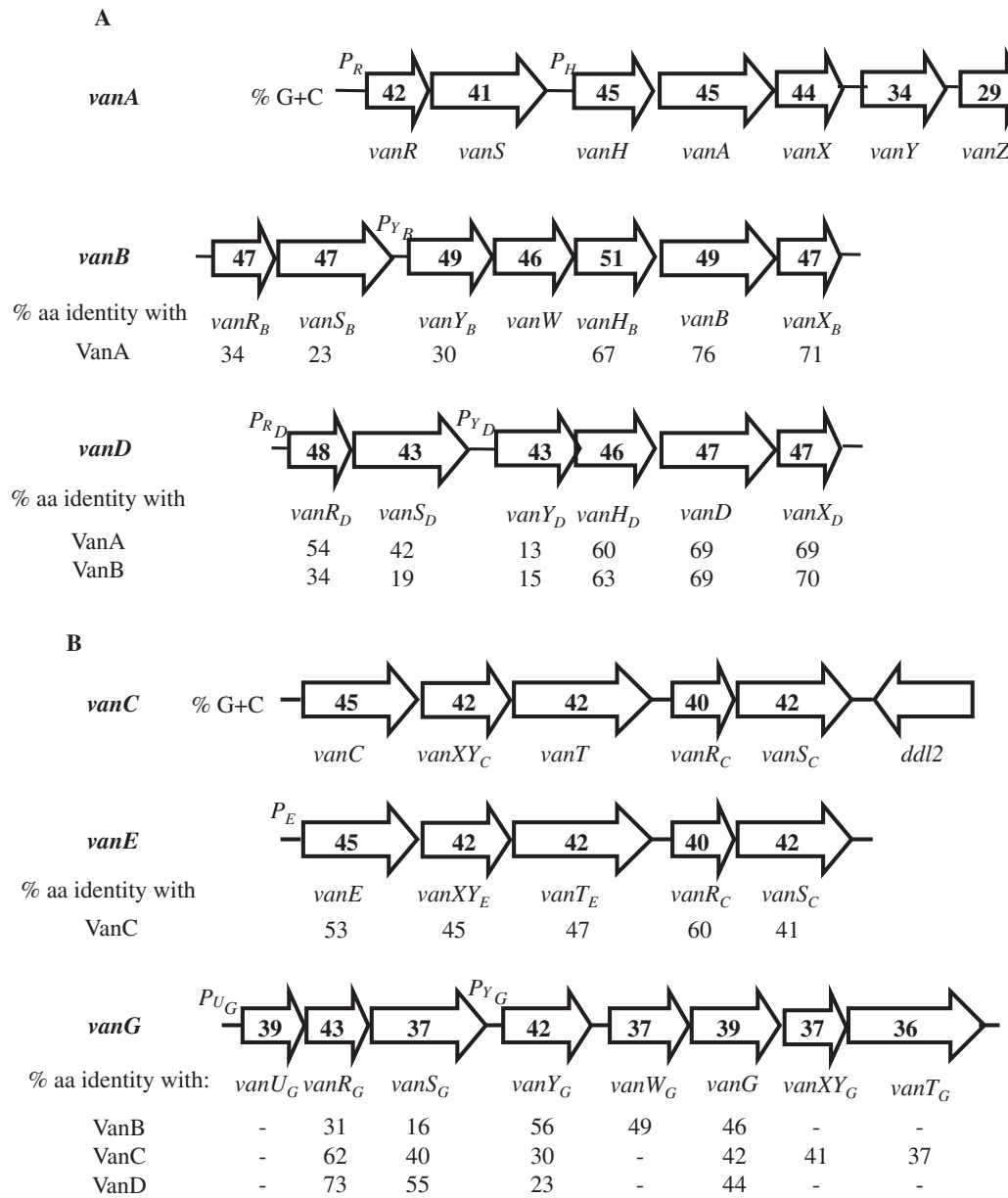


Fig. 20.5 Comparison of the prototype glycopeptide resistance gene clusters. Genes implicated in resistance due to synthesis of modified peptidoglycan precursors ending in (**A**) D-Ala-D-Lac (VanA-, VanB-, and VanD type) or (**B**) D-Ala-D-Ser (VanC, VanE, and VanG type). The number in the *open arrow* indicates the percentage of G+C. *Open arrows* represent coding sequences and direction of transcription

a histidine protein kinase and a phospho-$VanR_B$ phosphatase [87, 88]. In the presence of Vm, $VanS_B$ autophosphorylates, transfers its phosphate to $VanR_B$ [87] and $VanR_B$-P binds upstream from the P_{RB} regulatory promoter and from the P_{YB} resistance promoter [88]. $VanR_B$-P binds with higher affinity than $VanR_B$ to its targets resulting in enhanced transcription. $VanR_B$-P recruits the RNA polymerase and forms an open complex at P_{RB} and P_{YB} [88]. The P_{RB} and P_{YB} promoters are coordinately regulated, but in a different fashion. The P_{RB} regulatory promoter is able to recruit the RNA polymerase in the absence of $VanR_B$ and $VanR_B$-P leading to low-level transcription of the regulatory genes in the absence of induction which then allows to turn on the positive autoregulatory loop for expression of the resistance genes in the presence of vancomycin [88]. Despite the complex dual biochemical mechanism of resistance to vancomycin, in VanB-type resistance, biological cost in enterococci is negligible when noninduced; whereas a significant fitness reduction is observed when resistance is expressed in the presence of the inducer, the antibiotic itself [89]. Thus, due to inducibility, resistance is expressed exclusively when needed for bacterial survival.

Although VanB-type strains do not display teicoplanin resistance, mutations in the *vanSB* sensor gene were obtained in vitro, following selection on teicoplanin that led to constitutive or teicoplanin-inducible expression of the resistance genes [90], in animal models [91], and in humans [87]. Derivatives of VanB-type strains that are resistant to teicoplanin have been isolated from two patients following treatment with vancomycin [92] or teicoplanin [93], but the isolates were not studied further. Mutations leading to teicoplanin resistance also confer low-level resistance to the glycopeptide oritavancine [94]. In VanS-type sensors, five blocks (H, N, G1, F, and G2) of the kinase domains are highly conserved. Constitutive expression of the *vanB* cluster was due to substitutions at two specific positions located on either side of histidine 233, which corresponds to the putative autophosphorylation site of $VanS_B$ [90], or to a six amino acid deletion partially overlapping the conserved G2 ATP-binding domain of $VanS_B$ and leading to loss of phosphatase activity of the sensor [87]. Consequently, dephosphorylation of $VanR_B$ by $VanS_B$ is required to prevent transcription of the resistance genes [57]. Substitutions in the sensor domain of $VanS_B$ lead to inducible expression of resistance by vancomycin and teicoplanin [90]. The N-terminal domain of $VanS_B$ is thus involved in signal recognition and is associated with alterations of specificity that allow induction by teicoplanin [57, 95].

Three subtypes, *vanB1*, *vanB2*, and *vanB3* [96–98], of the *vanB* operon can be distinguished on the basis of specific nucleotide sequences in the $vanS_B$-$vanY_B$ intergenic region. There is no correlation between *vanB* subtype and the level of vancomycin resistance.

The *vanB* gene cluster is carried by large conjugative elements that are transferable from chromosome to chromosome [99]. One of these elements contains the composite transposon Tn*1547* found in a 250-kb genetic element and delineated by insertion sequences belonging to the IS*256* and IS*16* families [100]. The *vanB1* cluster has been associated with Tn*1547*. The *vanB* operon can also be located on plasmids (Table 20.1). Much of the dissemination of VanB resistance appears to result from the spread of *vanB2* clusters carried by Tn*916*-like conjugative transposons [96, 101–103]. Two related elements (27 and 34 kb in size) have been characterized and designated Tn*5382* [101, 104–106] and Tn*1549* [107, 108]. Tn*1549*, located on a plasmid related to pAD1 [107], contains 30 open reading frames organized into three functional regions as observed in the *Tn916* family of conjugative transposons [109]. These regions are implicated in (1) excision-integration, (2) vancomycin resistance, and (3) conjugative transfer. Interestingly, analysis of the base composition indicated that the origin of the left end of the transposon is different from that of the two other functional regions.

Insertion sequences seem to be integrated in *vanB* clusters far less often than within *vanA*. An IS*Enfa200* was identified between *vanSB* and *vanYB* in certain isolates of *E. faecium* with *vanB2* clusters from the United States [101]. An IS*150*-like element downstream from *vanXB* was found in several *vanB2*-type *E. faecium* [87, 106, 110].

5.1.3 VanD

VanD-type strains present moderate levels of resistance to vancomycin and teicoplanin. The organization of the *vanD* operon, which is chromosomally located, is similar to those of *vanA* and *vanB* (Fig. 20.5a) [111–116]. As in VanA- and VanB-type strains, VanD resistance is due to synthesis of peptidoglycan precursors that end quasi-exclusively in D-Ala-D-Lac. Although the biochemical mechanism of resistance is similar to those of VanA and VanB, VanD-type resistance displays some peculiarities. No genes homologous to *vanZ* from the *vanA* operon or *vanW* from the *vanB* operon are present in the *vanD* cluster. As opposed to VanA and VanB, VanD-type resistance is constitutively expressed and is not transferable by conjugation to other enterococci [112–116]. In VanD-type strains, $VanY_D$ D,D-carboxypeptidases belong to the PBP family of the catalytic serine enzymes that are susceptible to penicillin G, and are distinct from VanY and $VanY_B$ which are insensitive to penicillin G. All the VanD-type strains possess (1) an inactive host Ddl ligase, due to the presence of various mutations in the *ddl* gene, except one strain [113] and (2) a mutated $vanS_D$ or $vanR_D$ gene that is responsible for constitutive expression of glycopeptide resistance (Fig. 20.6) [113–116]. Absence of mutations in the Ddl of one *E. faecium* [113] and the fact that two *E. faecalis* strains harbor identical *vanD* operons but different mutations in their D-Ala:D-Ala ligase [114] strongly suggest that the mutations in the sensor or in the regulator were acquired before those in the Ddl ligase; otherwise the strain would have to be transiently glycopeptide-dependent for growth.

The $VanX_D$ D,D-dipeptidase activity is low in VanD-type strains despite the presence of a functional protein [113–115]. Lack of such activity should result in a glycopeptide susceptible phenotype, since these bacteria are unable to eliminate peptidoglycan precursors ending in D-Ala-D-Ala, the target for glycopeptides. However, in VanD-type strains the susceptible pathway does not function due to an inactive D-Ala:D-Ala ligase as the result of various mutations in the chromosomal *ddl* gene. In the strain with a functional Ddl enzyme, synthesis of $VanX_D$ and $VanY_D$ were high since their activities were required to eliminate the D-Ala-D-Ala produced by the chromosomal Ddl [113]. Another intriguing feature is that, in spite of synthesis of peptidoglycan precursors ending essentially in D-Ala-D-Lac, the level of resistance to teicoplanin remains low (Table 20.1).

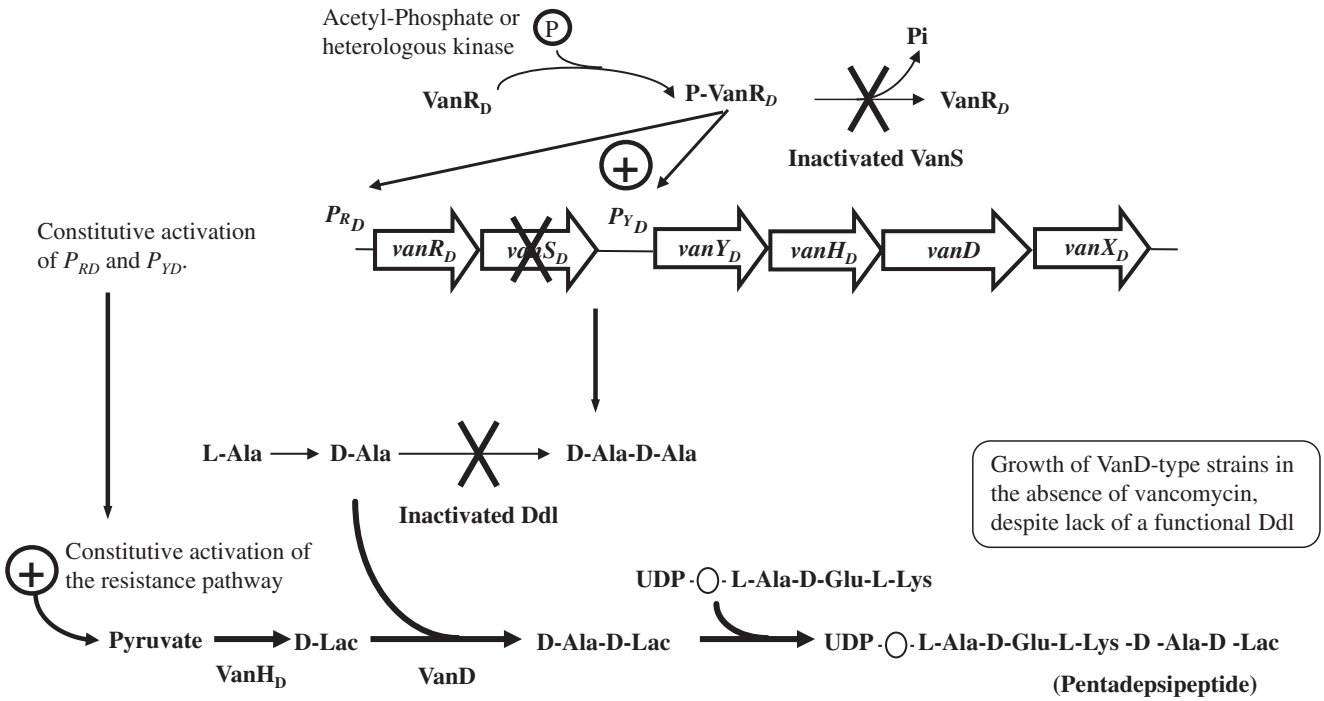


Fig. 20.6 Schematic representation of constitutive activation of P^{RD} and P^{YD} promoters of the *vanD* operon. VanD-type strains have an impaired D-Ala:D-Ala ligase and a mutation in the *vanSD* gene which allows growth in the absence of vancomycin

5.1.4 VanM

E. faecium VanM-type strain confers resistance at high levels of vancomycin (MIC, >256 μg/ml) and teicoplanin (MIC, 96 μg/ml) by inducible synthesis of precursors ending in D-Ala-D-Lac [34]. Although *vanM* sequence is closest to *vanA*, the organization of the *vanM* gene cluster is most similar to that of *vanD* (Fig. 20.5a). Glycopeptide resistance is transferable to *E. faecium*. Upstream from *vanRM* is an IS*1216*-like element encoding a transposase which may play a role in the dissemination of this resistant determinant.

5.2 Glycopeptide Resistance due to Synthesis of Modified Peptidoglycan Precursors Ending in D-Ala-D-Ser

5.2.1 VanC

E. gallinarum and *E. casseliflavus–E. flavescens* are intrinsically resistant to low levels of vancomycin but remain susceptible to teicoplanin (Table 20.1) [35, 36, 117, 118]. Production of peptidoglycan precursors ending in D-Ala-D-Ser is responsible for this type of resistance [29, 119, 120]. In *E. gallinarum*, expression of resistance can be inducible by vancomycin or constitutive due to mutations in the VanS$_C$ sensor [121, 122]. Three subtypes of the *vanC* genes are known: *vanC-1* for *E. gallinarum*, *vanC-2* for *E. casseliflavus*, and *vanC-3* for *E. flavescens* [35, 117, 118, 123]. The organization of the *vanC* operon, which is chromosomally located and not transferable, differs from those of *vanA*, *vanB*, and *vanD* (Fig. 20.5b). Three gene products, VanC, VanXYC, and VanTC, are required for resistance [124]. VanC is a ligase that synthesizes the dipeptide D-Ala-D-Ser which replaces D-Ala-D-Ala in late peptidoglycan precursors [29]. As already mentioned, in VanA- and VanB-type strains, hydrolysis of precursors ending in D-Ala is achieved by two enzymes, a D,D-dipeptidase and a D,D-carboxypeptidase, encoded by two separate genes (*vanX*/*vanX$_B$* and *vanY*/*vanYB*, respectively). In contrast, in VanC-type enterococci the two activities are encoded by a single gene, *vanXY$_C$* [125, 126]. Amino acid sequence comparison indicated that VanXY$_C$ is more closely related to VanY than to VanX [126]. VanT is a membrane-bound serine racemase with a cytoplasmic domain able to convert L-Ser to D-Ser [127, 128]. This enzyme also possesses alanine racemase activity [128]. It has been demonstrated that the transmembrane domain of VanT plays a crucial role in VanC-type resistance and that the protein is probably also involved in the uptake of L-Ser from the external medium [128]. Expression of the *vanC*, *vanXY$_C$*, and *vanT* genes is regulated by two genes located downstream from *vanT* that encode a two-component regulatory system, VanR$_C$S$_C$ [124]. The *vanC* cluster of *E. gallinarum* BM4174 is expressed constitutively [124] and is cotranscribed from a single P_C promoter located upstream from *vanC* [121]. An additional gene, *ddl2*, located downstream from the two regulatory genes and encoding a protein that has structural similarity to D-Ala:D-Ala ligases was

found in the VanC prototype strain BM4174 [129]. Thus, vancomycin-resistant *E. gallinarum* possess at least three ligase genes: two for D-Ala:D-Ala ligases and one for a D-Ala:D-Ser ligase. The *vanC-2* gene cluster of *E. casseliflavus* has been characterized [117]. The deduced proteins display high degrees of identity (from 71 to 91 %) to those encoded by the *vanC* operon. The *vanC-3* gene cluster displays extensive identity with *vanC-2*, from 97 to 100 %, including the intergenic regions [118]. It is therefore difficult to class *E. casseliflavus* and *E. flavescens* as distinct species [118].

5.2.2 VanE

The VanE-type strains exhibit low-level resistance to vancomycin and susceptibility to teicoplanin which is not transferable by conjugation (Table 20.1). The VanE phenotype is expressed inducibly or constitutively due to the synthesis of peptidoglycan precursors terminating in D-Ala-D-Ser [130, 131]. The organization of the *vanE* operon is identical to that of *vanC* (Fig. 20.5b) [130–132]. As in VanC-type resistance, three genes are required: *vanE*, $vanXY_E$, and $vanT_E$, encoding, respectively, a ligase responsible for synthesis of the dipeptide D-Ala-D-Ser, a D,D-peptidase, and a serine racemase and two genes, $vanR_E$ and $vanS_E$, coding for a two-component regulatory system are located downstream from *vanTE* [130]. The five genes are cotranscribed from a single P_E promoter located upstream from *vanE* [131]. Although the VanS sensor is likely to be inactive due to the presence of a stop codon in the 5′ portion of the gene, expression of vancomycin resistance is inducible in VanE prototype strain BM4405 [130]. Inducibility is probably due to cross-talk with another two-component regulatory system of the host. The constitutive expression in VanE-type strains is due to mutations in the $VanS_E$ sensor [131].

5.2.3 VanG

Acquired VanG-type resistance is characterized by a low level of resistance to vancomycin (MIC = 16 µg/ml) due to inducible production of modified precursors ending in D-Ala-D-Ser [31]. The chromosomal *vanG* cluster is composed of seven genes recruited from various *van* operons and its organization differs from that of the other *van* operons (Fig. 20.5b) [31, 133]. The mutated $vanY_G$ gene encodes a truncated inactive D,D-carboxypeptidase; $vanW_G$ encodes a protein of unknown function; the three resistance genes, *vanG*, $vanXY_G$, and $vanT_G$ code for a D-Ala:D-Ser ligase, a bifunctional D,D-peptidase, and a serine racemase, respectively [31]. Inducible expression of the resistance genes from the *PYG* resistance promoter is under the control of an unusual three-component regulatory system encoded by the *vanURSG* operon. In contrast to the other *van* operons in enterococci, *vanG* possesses the additional *vanUG* gene which encodes a transcriptional regulator [31, 133]. The three regulatory genes are co-transcribed, even in the absence of vancomycin, from the P_{UG} regulatory promoter whereas transcription of the resistance genes $vanY_GW_GGXY_GT_G$ is inducible and initiated from the P_{YG} resistance promoter [31]. This is the first *van* operon to be regulated in such a way. $VanS_G$ autophosphorylates, transfers its phosphate to $VanR_G$ but not to $VanU_G$. $VanU_G$, but not $VanR_G$, binds to *PUG* and negatively autoregulates the *vanURSG* operon, and also binds to *PYG* where it overlaps with $VanR_G$ [134]. In clinical isolate BM4518, the expression level of the resistance genes is dependent on vancomycin concentration whereas, in a Δ*vanUG* strain, resistance is expressed at a maximum level whatever the concentration of the inducer [134]. Thus, the binding competition between $VanU_G$ and $VanR_G$ on the *PYG* resistance promoter allows rheostatic activation of the resistance operon depending likely on the level of $VanR_G$ phosphorylation by the $VanS_G$ sensor.

VanG resistance is transferable to *E. faecalis* at a low frequency and transfer is associated with the movement, from chromosome to chromosome, of large genetic elements of *ca*. 240 kb conferring also *ermB*-encoded erythromycin resistance [31].

5.2.4 VanL

Acquired VanL-type resistance detected in Canada in *E. faecium* is characterized by a low level of resistance to vancomycin (MIC, 8 µg/ml) and susceptibility to teicoplanin (0.5 µg/ml) due to inducible production of modified precursors ending in D-Ala-D-Ser. The *vanL* gene cluster is similar in organization to the *vanC*, *vanE*, and *vanN* operons (Fig. 20.5b). However, the $VanT_L$ serine racemase is encoded by two separate genes, *vanTmL* and *vanTrL* corresponding to the membrane binding and racemase domains, respectively [30].

5.2.5 VanN

E. faecium UCN71, isolated from a blood culture, is resistant to low levels of vancomycin (MIC, 16 µg/ml) but susceptible to teicoplanin (MIC, 0.5 µg/ml) [33]. The organization of the *vanN* gene cluster detected on a plasmid is similar to that of the *vanC*, *vanE*, and *vanL* operons (Fig. 20.5b). The $vanR_N$ and $vanS_N$ regulatory genes coding for a two-component regulatory system are located downstream from the resistance genes which, in addition to the *vanN* ligase gene, include *vanXYN* and *vanTN*, which encode a D,D-carboxypeptidase and a serine racemase, respectively (Fig. 20.5b), that are cotranscribed from a unique promoter *PN* located upstream from *vanN* [33]. The deduced proteins of the *vanN* cluster are closely related (61–74 % of identity) to those of VanL. As opposed to the $VanT_L$ serine racemase, $VanT_N$ is not encoded by two separate genes. The VanN-type resistance is due to production of peptidoglycan precursors ending in D-Serine and is expressed constitutively following a mutation in the $VanS_N$ sensor near the autophosphorylation site that could affect its phosphatase activity. VanN-type

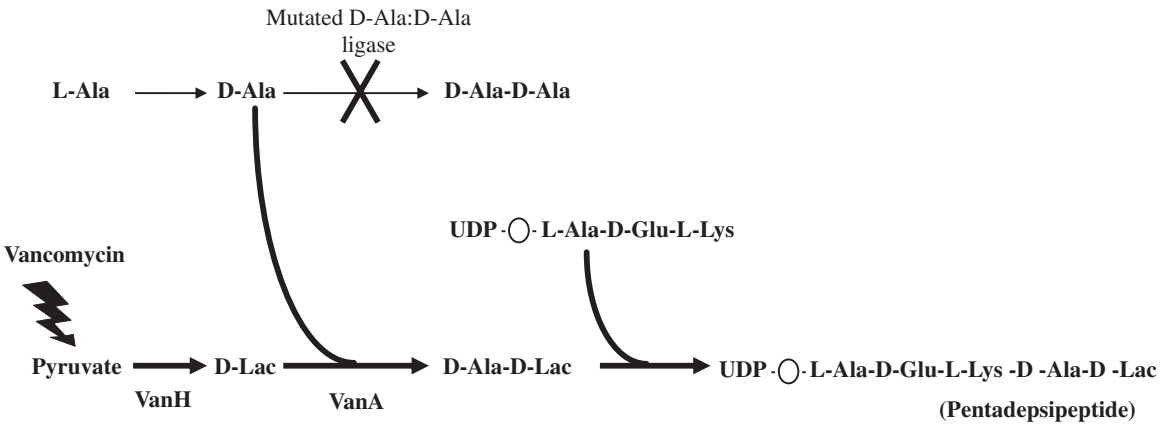


Fig. 20.7 Schematic representation of the synthesis of peptidoglycan precursors in a vancomycin-dependent strain. Presence of vancomycin in the culture medium is necessary to induce the synthesis of modified peptidoglycan precursors and to allow growth of the bacteria. ○, *N*-acetylmuramic acid

resistance is transferable by conjugation to *E. faecium*. This represents the first example of transferable D-Ala-D-Ser type resistance in *E. faecium*.

6 Vancomycin-Dependent Enterococci

Mutations in the host D-Ala:D-Ala ligase of enterococci are lethal unless an alternative pathway for cell wall synthesis is present (Fig. 20.7) [39, 90, 135]. Strains of enterococci that require the presence of vancomycin in the culture medium for growth have been isolated in vitro [39, 90, 135, 136], in animal models [91], and from patients treated for prolonged periods of time with vancomycin [137–141]. Strains containing a *vanA* or a *vanB* operon are able to survive by producing peptidoglycan precursors ending in D-Ala-D-Lac if a glycopeptide is present in the culture medium to induce expression of the *van* operon. Due to the fact that growth of these strains requires particular conditions, prevalence of vancomycin-dependent enterococci is probably underestimated in routine laboratories. Therefore, they could constitute a reservoir of vancomycin resistance genes which can be transferred to other bacteria. Furthermore, it has been observed that these strains can revert to a nondependent state, either by a mutation that leads to constitutive production of D-Ala-D-Lac and is thus also resistant to teicoplanin or by a mutation that restores the synthesis of D-Ala-D-Ala leading to a VanB phenotype inducible by vancomycin [90, 135, 137], or by a mutation in the transcription terminator of *vanRBSB* operon that leads to constitutive expression of the resistance genes from the regulatory promoter [142]. Suppression of transcription terminator represents the third mechanism of reversion from dependence after mutations in *vanSB* and reverse mutations in *ddl*. Thus disruption of vancomycin therapy may not be sufficient to cure patients infected with vancomycin-dependent enterococci, since they can revert to independence by three distinct mechanisms.

7 Origin of the Vancomycin Resistance Genes

7.1 Acquired D-Ala:D-Lac Ligases

Leuconostoc mesenteroides, *Pediococcus pentosaceus*, and *Lactobacillus casei*, which are intrinsically highly resistant to glycopeptides by production of peptidoglycan precursors ending in D-Lac [119, 143, 144], have been suspected to be the source of resistance ligases producing D-Ala-D-Lac. However, a phylogenetic tree based on the alignment of the deduced sequences of D-Ala:D-Ala ligases and related enzymes revealed that VanA, VanB, VanD, and VanM exhibit only limited identity with D-Ala:D-Lac ligases of these naturally resistant species (Fig. 20.8).

The glycopeptide-producing organisms, which harbor resistance genes to protect themselves against suicide, represent a source of resistance for human pathogens. Genes coding for homologues of VanH, VanA, and VanX have been found and with the same genetic organization in two glycopeptide-synthesizing organisms, *Amycolatopsis orientalis* C329.2, and *Streptomyces toyocaensis* NRRL15009, that produce vancomycin and the A47934 glycopeptide, respectively [145–148]. Furthermore, *vanHAX* homologues have also been detected in producers of chloro-eremomycin, ristocetin, vancomycin, and teicoplanin–avoparcin [146]. However, the base composition (G+C content) of the genes composing the *vanA*, *vanB*, *vanD*, and *vanM* clusters is significantly lower than that of the *vanHAX* homologues in the

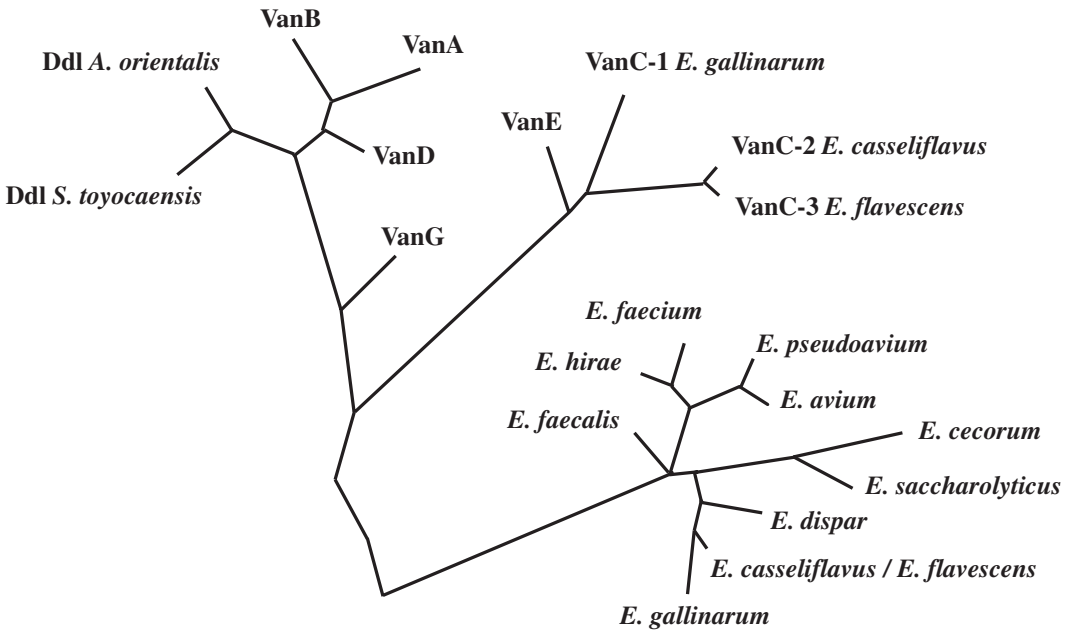


Fig. 20.8 Phylogenetic tree derived from the alignment of D-Ala:D-Ala, D-Ala:D-Lac, and D-Ala:D-Ser ligases

producers, suggesting that acquisition of the genes is probably not a recent event. A vancomycin resistance gene cluster, *vanF*, has been detected in the biopesticide *Paenibacillus popilliae*. This operon is composed of five genes encoding homologues of VanY, VanZ, VanH, VanA, and VanX [149, 150]. Orientation and alignment of the genes essential for resistance (*vanH*/*vanHF*, *vanA*/*vanF*, and *vanX*/*vanXF*) are identical in VanF and VanA. The base composition of the three resistance genes of *P. popilliae* is similar to that of the corresponding genes of *vanA* and *vanB*. *P. popilliae* could therefore represent an intermediate in the transfer from the producers to the clinical isolates. Such a transfer could have occurred through a long chain of related organisms so that the first and the last member of this chain are only distantly related. Glycopeptide resistance *vanA* operons were found in *Paenibacillus* isolated from soil [151]. Their level of identity with the enterococcal operons is markedly higher than that of *vanF*. The close similarity of these operons with that of *Enterococcus* suggests that the gene clusters have evolved from a common ancestor or that the *vanA* operons from soil organisms were acquired by enterococci.

The base composition differs also between the essential and the nonessential genes within the *van* operons, suggesting that the genes could originate from different species. The *van* gene clusters may thus have been composed by collecting genes from various sources.

Presence of the *vanB* operon on a Tn*1549*-like element in various anaerobes from the digestive tract was demonstrated [152]. Furthermore, transfer of the element from *Clostridium symbiosum* to *Enterococcus* spp. was obtained in vitro and in the digestive tract of gnotobiotic mice [153]. Anaerobic bacteria, which are also common in soil, could thus be an intermediate in the transfer of VanB-type vancomycin resistance from glycopeptide producers to enterococci.

7.2 Acquired D-Ala:D-Ser Ligases

No glycopeptide producers were found to synthesize peptidoglycan precursors ending in D-Ala-D-Ser suggesting that the origin of the VanC, E, G, L, and N type of resistance is different from that of VanA, B, and D.

The *vanC*, *vanE*, *vanL*, and *vanN* gene clusters present a high degree of identity (40–72 %) (Fig. 20.5). Thus, acquired resistance of these types could be due to transfer of a chromosomal operon from an other species of *Enterococcus* (*E. gallinarum*, *E. casseliflavus*/*flavescens*).

The *vanG* operon appears to be more heterogeneous. $VanR_G$ exhibits the highest identity (73 %) with $VanR_D$ and $VanS_G$ (55 % identity) with $VanS_D$; $VanY_G$ exhibits the highest identity with $VanY_B$ (56 %), and $vanW_G$ has 49 % identity with *vanW* which is present only in the *vanB* operon. The 3′ part of the *vanG* cluster (*vanG*, $vanXY_G$, $vanT_G$) is more closely related to *vanC* and *vanE* than to the corresponding genes in the other operons (Fig. 20.5) [31], except for the *vanG* gene for the D-Ala:D-Ser ligase which is phylogenetically closer to those for the D-Ala:D-Lac ligases. Thus, the *vanG* operon is composed of genes recruited from various *van* operons. However, the complete genome sequence of *Clostridium difficile* 630 revealed the presence of a *vanG*-like cluster that exhibits the higher degree of identity with *vanG* of *E. faecalis* and includes five open reading frames

encoding putative proteins similar to $VanR_G$, $VanS_G$, VanG, $VanXY_G$, and $VanT_G$ [154]. The *vanG*-like gene clusters are present in 85 % of *C. difficile* clinical isolates and acquisition occurred in a perfect 19-bp inverted repeat, in the absence of a detectable mobile structure [155]. To date, vancomycin resistance has not been reported in *C. difficile*, even if the *vanG*-like cluster is inducible by vancomycin and encodes functional proteins [156].

8 Transfer of VanA-Type Resistance to *S. aureus*

Since 2002, 11 strains of methicillin-resistant *S. aureus* (MRSA) exhibiting high or moderate levels of resistance to vancomycin and teicoplanin following acquisition of the *vanA* gene cluster from *Enterococcus* [157], designated VRSA, have been isolated in the USA [158–163]. Thus, transfer of glycopeptide resistance from enterococci to *S. aureus*, as already demonstrated in vitro [164], can also occur in vivo. A strong synergistic activity of vancomycin and methicillin against these strains has been demonstrated despite the fact that they are highly resistant to both drug classes suggesting that combination of a glycopeptide with a beta-lactam could be used to treat infections due to VRSA strains [165]. In each strain, the VanA-encoding genetic element Tn*1546* was found to be part of a plasmid [157], whether it had transposed from the incoming enterococcal plasmid into a resident plasmid (VRSA-1, -7, -8, -9, and -10) or, in certain instances, the enterococcal plasmid was maintained in the *S. aureus* recipient (VRSA-3, -5, and -6) [48, 166].

Two strains, VRSA-2 and VRSA-3, differ from the others in their levels of resistance to glycopeptides [158, 159]. They exhibit moderate resistance to vancomycin (MICs, 32 μg/ml and 64 μg/ml, respectively) and low resistance to teicoplanin (MICs, 4 μg/ml and 16 μg/ml, respectively). Expression of the *vanA* operon in VRSA-2 and VRSA-3 strains is similar to that in other VRSA strains [167]. Low-level glycopeptide resistance of VRSA-2 and VRSA-3 *S. aureus* is due to instability of the genetic element, plasmid or transposon, carrying the *vanA* operon associated with a longer lag phase before growth resumes after induction by vancomycin.

Three isolates, VRSA-7 [46, 161], VRSA-9 [45], and VRSA-11A [47], were found to be partially dependent on vancomycin for growth. Even in the absence of glycopeptides they mainly synthesize pentadepsipeptide precursors. Loose regulation of the *vanA* operon by VanR/VanS combined with a gene dosage effect due to the fact that the operon is borne by multicopy plasmids accounts for partial dependence. Substitution $N_{308}K$, which affects a critical active site residue in the Ddl of VRSA-7, $Q_{260}K$ and $A_{283}E$ in VRSA-9, and substitution $S_{183}A$ located in the first D-Ala subsite in VRSA-11A result, respectively, in a 1000-fold, 200-fold, and 600-fold decrease of enzymatic activity compared with a wild-type Ddl and the degree of Ddl impairment correlates with the levels of vancomycin dependence [45–47]. The crystal structure of VRSA-9 Ddl indicated that the $Q_{260}K$ substitution does not induce any significant conformational change, whereas the $A_{283}E$ mutation is responsible for new ion-pair/hydrogen bond interactions leading to an asymmetric rearrangement of side chains in the dimer interface and to a more closed conformation of the active site which may be responsible for reduced enzymatic activity [102]. VRSA-11A and VRSA-11B *S. aureus* were isolated from the same patient. VRSA-11A is partially dependent on glycopeptide for growth as discussed above, whereas VRSA-11B is constitutively resistant due to an additional mutation $P_{216}T$, in the transcriptional regulator [47]. It is thus likely that VRSA-11B is a constitutive derivative of VRSA-11A selected during prolonged vancomycin therapy.

In contrast to VanB-type *E. faecalis* [89], there is a slight fitness burden due to the presence in *vanA*-type MRSA of noninduced transposon Tn*1546* [168]. In these strains, and as opposed to the *vanB* operon in *Enterococcus* spp., expression of the *vanA* gene cluster is loosely regulated, and its carriage on multicopy plasmids results in a gene dosage effect that enhances the effect of loose regulation. These two findings result in a slight fitness disadvantage on the host that, in the absence of selective pressure, may account for the lack of dissemination of VanA-type vancomycin-resistant MRSA.

References

1. Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat Rev Microbiol. 2012;10:266–78.
2. Bonten MJ, Willems R, Weinstein RA. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis. 2001;1:314–25.
3. Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. Clin Microbiol Rev. 2000;13:686–707.
4. Schouten MA, Hoogkamp-Korstanje JA, Meis JF, Voss A. Prevalence of vancomycin-resistant enterococci in Europe. Eur J Clin Microbiol Infect Dis. 2000;19:816–22.
5. Low DE, Keller N, Barth A, Jones RN. Clinical prevalence, antimicrobial susceptibility, and geographic resistance patterns of enterococci: results from the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin Infect Dis. 2001;32 Suppl 2:S133–45.
6. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infect Control Hosp Epidemiol. 2008;29:996–1011.
7. Fontana R, Ligozzi M, Pittaluga F, Satta G. Intrinsic penicillin resistance in enterococci. Microb Drug Resist. 1996;2:209–13.
8. Grayson ML, Eliopoulos GM, Wennersten CB, Ruoff KL, De Girolami PC, Ferraro MJ, Moellering Jr RC. Increasing resistance to β-lactam antibiotics among clinical isolates of *Enterococcus*

faecium: a 22-year review at one institution. Antimicrob Agents Chemother. 1991;35:2180–4.
9. Moellering Jr RC. The *enterococcus*: a classic example of the impact of antimicrobial resistance on therapeutic options. J Antimicrob Chemother. 1991;28:1–12.
10. Rybkine T, Mainardi JL, Sougakoff W, Collatz E, Gutmann L. Penicillin-binding protein 5 sequence alterations in clinical isolates of *Enterococcus faecium* with different levels of beta-lactam resistance. J Infect Dis. 1998;178:159–63.
11. Murray BE. Beta-lactamase-producing enterococci. Antimicrob Agents Chemother. 1992;36:2355–9.
12. Gold HS. Vancomycin-resistant enterococci: mechanisms and clinical observations. Clin Infect Dis. 2001;33:210–9.
13. Murray BE. The life and times of the *Enterococcus*. Clin Microbiol Rev. 1990;3:46–65.
14. Barna JCJ, Williams DH. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Ann Rev Microbiol. 1984;38:339–57.
15. Reynolds PE. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis. 1989;8:943–50.
16. Arthur M, Reynolds P, Courvalin P. Glycopeptide resistance in enterococci. Trends Microbiol. 1996;4:401–7.
17. Nieto M, Perkins HR. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. Biochem J. 1971;123:789–803.
18. Leclercq R, Derlot E, Duval J, Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med. 1988;319:157–61.
19. Uttley AH, Collins CH, Naidoo J, Georges RC. Vancomycin resistant enterococci. Lancet. 1988;1(8575–6):57–8.
20. Ramsey AM, Zilberberg MD. Secular trends of hospitalization with vancomycin-resistant enterococcus infection in the United States, 2000–2006. Infect Control Hosp Epidemiol. 2009;30:184–6.
21. Arias CA, Murray BE. Emergence and management of drug-resistant enterococcal infections. Expert Rev Anti Infect Ther. 2008;6:637–55.
22. McCracken M, Wong A, Mitchell R, Gravel D, Conly J, Embil J, Johnston L, Matlow A, Ormiston D, Simor AE, Smith S, Du T, Hizon R, Mulvey MR. Molecular epidemiology of vancomycin-resistant enterococcal bacteraemia: results from the Canadian Nosocomial Infection Surveillance Program, 1999–2009. J Antimicrob Chemother. 2013;68:1505–9.
23. Bourdon N, Fines-Guyon M, Thiolet JM, Maugat S, Coignard B, Leclercq R, Cattoir V. Changing trends in vancomycin-resistant enterococci in French hospitals, 2001–08. J Antimicrob Chemother. 2011;66:713–21.
24. Deshpande LM, Fritsche TR, Moet GJ, Biedenbach DJ, Jones RN. Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. Diagn Microbiol Infect Dis. 2007;58:163–70.
25. Lester CH, Sandvang D, Olsen SS, Schonheyder HC, Jarlov JO, Bangsborg J, Hansen DS, Jensen TG, Frimodt-Moller N, Hammerum AM. Emergence of ampicillin-resistant *Enterococcus faecium* in Danish hospitals. J Antimicrob Chemother. 2008;62:1203–6.
26. Top J, Willems R, van der Velden S, Asbroek M, Bonten M. Emergence of clonal complex 17 *Enterococcus faecium* in The Netherlands. J Clin Microbiol. 2008;46:214–9.
27. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, Klare I, Kristinsson KG, Leclercq R, Lester CH, Lillie M, Novais C, Olsson-Liljequist B, Peixe LV, Sadowy E, Simonsen GS, Top J, Vuopio-Varkila J, Willems RJ, Witte W, Woodford N. Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill. 2008;13.
28. Bugg TDH, Wright GD, Dutka-Malen S, Arthur M, Courvalin P, Walsh CT. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry. 1991;30:10408–15.
29. Reynolds PE, Snaith HA, Maguire AJ, Dutka-Malen S, Courvalin P. Analysis of peptidoglycan precursors in vancomycin-resistant *Enterococcus gallinarum* BM4174. Biochem J. 1994;301:5–8.
30. Boyd DA, Willey BM, Fawcett D, Gillani N, Mulvey MR. Molecular characterization of *Enterococcus faecalis* N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, *vanL*. Antimicrob Agents Chemother. 2008;52:2667–72.
31. Depardieu F, Bonora MG, Reynolds PE, Courvalin P. The *vanG* glycopeptide resistance operon from *Enterococcus faecalis* revisited. Mol Microbiol. 2003;50:931–48.
32. Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev. 2007;20:79–114.
33. Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V. D-Ala-D-Ser VanN-type transferable vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother. 2011;55:4606–12.
34. Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M. *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob Agents Chemother. 2010;54:4643–7.
35. Leclercq R, Dutka-Malen S, Duval J, Courvalin P. Vancomycin resistance gene *vanC* is specific to *Enterococcus gallinarum*. Antimicrob Agents Chemother. 1992;36:2005–8.
36. Navarro F, Courvalin P. Analysis of genes encoding D-alanine:D-alanine ligase-related enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens*. Antimicrob Agents Chemother. 1994;38:1788–93.
37. Arthur M, Molinas C, Depardieu F, Courvalin P. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol. 1993;175:117–27.
38. Handwerger S, Skoble J. Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother. 1995;39:2446–53.
39. Rosato A, Pierre J, Billot-Klein D, Buu-Hoi A, Gutmann L. Inducible and constitutive expression of resistance to glycopeptides and vancomycin dependence in glycopeptide-resistant *Enterococcus avium*. Antimicrob Agents Chemother. 1995;39:830–3.
40. Cercenado E, Unal S, Eliopoulos CT, Rubin LG, Isenberg HD, Moellering Jr RC, Eliopoulos GM. Characterization of vancomycin resistance in *Enterococcus durans*. J Antimicrob Chemother. 1995;36:821–5.
41. Hall LMC, Chen HY, Williams RJ. Vancomycin-resistant *Enterococcus durans*. Lancet. 1992;340:1105.
42. Torres C, Reguera JA, Sanmartin MJ, Perez-Diaz JC, Baquero F. *vanA*-mediated vancomycin-resistant *Enterococcus* spp. in sewage. J Antimicrob Chemother. 1994;33:553–61.
43. Dutka-Malen S, Blaimont B, Wauters G, Courvalin P. Emergence of high-level resistance to glycopeptides in *Enterococcus gallinarum* and *Enterococcus casseliflavus*. Antimicrob Agents Chemother. 1994;38:1675–7.
44. Ligozzi M, Lo Cascio G, Fontana R. *vanA* gene cluster in a vancomycin-resistant clinical isolate of *Bacillus circulans*. Antimicrob Agents Chemother. 1998;42:2055–9.

45. Meziane-Cherif D, Saul FA, Moubareck C, Weber P, Haouz A, Courvalin P, Perichon B. Molecular basis of vancomycin dependence in VanA-type *Staphylococcus aureus* VRSA-9. J Bacteriol. 2010;192:5465–71.
46. Moubareck C, Meziane-Cherif D, Courvalin P, Perichon B. VanA-type *Staphylococcus aureus* strain VRSA-7 is partially dependent on vancomycin for growth. Antimicrob Agents Chemother. 2009; 53:3657–63.
47. Perichon B, Courvalin P. *Staphylococcus aureus* VRSA-11B is a constitutive vancomycin-resistant mutant of vancomycin-dependent VRSA-11A. Antimicrob Agents Chemother. 2012; 56(9):4693–6.
48. Perichon B, Courvalin P. VanA-type vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2009;53: 4580–7.
49. Arthur M, Molinas C, Bugg TDH, Wright GD, Walsh CT, Courvalin P. Evidence for in vivo incorporation of D-lactate into peptidoglycan precursors of vancomycin-resistant enterococci. Antimicrob Agents Chemother. 1992;36:867–9.
50. Reynolds PE, Depardieu F, Dutka-Malen S, Arthur M, Courvalin P. Glycopeptide resistance mediated by enterococcal transposon Tn*1546* requires production of VanX for hydrolysis of D-alanyl-D-alanine. Mol Microbiol. 1994;13:1065–70.
51. Wu Z, Wright GD, Walsh CT. Overexpression, purification and characterization of VanX, a D, D-dipeptidase which is essential for vancomycin resistance in Enterococcus faecium BM4147. Biochemistry. 1995;34:2455–63.
52. Arthur M, Depardieu F, Cabanié L, Reynolds P, Courvalin P. Requirement of the VanY and VanX D, D-peptidases for glycopeptide resistance in enterococci. Mol Microbiol. 1998;30: 819–30.
53. Arthur M, Depardieu F, Snaith HA, Reynolds PE, Courvalin P. Contribution of VanY D, D-carboxypeptidase to glycopeptide resistance in Enterococcus faecalis by hydrolysis of peptidoglycan precursors. Antimicrob Agents Chemother. 1994;38:1899–903.
54. Arthur M, Depardieu F, Molinas C, Reynolds P, Courvalin P. The *vanZ* gene of Tn*1546* from *Enterococcus faecium* BM4147 confers resistance to teicoplanin. Gene. 1995;154:87–92.
55. Arthur M, Molinas C, Courvalin P. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol. 1992;174:2582–91.
56. Wright GD, Holman TR, Walsh CT. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry. 1993;32:5057–63.
57. Arthur M, Depardieu F, Courvalin P. Regulated interactions between partner and non partner sensors and responses regulators that control glycopeptide resistance gene expression in enterococci. Microbiology. 1999;145:1849–58.
58. Arthur M, Depardieu F, Gerbaud G, Galimand M, Leclercq R, Courvalin P. The VanS sensor negatively controls VanR-mediated transcriptional activation of glycopeptide resistance genes of Tn*1546* and related elements in the absence of induction. J Bacteriol. 1997;179:97–106.
59. Holman TR, Wu Z, Wanner BL, Walsh CT. Identification of the DNA-binding site for the phosphorylated VanR protein required for vancomycin resistance in *Enterococcus faecium*. Biochemistry. 1994;33:4625–31.
60. Clark NC, Cooksey RC, Hill BC, Swenson JM, Tenover FC. Characterization of glycopeptide-resistant enterococci from U.S. hospitals. Antimicrob Agents Chemother. 1993;37:2311–7.
61. Dutka-Malen S, Leclercq R, Coutant V, Duval J, Courvalin P. Phenotypic and genotypic heterogeneity of glycopeptide resistance determinants in gram-positive bacteria. Antimicrob Agents Chemother. 1990;34:1875–9.
62. Handwerger S, Pucci MJ, Kolokathis A. Vancomycin resistance is encoded on a pheromone response plasmid in *Enterococcus faecium* 228. Antimicrob Agents Chemother. 1990;34:358–60.
63. Hashimoto Y, Tanimoto K, Ozawa Y, Murata T, Ike Y. Amino acid substitutions in the VanS sensor of the VanA-type vancomycin-resistant *Enterococcus* strains result in high-level vancomycin resistance and low-level teicoplanin resistance. FEMS Microbiol Lett. 2000;185:247–54.
64. Jensen LB, Ahrens P, Dons L, Jones RN, Hammerum AM, Aarestrup FM. Molecular analysis of Tn*1546* in *Enterococcus faecium* isolated from animals and humans. J Clin Microbiol. 1998;36:437–42.
65. Lauderdale TL, McDonald LC, Shiau YR, Chen PC, Wang HY, Lai JF, Ho M. Vancomycin-resistant enterococci from humans and retail chickens in Taiwan with unique VanB phenotype-*vanA* genotype incongruence. Antimicrob Agents Chemother. 2002;46: 525–7.
66. Palepou MF, Adebiyi AM, Tremlett CH, Jensen LB, Woodford N. Molecular analysis of diverse elements mediating VanA glycopeptide resistance in enterococci. J Antimicrob Chemother. 1998;42:605–12.
67. Willems RJ, Top J, van den Braak N, van Belkum A, Mevius DJ, Hendriks G, van Santen-Verheuvel M, van Embden JD. Molecular diversity and evolutionary relationships of Tn*1546*-like elements in enterococci from humans and animals. Antimicrob Agents Chemother. 1999;43:483–91.
68. Oh JY, An S, Jin JS, Lee YC, Cho DT, Lee JC. Phenotypic and genotypic differences of the vancomycin-resistant *Enterococcus faecium* isolates from humans and poultry in Korea. J Microbiol. 2007;45:466–72.
69. Song JH, Ko KS, Oh WS, Park S, Heo ST, Kwon KT, Ryu SY, Peck KR, Lee NY. High frequency of vancomycin-resistant *Enterococcus faecium* isolates with VanB phenotype and *vanA* genotype in Korean hospitals. Diagn Microbiol Infect Dis. 2006;56:401–6.
70. Handwerger S, Skoble J, Discotto LF, Pucci MJ. Heterogeneity of the *vanA* gene cluster in clinical isolates of enterococci from the northeastern United States. Antimicrob Agents Chemother. 1995;39:362–8.
71. Sung K, Khan SA, Nawaz MS. Genetic diversity of Tn*1546*-like elements in clinical isolates of vancomycin-resistant enterococci. Int J Antimicrob Agents. 2008;31:549–54.
72. Simonsen GS, Myhre MR, Dahl KH, Olsvik O, Sundsfjord A. Typeability of Tn*1546*-like elements in vancomycin-resistant enterococci using long-range PCRs and specific analysis of polymorphic regions. Microb Drug Resist. 2000;6:49–57.
73. Werner G, Klare I, Fleige C, Witte W. Increasing rates of vancomycin resistance among *Enterococcus faecium* isolated from German hospitals between 2004 and 2006 are due to wide clonal dissemination of vancomycin-resistant enterococci and horizontal spread of *vanA* clusters. Int J Med Microbiol. 2008; 298:515–27.
74. Darini AL, Palepou MF, Woodford N. Nucleotide sequence of IS*1542*, an insertion sequence identified within VanA glycopeptide resistance elements of enterococci. FEMS Microbiol Lett. 1999;173:341–6.
75. Gu L, Cao B, Liu Y, Guo P, Song S, Li R, Dai H, Wang C. A new Tn*1546* type of VanB phenotype-*vanA* genotype vancomycin-resistant *Enterococcus faecium* isolates in mainland China. Diagn Microbiol Infect Dis. 2009;63:70–5.
76. Lee WG, Huh JY, Cho SR, Lim YA. Reduction in glycopeptide resistance in vancomycin-resistant enterococci as a result of *vanA* cluster rearrangements. Antimicrob Agents Chemother. 2004;48: 1379–81.
77. Woodford N, Adebiyi AM, Palepou MF, Cookson BD. Diversity of VanA glycopeptide resistance elements in enterococci from

humans and nonhuman sources. Antimicrob Agents Chemother. 1998;42:502–8.
78. MacKinnon MG, Drebot MA, Tyrrell GJ. Identification and characterization of IS*1476*, an insertion sequence-like element that disrupts VanY function in a vancomycin-resistant *Enterococcus faecium* strain. Antimicrob Agents Chemother. 1997;41:1805–7.
79. Park IJ, Lee WG, Lim YA, Cho SR. Genetic rearrangements of Tn*1546*-like elements in vancomycin-resistant *Enterococcus faecium* isolates collected from hospitalized patients over a seven-year period. J Clin Microbiol. 2007;45:3903–8.
80. Darini AL, Palepou MF, James D, Woodford N. Disruption of *vanS* by IS*1216V* in a clinical isolate of *Enterococcus faecium* with VanA glycopeptide resistance. Antimicrob Agents Chemother. 1999;43:995–6.
81. Park IJ, Lee WG, Shin JH, Lee KW, Woo GJ. VanB phenotype-*vanA* genotype *Enterococcus faecium* with heterogeneous expression of teicoplanin resistance. J Clin Microbiol. 2008;46:3091–3.
82. Schouten MA, Willems RJ, Kraak WA, Top J, Hoogkamp-Korstanje JA, Voss A. Molecular analysis of Tn*1546*-like elements in vancomycin-resistant enterococci isolated from patients in Europe shows geographic transposon type clustering. Antimicrob Agents Chemother. 2001;45:986–9.
83. Huh JY, Lee WG, Lee K, Shin WS, Yoo JH. Distribution of insertion sequences associated with Tn*1546*-like elements among *Enterococcus faecium* isolates from patients in Korea. J Clin Microbiol. 2004;42:1897–902.
84. Quintiliani Jr R, Evers S, Courvalin P. The *vanB* gene confers various levels of self-transferable resistance to vancomycin in enterococci. J Infect Dis. 1993;167:1220–3.
85. Arthur M, Depardieu F, Reynolds P, Courvalin P. Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. Mol Microbiol. 1996;21:33–44.
86. Evers S, Courvalin P. Regulation of VanB-type vancomycin resistance gene expression by the $VanS_B$-$VanR_B$ two-component regulatory system in *Enterococcus faecalis* V583. J Bacteriol. 1996;178:1302–9.
87. Depardieu F, Courvalin P, Msadek T. A six amino acid deletion, partially overlapping the $VanS_B$ G2 ATP-binding motif, leads to constitutive glycopeptide resistance in VanB-type *Enterococcus faecium*. Mol Microbiol. 2003;50:1069–83.
88. Depardieu F, Courvalin P, Kolb A. Binding sites of $VanR_B$ and sigma70 RNA polymerase in the *vanB* vancomycin resistance operon of *Enterococcus faecium* BM4524. Mol Microbiol. 2005;57:550–64.
89. Foucault ML, Depardieu F, Courvalin P, Grillot-Courvalin C. Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. Proc Natl Acad Sci U S A. 2010;107:16964–9.
90. Baptista M, Depardieu F, Reynolds P, Courvalin P, Arthur M. Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type enterococci. Mol Microbiol. 1997;25:93–105.
91. Aslangul E, Baptista M, Fantin B, Depardieu F, Arthur M, Courvalin P, Carbon C. Selection of glycopeptide-resistant mutants of VanB-type *Enterococcus faecalis* BM4281 in vitro and in experimental endocarditis. J Infect Dis. 1997;175:598–605.
92. Hayden MK, Trenholme GM, Schultz JE, Sahm DF. *In vivo* development of teicoplanin resistance in a VanB *Enterococcus faecium* isolate. J Infect Dis. 1993;167:1224–7.
93. Kawalec M, Gniadkowski M, Kedzierska J, Skotnicki A, Fiett J, Hryniewicz W. Selection of a teicoplanin-resistant *Enterococcus faecium* mutant during an outbreak caused by vancomycin-resistant enterococci with the VanB phenotype. J Clin Microbiol. 2001;39:4274–82.
94. Arthur M, Depardieu F, Reynolds P, Courvalin P. Moderate-level resistance to glycopeptide LY333328 mediated by genes of the *vanA* and *vanB* clusters in enterococci. Antimicrob Agents Chemother. 1999;43:1875–80.
95. Baptista M, Depardieu F, Courvalin P, Arthur M. Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. Antimicrob Agents Chemother. 1996;40:2291–5.
96. Dahl KH, Simonsen GS, Olsvik O, Sundsfjord A. Heterogeneity in the *vanB* gene cluster of genomically diverse clinical strains of vancomycin-resistant enterococci. Antimicrob Agents Chemother. 1999;43:1105–10.
97. Gold HS, Unal S, Cercenado E, Thauvin-Eliopoulos C, Eliopoulos GM, Wennersten CB, Moellering Jr RC. A gene conferring resistance to vancomycin but not teicoplanin in isolates of *Enterococcus faecalis* and *Enterococcus faecium* demonstrates homology with *vanB*, *vanA*, and *vanC* genes of enterococci. Antimicrob Agents Chemother. 1993;37:1604–9.
98. Patel R, Uhl JR, Kohner P, Hopkins MK, Steckelberg JM, Kline B, Cockerill 3rd FR. DNA sequence variation within *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes of clinical *Enterococcus* isolates. Antimicrob Agents Chemother. 1998;42:202–5.
99. Quintiliani Jr R, Courvalin P. Conjugal transfer of the vancomycin resistance determinant *vanB* between enterococci involves the movement of large genetic elements from chromosome to chromosome. FEMS Microbiol Lett. 1994;119:359–64.
100. Quintiliani Jr R, Courvalin P. Characterization of Tn*1547*, a composite transposon flanked by the IS*16* and IS*256*-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. Gene. 1996;172:1–8.
101. Dahl KH, Lundblad EW, Rokenes TP, Olsvik O, Sundsfjord A. Genetic linkage of the *vanB2* gene cluster to Tn*5382* in vancomycin-resistant enterococci and characterization of two novel insertion sequences. Microbiology. 2000;146:1469–79.
102. McGregor KF, Nolan C, Young HK, Palepou MF, Tysall L, Woodford N. Prevalence of the *vanB2* gene cluster in VanB glycopeptide-resistant enterococci in the United Kingdom and the Republic of Ireland and its association with a Tn*5382*-like element. Antimicrob Agents Chemother. 2001;45:367–8.
103. Rice LB. Emergence of vancomycin-resistant enterococci. Emerg Infect Dis. 2001;7:183–7.
104. Carias LL, Rudin SD, Donskey CJ, Rice LB. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J Bacteriol. 1998;180:4426–34.
105. Lopez M, Hormazabal JC, Maldonado A, Saavedra G, Baquero F, Silva J, Torres C, del Campo R. Clonal dissemination of *Enterococcus faecalis* ST201 and *Enterococcus faecium* CC17-ST64 containing Tn*5382-vanB2* among 16 hospitals in Chile. Clin Microbiol Infect. 2009;15:586–8.
106. Lu JJ, Chang TY, Perng CL, Lee SY. The *vanB2* gene cluster of the majority of vancomycin-resistant *Enterococcus faecium* isolates from Taiwan is associated with the *pbp5* gene and is carried by Tn*5382* containing a novel insertion sequence. Antimicrob Agents Chemother. 2005;49:3937–9.
107. Garnier F, Taourit S, Glaser P, Courvalin P, Galimand M. Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. Microbiology. 2000;146:1481–9.
108. Umeda A, Garnier F, Courvalin P, Galimand M. Association between the *vanB2* glycopeptide resistance operon and Tn*1549* in enterococci from France. J Antimicrob Chemother. 2002;50:253–6.
109. Clewell DB, Flannagan SE, Jaworski DD. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. Trends Microbiol. 1995;3:229–36.

110. Lee WG, Kim W. Identification of a novel insertion sequence in *vanB2*-containing *Enterococcus faecium*. Lett Appl Microbiol. 2003;36:186–90.
111. Boyd DA, Kibsey P, Roscoe D, Mulvey MR. *Enterococcus faecium* N03-0072 carries a new VanD-type vancomycin resistance determinant: characterization of the VanD5 operon. J Antimicrob Chemother. 2004;54:680–3.
112. Casadewall B, Courvalin P. Characterization of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. J Bacteriol. 1999;181:3644–8.
113. Depardieu F, Foucault ML, Bell J, Dubouix A, Guibert M, Lavigne JP, Levast M, Courvalin P. New combinations of mutations in VanD-Type vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus avium* strains. Antimicrob Agents Chemother. 2009;53:1952–63.
114. Depardieu F, Kolbert M, Pruul H, Bell J, Courvalin P. VanD-type vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. Antimicrob Agents Chemother. 2004;48:3892–904.
115. Depardieu F, Reynolds PE, Courvalin P. VanD-type vancomycin-resistant *Enterococcus faecium* 10/96A. Antimicrob Agents Chemother. 2003;47:7–18.
116. Perichon B, Casadewall B, Reynolds P, Courvalin P. Glycopeptide-resistant *Enterococcus faecium* BM4416 is a VanD-type strain with an impaired D-Alanine:D-Alanine ligase. Antimicrob Agents Chemother. 2000;44:1346–8.
117. Dutta I, Reynolds PE. Biochemical and genetic characterization of the *vanC-2* vancomycin resistance gene cluster of *Enterococcus casseliflavus* ATCC 25788. Antimicrob Agents Chemother. 2002;46:3125–32.
118. Dutta I, Reynolds PE. The *vanC-3* vancomycin resistance gene cluster of *Enterococcus flavescens* CCM439. J Antimicrob Chemother. 2003;51:703–6.
119. Billot-Klein D, Gutmann L, Sablé S, Guittet E, van Heijenoort J. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J Bacteriol. 1994;176:2398–405.
120. Grohs P, Gutmann L, Legrand R, Schoot B, Mainardi JL. Vancomycin resistance is associated with serine-containing peptidoglycan in *Enterococcus gallinarum*. J Bacteriol. 2000;182:6228–32.
121. Panesso D, Abadia-Patino L, Vanegas N, Reynolds PE, Courvalin P, Arias CA. Transcriptional analysis of the *vanC* cluster from *Enterococcus gallinarum* strains with constitutive and inducible vancomycin resistance. Antimicrob Agents Chemother. 2005;49:1060–6.
122. Sahm DF, Free L, Handwerger S. Inducible and constitutive expression of *vanC-1* encoded resistance to vancomycin in *Enterococcus gallinarum*. Antimicrob Agents Chemother. 1995;39:1480–4.
123. Clark NC, Teixeira LM, Facklam RR, Tenover FC. Detection and differentiation of *vanC-1*, *vanC-2*, and *vanC-3* glycopeptide resistance genes in enterococci. J Clin Microbiol. 1998;36:2294–7.
124. Arias CA, Courvalin P, Reynolds PE. *vanC* cluster of vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrob Agents Chemother. 2000;44:1660–6.
125. Meziane-Cherif D, Stogios PJ, Evdokimova E, Savchenko A, Courvalin P. Structural basis for the evolution of vancomycin resistance D, D-peptidases. Proc Natl Acad Sci U S A. 2014;111(16):5872–7.
126. Reynolds PE, Arias CA, Courvalin P. Gene vanXYC encodes D, D -dipeptidase (VanX) and D, D-carboxypeptidase (VanY) activities in vancomycin-resistant Enterococcus gallinarum BM4174. Mol Microbiol. 1999;34:341–9.
127. Arias CA, Martin-Martinez M, Blundell TL, Arthur M, Courvalin P, Reynolds PE. Characterization and modelling of VanT: a novel, membrane-bound, serine racemase from vancomycin-resistant *Enterococcus gallinarum* BM4174. Mol Microbiol. 1999;31:1653–64.
128. Arias CA, Weisner J, Blackburn JM, Reynolds PE. Serine and alanine racemase activities of VanT: a protein necessary for vancomycin resistance in *Enterococcus gallinarum* BM4174. Microbiology. 2000;146:1727–34.
129. Ambur OH, Reynolds PE, Arias CA. D-Ala:D-Ala ligase gene flanking the *vanC* cluster: evidence for presence of three ligase genes in vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrob Agents Chemother. 2002;46:95–100.
130. Abadia Patino L, Courvalin P, Perichon B. *vanE* gene cluster of vancomycin-resistant *Enterococcus faecalis* BM4405. J Bacteriol. 2002;184:6457–64.
131. Abadia-Patino L, Christiansen K, Bell J, Courvalin P, Perichon B. VanE-type vancomycin-resistant *Enterococcus faecalis* clinical isolates from Australia. Antimicrob Agents Chemother. 2004;48:4882–5.
132. Boyd DA, Cabral T, Van Caeseele P, Wylie J, Mulvey MR. Molecular characterisation of the *vanE* gene cluster in vancomycin-resistant *Enterococcus faecalis* N00-410 isolated in Canada. Antimicrob Agents Chemother. 2002;46:1977–9.
133. Boyd DA, Du T, Hizon R, Kaplen B, Murphy T, Tyler S, Brown S, Jamieson F, Weiss K, Mulvey MR. VanG-type vancomycin-resistant *Enterococcus faecalis* strains isolated in Canada. Antimicrob Agents Chemother. 2006;50:2217–21.
134. Depardieu F, Mejean V, Courvalin P. Competition between $VanU_G$ and $VanR_G$ activator leads to rheostatic control of vancomycin resistance operon. PLoS Genet. 2015;11:e1005170.
135. Van Bambeke F, Chauvel M, Reynolds PE, Fraimow HS, Courvalin P. Vancomycin-dependent *Enterococcus faecalis* clinical isolates and revertant mutants. Antimicrob Agents Chemother. 1999;43:41–7.
136. Sifaoui F, Gutmann L. Vancomycin dependence in a *vanA*-producing *Enterococcus avium* strain with a nonsense mutation in the natural D-Ala:D-Ala ligase gene. Antimicrob Agents Chemother. 1997;41:1409.
137. Dever LL, Smith SM, Handwerger S, Eng RH. Vancomycin-dependent *Enterococcus faecium* isolated from stool following oral vancomycin therapy. J Clin Microbiol. 1995;33:2770–3.
138. Farrag N, Eltringham I, Liddy H. Vancomycin-dependent *Enterococcus faecalis*. Lancet. 1996;348:1581–2.
139. Fraimow HS, Jungkind DL, Lander DW, Delso DR, Dean JL. Urinary tract infection with an *Enterococcus faecalis* isolate that requires vancomycin for growth. Ann Intern Med. 1994;121:22–6.
140. Green M, Shlaes JH, Barbadora K, Shlaes DM. Bacteremia due to vancomycin-dependent *Enterococcus faecium*. Clin Infect Dis. 1995;20:712–4.
141. Stewart B, Hall L, Duke B, Ball D. Vancomycin-dependent enterococci: curious phenomenon or serious threat? J Antimicrob Chemother. 1997;40:734–5.
142. San Millan A, Depardieu F, Godreuil S, Courvalin P. VanB-type *Enterococcus faecium* clinical isolate successively inducibly resistant to, dependent on, and constitutively resistant to vancomycin. Antimicrob Agents Chemother. 2009;53:1974–82.
143. Gay Elisha B, Courvalin P. Analysis of genes encoding D-alanine:D-alanine ligase-related enzymes in *Leuconostoc mesenteroides* and *Lactobacillus* spp. Gene. 1995;152:79–83.
144. Handwerger S, Pucci MJ, Volk KJ, Liu J, Lee M. Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic peptidoglycan precursors that terminate in lactate. J Bacteriol. 1994;176:260–4.

145. Marshall CG, Broadhead G, Leskiw BK, Wright GD. D-Ala:D-Ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin -resistance ligases VanA and VanB. Proc Natl Acad Sci U S A. 1997; 94:6480–3.
146. Marshall CG, Lessard IA, Park I, Wright GD. Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. Antimicrob Agents Chemother. 1998;42:2215–20.
147. Marshall CG, Wright GD. DdlN from vancomycin-producing *Amycolatopsis orientalis* C329.2 is a VanA homologue with D-alanyl-D-lactate ligase activity. J Bacteriol. 1998;180:5792–5.
148. Marshall CG, Wright GD. The glycopeptide antibiotic producer *Streptomyces toyocaensis* NRRL15009 has both D-alanyl:D-alanine and D-alanyl:D-lactate ligases. FEMS Microbiol Lett. 1997;157:295–9.
149. Patel R, Piper K, Cockerill 3rd FR, Steckelberg JM, Yousten AA. The biopesticide *Paenibacillus popilliae* has a vancomycin resistance gene cluster homologous to the enterococcal VanA vancomycin resistance gene cluster. Antimicrob Agents Chemother. 2000;44:705–9.
150. Rippere K, Patel R, Uhl JR, Piper KE, Steckelberg JM, Kline BC, Cockerill 3rd FR, Yousten AA. DNA sequence resembling *vanA* and *vanB* in the vancomycin-resistant biopesticide *Bacillus popilliae*. J Infect Dis. 1998;178:584–8.
151. Guardabassi L, Perichon B, van Heijenoort J, Blanot D, Courvalin P. Glycopeptide resistance *vanA* operons in *Paenibacillus* strains isolated from soil. Antimicrob Agents Chemother. 2005;49: 4227–33.
152. Ballard SA, Pertile KK, Lim M, Johnson PD, Grayson ML. Molecular characterization of *vanB* elements in naturally occurring gut anaerobes. Antimicrob Agents Chemother. 2005;49: 1688–94.
153. Launay A, Ballard SA, Johnson PD, Grayson ML, Lambert T. Transfer of vancomycin resistance transposon Tn*1549* from *Clostridium symbiosum* to *Enterococcus* spp. in the Gut of Gnotobiotic Mice. Antimicrob Agents Chemother. 2006;50: 1054–62.
154. Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeno-Tarraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Dougan G, Barrell B, Parkhill J. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat Genet. 2006;38: 779–86.
155. Ammam F, Marvaud JC, Lambert T. Distribution of the *vanG*-like gene cluster in *Clostridium difficile* clinical isolates. Can J Microbiol. 2012;58:547–51.
156. Ammam F, Meziane-Cherif D, Mengin-Lecreulx D, Blanot D, Patin D, Boneca IG, Courvalin P, Lambert T, Candela T. The functional *vanGCd* cluster of *Clostridium difficile* does not confer vancomycin resistance. Mol Microbiol. 2013;89:612–25.
157. Zhu W, Murray PR, Huskins WC, Jernigan JA, McDonald LC, Clark NC, Anderson KF, McDougal LK, Hageman JC, Olsen-Rasmussen M, Frace M, Alangaden GJ, Chenoweth C, Zervos MJ, Robinson-Dunn B, Schreckenberger PC, Reller LB, Rudrik JT, Patel JB. Dissemination of an *Enterococcus* Inc18-Like *vanA* plasmid associated with vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2010;54:4314–20.
158. Kacica M, McDonald LC. Vancomycin-resistant *Staphylococcus aureus*-New York, 2004. Morb Mortal Wkly Rep. 2004;53: 322–3.
159. Miller D, Urdaneta V, Weltman A, Park S. Vancomycin-resistant *Staphylococcus aureus*-Pennsylvania, 2002. Morb Mortal Wkly Rep. 2002;51:902.
160. Sievert DM, Boulton ML, Stolman G, Johnson D, Stobierski MG, Downes FP, Somsel PA, Rudrik JT, Brown WJ, Hafeez W, Lundstrom T, Flanagan E, Johnson R, Mitchell J, Chang S. *Staphylococcus aureus* resistant to vancomycin—United States, 2002. MMWR Morb Mortal Wkly Rep. 2002;51:565–7.
161. Sievert DM, Rudrik JT, Patel JB, McDonald LC, Wilkins MJ, Hageman JC. Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002–2006. Clin Infect Dis. 2008;46:668–74.
162. Tenover FC. Vancomycin-resistant *Staphylococcus aureus*: a perfect but geographically limited storm? Clin Infect Dis. 2008;46: 675–7.
163. Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science. 2003;302:1569–71.
164. Noble WC, Virani Z, Cree RGA. Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol Lett. 1992;93:195–8.
165. Perichon B, Courvalin P. Synergism between beta-lactams and glycopeptides against VanA-type methicillin-resistant *Staphylococcus aureus* and heterologous expression of the *vanA* operon. Antimicrob Agents Chemother. 2006;50:3622–30.
166. Zhu W, Clark NC, McDougal LK, Hageman J, McDonald LC, Patel JB. Vancomycin-resistant *Staphylococcus aureus* isolates associated with Inc18-like *vanA* plasmids in Michigan. Antimicrob Agents Chemother. 2008;52:452–7.
167. Perichon B, Courvalin P. Heterologous expression of the enterococcal *vanA* operon in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2004;48:4281–5.
168. Foucault ML, Courvalin P, Grillot-Courvalin C. Fitness cost of VanA-type vancomycin resistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2009;53: 2354–9.

Daptomycin Resistance

21

Jordan R. Smith, Kimberly C. Claeys, Evan J. Zasowski, Juwon Yim, and Michael J. Rybak

1 Introduction

Daptomycin is a cyclic lipopeptide antibiotic and the first member of this novel class of antimicrobials to gain approval for commercial use. Initially isolated in the 1980s, daptomycin is a component of an antibiotic complex naturally synthesized by *Streptomyces roseosporus* [1]. Like the other lipopeptide components of this complex, daptomycin comprises a thirteen amino acid hydrophilic peptide core with a lipophilic fatty acid "tail" which acrylates the *N*-terminus of the exocyclic side chain [2]. It is the lipophilic "tail" that is believed to be essential to the antibiotic activity of these compounds, and daptomycin's unique decanoic acid "tail" is its distinguishing characteristic [2].

Daptomycin's mechanism of action centers on its ability to insert itself into the gram-positive bacterial cytoplasmic membrane (CM) via the lipophilic "tail" moiety [3]. This is promoted by the presence of physiologic levels of calcium, which results in the formation of calcium-complexed daptomycin and conformational change [4]. Although it is known that daptomycin's bactericidal effect is related to leakage of intracellular potassium ions and membrane depolarization, the precise molecular mechanism is not fully elucidated [5, 6]. Recent data suggest daptomycin oligomerization in the CM results in localized changes in membrane curvature [7]. In addition to the disruption of membrane integrity, this also appears to result in recruitment of essential cell division proteins to the areas of membrane distortion, which in turn alters peptidoglycan synthesis and cell wall structure [7]. The resultant bactericidal activity is concentration-dependent, with enhancement of effect with increasing drug concentrations. The pharmacokinetic/pharmacodynamic (PK/PD) index that best predicts daptomycin's effect is the area under the curve (AUC) to minimum inhibitory concentration (MIC) ratio (AUC/MIC) [8]. This concept is vital to daptomycin dose optimization for both efficacy and prevention of resistance development [8, 9].

Daptomycin is active against a majority of clinically important gram-positive pathogens such *as Staphylococcus aureus*, *Enterococcus* spp., and *Streptococcus* spp. Daptomycin was first FDA approved in 2003 for complicated skin and skin structure infections, and it was later approved for *Staphylococcus aureus* bacteremia, including right-sided infective endocarditis [10, 11]. Given its potent gram-positive activity, including activity against many multidrug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), daptomycin is often used for off-label indications such as bone and joint infections, urinary tract infections, and enterococcal bacteremia [12–14]. Of note, daptomycin is not effective in the treatment of lower respiratory infections as it is inactivated in the lungs by pulmonary surfactant [15, 16]. Daptomycin's ability to treat serious infections caused by multidrug-resistant organisms make it a valuable member of the antibiotic armamentarium. However, bacteria have begun to acquire the ability to evade and withstand attack from daptomycin, and daptomycin non-susceptibility has become a reality [17–20].

J.R. Smith, Pharm. D.
High Point University School of Pharmacy, High Point, NC, USA
e-mail: jsmith5@highpoint.edu

K.C. Claeys, Pharm. D. • E.J. Zasowski, Pharm. D., M.P.H.
J. Yim, Pharm. D
Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy, Wayne State University, Detroit, MI, USA

M.J. Rybak, Pharm. D., M.P.H. (✉)
Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, USA

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_21

2 Daptomycin Nonsusceptibility in *S. aureus*

The exact mechanisms underlying daptomycin nonsusceptibility, or "resistance," in *Staphylococcus aureus* have not been fully elucidated and are an ongoing area of research. It is clear, however, that multiple pathways to phenotypic resistance exist, including changes in the cell membrane composition and cell wall architecture. Additionally, resistance to daptomycin shares similar mechanisms as resistance to vancomycin, with multiple studies demonstrating a correlation. Thankfully, despite clinical description of DNS *Staphylococcus aureus*, trends in surveillance data suggest that this phenotype remains rare among staphylococci, with 0.11 and 0.05 % of tested isolates considered nonsusceptible by CLSI standards from 2005 to 2010 and 2005 to 2012, respectively [21–23].

One of the most frequently identified and cited mutations leading to daptomycin resistance is single nucleotide polymorphisms (SNPs) of the nonessential integral membrane protein termed multiple peptide resistance factor (*mprF*) gene open reading frame (ORF) [24–27]. These SNPs result in a gain-of-function mutation in the *mprF* gene [28]. In a study by Freidman et al., it was demonstrated that serial passage of *S. aureus* through sub-inhibitory concentrations of daptomycin resulted in a number of genetic mutations, with SNPs in the *mprF* ORF occurring first. [24] This *mprF* gene is involved in the lysinylation of phosphatidylglycerol (PG) as well as the "flipping" or inner-outer translocation of lysylphosphatidylglycerol (L-PG) across the CM [27, 29–31]. Both of these functions serve to change the CM from more negatively charged to more positively charged. Investigators have proposed that the translocation of L-PG contributes more to the effects of CM charge than the production of new L-PG [27, 32, 33]. The relative change in charge of the CM results in a charge repulsion resistance wherein the net positively charged calcium-complexed daptomycin no longer has a docking site within the CM [33]. Currently there is not a consensus regarding whether *mprF* SNPs are causal of daptomycin resistance. Rubio et al. were able to demonstrate the reversal of daptomycin resistance via blockage of mutated MprF [28]. In contrast, other investigators have found daptomycin resistance and increased CM positive charge without the presence of these SNPs [34, 35].

The characteristics of reduced daptomycin binding and increased positive surface charge have been demonstrated to be consistent phenotypes [33]. Mishra et al. recently observed daptomycin-resistant strains with more relatively negative surface charges, concluding that the charge repulsion mechanism cannot fully account for all daptomycin resistance phenotypes [36]. Cytoplasmic membrane fluidity/composition also plays a role in the development of resistance. Previous publications have noted both increased and decreased CM fluidity [37, 38]. Of note, changes in lipid composition include increased carotenoids, which have been linked to increased CM rigidity and daptomycin MICs [39]. The YycG sensor kinase is related to the regulation of fatty acid biosynthesis, altering the CM composition [24, 40].

The enhanced positive surface charge resulting in a daptomycin-resistant phenotype may also be caused by upregulation of *dltABCD* operon, which controls alanylation of cell wall (CW) teichoic acid and reduces autolytic activity, which in turn increases cell wall positive charge [41–43]. Yang et al. were able to demonstrate daptomycin resistance associated with increased positive surface charge without a significant increase in SNPs in the *mprF* gene or L-PG, but notably enhanced transcription of the *dltABCD* operon occurred [35]. These findings were supported by Cafiso et al., who also went further and proposed that increased expression of the *dltABCD* operon is a strain-independent keystone mutation [44].

Daptomycin has also been shown to be a strong inducer of the CW "stress stimulon," which includes the two-component regulator *vraSR* (vancomycin-resistance associated sensor/regulator) [45]. Vra is part of the intermembrane-sensing histidine kinase family. The sensor kinase (VraS) detects threats from the outside environment, leading to the activation of the response regulator (VraR) and subsequent expression of target genes that enhance cell wall synthesis and a thickened cell wall [46, 47]. The thickened CW has been associated with daptomycin resistance [48]. The induction of *vraSR* has been more commonly studied with cell wall active agents such as beta-lactams and vancomycin, and inactivation of *vraSR* leads to a reduction in beta-lactam and vancomycin resistance [49, 50]. Muthaiyan et al. also demonstrated that daptomycin is an inducer of the CW stress stimulon and suggested that inhibition of cell wall biosynthesis is a component of daptomycin's mechanism of action [51]. This has been verified by Mehta et al. who also demonstrated that inactivation of *vraSR* resulted in increased susceptibility to daptomycin [52].

Resistance to daptomycin can occur in patients who have not been previously exposed to the antimicrobial [53]. Numerous studies have demonstrated an association between vancomycin susceptibilities and daptomycin susceptibilities [48, 53–56]. A report by the Centers for Disease Control and Prevention noted an association between increased vancomycin MICs and increased daptomycin MICs [55]. Sakoulas et al. were able to demonstrate heterogeneity in daptomycin susceptibility in isolates exposed to vancomycin-containing media [56]. In 2006, Cui et al. reported the positive correlation between the presence of a vancomycin intermediate phenotype and decreased daptomycin susceptibility. It was hypothesized that the thickened cell wall created a barrier that daptomycin could not overcome to reach its site of action at the CM [54]. Mutational changes of the *mprF* and *yycG* genes have also been shown to affect vancomycin susceptibilities; however, it has been previously demonstrated

that these mutations are not necessary in the development of reduced susceptibility to both vancomycin and daptomycin [57–60]. In a study by Mwangi et al., a series of MRSA blood isolates collected from a patient being treated with vancomycin for infective endocarditis demonstrated the development of VISA while on therapy. These isolates were evaluated for antibiotic susceptibility changes and underwent full genome sequencing to evaluate potential mutations consistent with antibiotic resistance. Of interest, while vancomycin MICs increased over the 2 month treatment period leading to VISA with a final vancomycin MIC of 8 μg/mL, an eightfold increase in daptomycin MIC from 0.012 to 1 μg/mL was also observed, even though the patient had not received daptomycin during therapy. Sequencing results revealed 35 point mutations, including mutations in *rpoC* and *yycH*, mutations in genes previously implicated in daptomycin nonsusceptibility [53]. The same parent, vancomycin-susceptible strain from this patient was further evaluated in an in vitro PK/PD model with simulated endocardial vegetations over a 30-day vancomycin simulated dosing exposure [61]. Once again, VISA emerged during the simulated vancomycin dosing regimen with a corresponding increase in the daptomycin MIC to 1 μg/mL. The VISA strain possessed reduced negative cell surface charge and thicker cell walls, phenotypic traits associated with daptomycin nonsusceptibility. These in vitro data further corroborate the potential correlation between vancomycin and daptomycin nonsusceptibility. From these observations we can conclude that vancomycin exposure can lead to later daptomycin resistance, with a thickened cell wall and/or multiple possible genetic changes [53, 61].

Recently, there have been significant advances and insight into the role of the endogenous innate immune system, in particular cationic host defense peptides (HDPs), in daptomycin treatment failure. Cationic HDPs originate from hematogenous sources such as platelets and neutrophils and interact with bacterial cell membranes [62, 63]. The bactericidal effects of cationic HDPs share similar mechanisms as calcium-complexed daptomycin; therefore the mechanisms of resistance to daptomycin also affect the activity of cationic HDPs. The cross-resistance between HDPs and daptomycin has been demonstrated in previous studies wherein, in vitro, daptomycin-resistant *S. aureus* isolates were also resistant to several HDPs, including those of white blood cell (hNP-1) or platelet (tPMP-1) origin [33, 38]. The causes of cross-resistance are many and still not fully defined but include mechanisms previously noted, such as cell wall thickening, cell membrane changes, and SNPs. Bayer et al. have demonstrated that there is a high degree of heterogeneity in the expression of *mprF* SNPs that results in higher daptomycin MICs and decreased killing by cationic HDPs [64]. An *in vivo* rabbit model has demonstrated that daptomycin MICs can become increased in daptomycin-naive hosts, and this resistance may be secondary to exposure to endogenous cationic HDPs [32]. Persistent endovascular infections, such as infective endocarditis, are of primary concern secondary to the high inoculum and exposure to PMNs and platelets [65]. The clinical significance and exact mechanisms behind cross-resistance between daptomycin and cationic HDPs is an ongoing area of investigation.

3 Overcoming Daptomycin Nonsusceptibility in Staphylococci

The development of daptomycin nonsusceptibility among staphylococci is an area of concern, especially since daptomycin is frequently used as a last-line antibiotic against multidrug-resistant gram-positive organisms. Recently, several publications have demonstrated creative, effective means of combating staphylococci with elevated daptomycin MICs. In a 2008 study, Rose and colleagues evaluated high-dose daptomycin versus standard-dose against daptomycin-susceptible and daptomycin-nonsusceptible *S. aureus* [66]. The authors demonstrated that although daptomycin MIC increases occurred with standard, 6-mg/kg/day dosing, 10-mg/kg/day dosing was able to prevent these MIC increases. They also demonstrated that combination regimens including gentamicin or rifampin with high-dose daptomycin were able to produce bactericidal activity against daptomycin-nonsusceptible strains. Outside of simply increasing the daptomycin dose, the combinations employed in this study presented an interesting means to combat daptomycin-nonsusceptibility. A common principle taken advantage of in recent literature is the "seesaw effect," a phenomenon first described in MRSA with reduced susceptibility to vancomycin [67]. The "seesaw effect" is demonstrated by the steadily decreasing beta-lactam MIC values as vancomycin MICs continue to rise, owing to reduced expression of the *mecA* gene [68]. Expanding upon this idea, several studies have demonstrated synergy between beta-lactams and vancomycin against MRSA isolates harboring reduced susceptibilities to vancomycin [69–73]. In the case of daptomycin nonsusceptibility, pioneering work by Yang and colleagues demonstrated the efficacy of daptomycin in combination with oxacillin against daptomycin-nonsusceptible MRSA in a rabbit model of endocarditis [74]. In vitro MIC testing demonstrated a "seesaw" phenomenon in six MRSA strain pairs, and in vitro time-kill testing demonstrated synergy between daptomycin and oxacillin against two of the strains. Most impressively, the combination of daptomycin and oxacillin produced significantly greater kill compared to either agent alone, illustrating the therapeutic enhancement present between these agents. Several authors have expanded upon this research and evaluated daptomycin synergy with other agents. Dhand and colleagues described seven cases of persistent MRSA bacteremia that were cleared with the combination of daptomycin and an anti-staphylococcal beta-lactam [75]. The authors provided in

vitro evidence that against daptomycin-nonsusceptible MRSA, nafcillin exposure increases daptomycin binding and decreases cell surface charge compared to cells unexposed to nafcillin, demonstrating a potential mechanism for enhanced daptomycin efficacy. Other authors have corroborated these results with combinations of daptomycin and ceftobiprole, ceftaroline, oxacillin, meropenem, cefazolin, or cefepime [76–78]. Owing to the anti-MRSA activity of ceftaroline, its combination with daptomycin warrants special mention. Several pharmacokinetic/pharmacodynamic model experiments have demonstrated therapeutic enhancement with this combination [78–80]. In addition to therapeutic enhancement with the combination, Werth and colleagues demonstrated that ceftaroline enhanced daptomycin-induced cell depolarization, reduced cell wall thickness, increased daptomycin binding, and increased killing by cathelicidin LL37, a biological antimicrobial HDP that mimics daptomycin's mechanism [78]. The combination has been evaluated clinically, as well. In a series of 26 patients, Sakoulas and colleagues reported the successful clearance of refractory staphylococcal bacteremia with the combination of daptomycin and ceftaroline [81]. Although none of the strains in this series was daptomycin-nonsusceptible, these results combined with previous data lend credence to the viability of this combination in refractory staphylococcal bacteremia.

Beta-lactams are not the only agents to have demonstrated synergy with daptomycin against daptomycin-nonsusceptible MRSA. Several studies have reported synergistic activity between trimethoprim-sulfamethoxazole and daptomycin [82–84]. In an in vitro model of simulated endocardial vegetations, Steed and colleagues demonstrated superior efficacy with the combination of daptomycin and trimethoprim-sulfamethoxazole compared to daptomycin in combination with cefepime, linezolid, or nafcillin [82]. These data are further supported by a report of two patients with daptomycin-nonsusceptible, vancomycin-intermediate *S. aureus* who were successfully cleared of bacteremia with the daptomycin and trimethoprim-sulfamethoxazole combination [84]. A recent case series also evaluated this combination in cases of refractory staphylococcal bacteremia and confirmed the synergistic activity present with in vitro time-kill studies [85].

3.1 Alternative Agents with Activity Against DNS *S. aureus*

Outside of combination therapy, there are several alternative options with the potential for activity against daptomycin-nonsusceptible *S. aureus*. These agents include ceftaroline, mentioned above, as well as linezolid, tedizolid, telavancin, dalbavancin, and oritavancin [86]. Ceftaroline has been mentioned previously and is the only cephalosporin available with anti-MRSA activity. As discussed earlier, ceftaroline possesses impressive synergy with daptomycin against daptomycin-nonsusceptible *S. aureus*. However, ceftaroline also possesses bactericidal activity on its own against daptomycin-nonsusceptible *S. aureus* in the same in vitro model [80]. Ceftaroline MICs have also been demonstrated to decrease with subsequent increases in daptomycin MICs, possibly rendering ceftaroline more effective against *S. aureus* with reduced daptomycin susceptibility [87]. Recent surveillance data also demonstrate that ceftaroline maintains an MIC range of 0.03–0.12 μg/mL against daptomycin-nonsusceptible *S. aureus* [88]. Clinically, ceftaroline has demonstrated efficacy in refractory *S. aureus* infection. In a recent, retrospective study, Casapao and colleagues reported on the successful use of ceftaroline in patients with several disease states, including bacteremia [89]. Only 14 isolates demonstrated reduced daptomycin susceptibility, but the authors report successful ceftaroline use in these refractory cases. Going forward, ceftaroline will be an important agent against daptomycin-nonsusceptible *S. aureus*, and further clinical data will better define its role.

Tedizolid and linezolid, both members of the oxazolidinone antibiotic class, maintain excellent activity against *S. aureus*. Owing to their distinct mechanisms of action, cross-resistance is extremely uncommon [90]. Population-based data demonstrate that tedizolid inhibits 99.9 % of *S. aureus* at concentrations of 0.5 μg/mL, and resistance rates to linezolid among *S. aureus* have ranged from only 0.03 to 0.15 % over from 2004 to 2013 [91, 92]. Each of these agents is bacteriostatic, however, possibly limiting their utility in deep-seated infections such as endocarditis. Even so, for a large portion of infections, these agents remain an important alternative in the setting of daptomycin nonsusceptibility.

Three remaining agents, telavancin, dalbavancin, and oritavancin, are lipoglycopeptides that combine structural and functional features of lipopeptides and glycopeptides [86]. Recent data demonstrated that in a population sample of 9610 *S. aureus* isolates, 100 % were susceptible to telavancin with MIC values of 0.125 μg/mL or lower [93, 94]. Among these isolates, 43 were daptomycin-nonsusceptible, and all were susceptible to telavancin. Telavancin has been evaluated in an in vitro model of simulated endocardial vegetations against daptomycin-nonsusceptible *S. aureus* [95]. In this study, telavancin was superior to daptomycin in killing the daptomycin-nonsusceptible strain. In vivo data from rabbit models of endocarditis also demonstrate the bactericidal activity of telavancin against daptomycin-nonsusceptible MRSA strains [96]. Although clinical data are limited regarding the activity of telavancin against daptomycin-nonsusceptible MRSA, one case report has been published describing effective telavancin therapy of mitral valve endocarditis following daptomycin failure [97]. Going forward, telavancin is an intriguing therapeutic

option for daptomycin-nonsusceptible MRSA infections and warrants further study.

Oritavancin demonstrates in vitro activity against daptomycin-nonsusceptible strains as well, producing bactericidal activity in time-kill studies [98]. In 24-h in vitro time-kill studies, oritavancin demonstrated more rapid, bactericidal activity compared to daptomycin, vancomycin, teicoplanin, or linezolid against MSSA, MRSA, vancomycin-resistant MRSA, and vancomycin-intermediate MRSA [99]. In a recent surveillance study evaluating 9115 *S. aureus* isolates from invasive infections, 9038 (99.1 %) were inhibited by oritavancin at 0.125 μg/mL, the current CLSI resistance cutoff, and the MIC_{90} was 0.063 μg/mL [100]. In this study, 100 isolates possessed daptomycin MIC values ≥1 μg/mL, and all but six of these isolates possessed oritavancin MIC values ≤0.125 μg/mL. As another new agent against multidrug-resistant MRSA, oritavancin presents an important option for further study.

Dalbavancin, the third approved lipoglycopeptide, maintains excellent antistaphylococcal activity as well, with MIC values consistently eightfold lower than daptomycin in surveillance data [101]. However, there are limited data regarding its efficacy against *S. aureus* with daptomycin MICs >1 μg/mL. Still, dalbavancin provides another potentially viable alternative for MRSA infections with reduced daptomycin susceptibility. Although clinical data are limited owing to the rarity of daptomycin nonsusceptibility clinically, each of these agents and combination therapies is an important piece of the arsenal when daptomycin nonsusceptibility inevitably arises.

4 Daptomycin Nonsusceptibility in Enterococci

Daptomycin is considered a key antibiotic for the treatment of multidrug-resistant enterococcal infections. Although daptomycin-nonsusceptible enterococci have been well described in the literature, worldwide surveillance data indicate only 0.8 % of tested enterococcal isolates from 2005 to 2012 were considered nonsusceptible per the CLSI breakpoint (≥8 μg/mL) [22, 23]. However, when resistance does emerge, therapeutic options are limited [102, 103]. The precise mechanism of daptomycin resistance in enterococci is not fully understood. Recent studies have focused primarily on mutations in two groups of genes.

A group of genes associated with daptomycin nonsusceptibility in enterococci encodes LiaFSR (lipid-II cycle interfering antibiotics protein), a three-component regulatory system, which regulates the response of the cell envelope to cell membrane-disrupting antibiotics such as vancomycin and daptomycin in some gram-positive bacteria [104–106]. Another group of genes, *gdpD* (glycerophosphoryl diester phosphodiesterase) and *cls* (cardiolipin synthetase), are presumed to encode enzymes associated with cell membrane phospholipid metabolism. Specifically, *gdpD* was previously found to be involved in hydrolysis of glycerophosphodiesters in other bacteria in the process of glycerol metabolism [107]. Cardiolipin is a major component of cell membrane domains in bacteria, which is highly involved in regulation of various cellular functions such as bacterial cell division and membrane transport [108]. It has been shown that bacterial cells deficient in cardiolipin have longer doubling times, are less likely to survive in the stationary phase, and demonstrate altered antibiotic sensitivity. Cardiolipin content is modified to adapt to changes in harsh environmental stress [109, 110].

In comparative whole-genome sequencing by Arias and colleagues, presence of mutations in these genes and emergence of daptomycin resistance in *E. faecalis* was explored using a pair of daptomycin-susceptible and daptomycin-resistant clinical isolate from a patient before and after treatment with daptomycin. In-frame deletions in three genes were observed in the resistant isolate compared to the susceptible isolate. Of these, one gene encodes LiaF, which is a negative regulator of LiaRS-mediated signal transduction [104]. Mutation in *liaF* appears to activate this three-component system and is presumed to alter transcription of several genes that can help maintain homeostasis of the cell envelope. Concurrent mutations in genes encoding for GdpD and Cls were observed as well, which were shown to change the composition and distribution of phospholipids in the cell membrane [111]. Subsequently, Tran and colleagues performed whole genome sequencing using a pair of daptomycin-susceptible and daptomycin-resistant *E. faecium* clinical isolates obtained from a patient during daptomycin therapy, and demonstrated that daptomycin resistance was associated with mutations in two genes encoding proteins responsible for cell membrane phospholipid metabolism: cyclopropane fatty acid synthase enzyme along with *cls*, as seen in *E. faecalis*. In addition, mutations were also observed in histidine kinase YycG, a member of the YycFG system, which regulates cell envelope homeostasis similarly to LiaFSR and has been shown to influence daptomycin susceptibility in *S. aureus* [112].

Involvement of the LiaFSR system and Cls in development of daptomycin resistance in enterococci was bolstered by consistent findings from other studies. In time-kill assays by Munita and colleagues it was demonstrated that *liaF* codon deletion may lead to the development of the daptomycin-resistant phenotype in *E. faecalis* [113]. In the same context, Reyes and colleagues demonstrated that deletion of *liaR*, a putative gene of the response regulator of the LiaFSR system from daptomycin-nonsusceptible *E. faecalis*, may restore daptomycin activity by interfering with the LiaFSR pathway [114]. Palmer and colleagues conducted

whole-genome sequencing on three daptomycin-resistant variants of *E. faecalis*, which was generated by exposure to increasing levels of daptomycin in vitro, to identify occurrence of genetic changes that may contribute to emergence of daptomycin resistance. Mutations in *cls* were consistently observed in each of the daptomycin-resistant variants. The role of *cls* mutation in daptomycin resistance was confirmed by the finding that insertion of the *cls* mutant allele from resistant variants conferred daptomycin resistance to formerly daptomycin-susceptible strains [115].

Tran and colleagues proposed a novel mechanism of daptomycin resistance in enterococci, which could connect all the genetic mutations described above in a systematic manner. In their study, binding of daptomycin-calcium complex to cell surface was not compromised in a daptomycin-nonsusceptible strain compared to a daptomycin-susceptible strain, suggesting that there may be additional mechanisms of daptomycin nonsusceptibility among *E. faecalis* other than repulsion of antimicrobial molecules from the cell envelope as described in other gram-positive bacteria. The difference between daptomycin-susceptible and daptomycin-resistant strains was in the distribution of antibiotic molecules bound to the cell membrane. Daptomycin-resistant *E. faecalis* appears to divert daptomycin-calcium complex away from the septum, the drug target. The authors proposed that all these morphologic changes observed in daptomycin-resistant *E. faecalis* start from activation of the LiaFSR three-component regulatory system, which leads to altered distribution of cardiolipin-enriched microdomains, specifically away from the septum. Changes in phospholipid synthesis enzymes such as GdpD and Cls affect phospholipid content of the cell membrane, especially reducing phosphatidylglycerol to a great extent. As a result, daptomycin-calcium complex binding to the septum is further distorted, and is redirected to the cell membrane with reduced amount of phosphatidylglycerol that plays an important role directing oligomerization of daptomycin [3, 116]. The end result is that daptomycin fails to disrupt plasma membrane function in enterococci and loses its antimicrobial effect. This diversion of calcium-bound daptomycin molecules from the drug target has been observed in daptomycin-nonsusceptible *E. faecium* as well. Diaz and colleagues assessed the interactions of daptomycin with the cell membrane in *E. faecium* clinical isolates with different MICs and genetic mutations, including mutations within the LiaFSR and YycFG pathways, using fluorescently labeled daptomycin [117]. Although the authors described decreased binding of daptomycin molecules to the cell membrane in several isolates with daptomycin nonsusceptibility, one *E. faecium* strain did not possess altered daptomycin binding. In this strain, daptomycin was diverted from the division septum, similar to the mechanism inherent to *E. faecalis*. Repulsion of the daptomycin-calcium complex from the cell membrane, well described in daptomycin-nonsusceptible *S. aureus*, has also been demonstrated in enterococci. Humphries and colleagues demonstrated that daptomycin-nonsusceptible *E. faecium* possessed higher net surface charge, and subsequent daptomycin repulsion, compared to daptomycin-susceptible *E. faecium* [19]. Steed and colleagues corroborated these findings [118]. In their study, the authors compared three isogenic strain pairs of vancomycin-resistant *E. faecium* strains and found that membrane surface charge was higher in daptomycin-nonsusceptible mutants compared to their susceptible parent strains. In addition, increased cell wall thickness and decreased membrane depolarization by daptomycin were observed among the daptomycin-nonsusceptible VRE strains.

The definite mechanism(s) of daptomycin resistance in enterococcus are still uncertain. Further studies are necessary to fully comprehend the mechanisms.

5 Overcoming Daptomycin Nonsusceptibility in Enterococci

Daptomycin nonsusceptibility among enterococci is a growing concern, and novel therapeutic regimens are necessary. Perhaps most importantly, measures must be taken to avoid enterococcal resistance before it occurs. Werth and colleagues demonstrated that daptomycin doses of 10 mg/kg/day may be necessary to successfully prevent the emergence of daptomycin nonsusceptibility in enterococci, well above the FDA-approved dose of 6 mg/kg/day [9]. The combination regimen of daptomycin and ceftriaxone has also been employed as initial therapy in vitro, and this combination was able to successfully prevent the emergence of resistance, while daptomycin alone was not [119]. Against *E. faecium* strains in the high end of the susceptible range to daptomycin, LiaFSR mutations are frequent, as described above [120]. These mutations render daptomycin ineffective, even though MIC values remain in the susceptible range. The importance of these mutations is described in a recent case report, where an *E. faecium* isolate with a susceptible daptomycin MIC of 3 μg/mL but with a *liaFSR* mutation developed a daptomycin MIC >256 μg/mL during the course of therapy [20]. Against a set of isolates possessing *liaFSR* mutations, Diaz and colleagues demonstrated restored daptomycin activity in the presence of ampicillin [117]. Unfortunately, against strains with mutations in YycFG system, another frequent resistance mutation, ampicillin was unable to restore daptomycin activity. Sakoulas and colleagues have demonstrated synergy between beta-lactams and daptomycin against both *E. faecalis* and *E. faecium*, although in both cases the isolates were in the susceptible daptomycin range [121, 122]. The importance of these studies lies in their descriptions of beta-lactam effects on daptomycin against these enterococci. Against *E. faecalis*, ceftaroline enhanced binding of daptomycin and sensitized

the bacteria to killing by LL37, a cationic HDP described earlier [121]. Against *E. faecium*, ampicillin reduced daptomycin MIC values roughly 2.5-fold, reduced positive surface charge, enhanced daptomycin binding, and sensitized the bacteria to killing by LL37 [122]. The ability of beta-lactams to enhance daptomycin activity against *E. faecalis* and *E. faecium* has been corroborated recently by Smith and colleagues, who demonstrated synergistic activity between daptomycin and ceftaroline, ertapenem, and ampicillin against in in vitro time-kill studies [123]. Higher doses of daptomycin and combination therapies may prevent the emergence of daptomycin nonsusceptibility and warrant further evaluation clinically, especially when daptomycin MIC values are in the 3–4 μg/mL range. When daptomycin nonsusceptibility arises, however, both options and evidence are limited. Against an *E. faecium* strain with a daptomycin MIC of 32 μg/mL, Sakoulas and colleagues demonstrated the beneficial effects of ceftaroline in providing synergistic activity in time-kill experiments [124]. Ampicillin and ceftriaxone were also evaluated, but ceftaroline alone was able to provide synergistic activity. Similar to experiments involving susceptible isolates, ceftaroline was able to increase daptomycin surface binding, increase membrane fluidity, and decrease net cell surface charge, demonstrating possible mechanistic reasons for the synergy. Werth and colleagues observed similar activity with the combination of ceftobiprole and daptomycin against daptomycin-nonsusceptible *E. faecium* [125]. It is possible that the unique ability among cephalosporins of ceftaroline and ceftobiprole to bind PBP5 in enterococci is integral to their synergistic activity with daptomycin against these isolates [126].

5.1 Alternative Agents with Activity Against DNS *Enterococcus* spp.

Similar to staphylococci, several antimicrobials possess in vitro activity against enterococci and may be useful when daptomycin nonsusceptibility occurs. Among them are, again, linezolid, tedizolid, and oritavancin. Telavancin and dalbavancin, although active against enterococci with the *vanB* vancomycin resistance gene, lack reliable activity against VRE that contain the much more common *vanA*, and cannot therefore be counted on to have activity against a majority of VRE isolates [127].

Linezolid has been the subject of several meta-analyses in comparison to daptomycin in the setting of enterococcal bacteremia, and the data suggest that the two agents are likely similar, although one meta-analysis suggests that daptomycin may be associated with higher mortality [128–130]. Recent United States surveillance data demonstrate linezolid resistance rates among enterococci of only 0.65 %, suggesting that this agent possesses activity against the vast majority of enterococcal isolates encountered clinically [91]. Tedizolid, as well, possesses excellent activity against *E. faecalis* and *E. faecium*. Among a survey of 855 clinical enterococcal isolates, 850 (99.4 %) were inhibited by tedizolid at ≤0.5 μg/mL, the resistance cutoff for *E. faecalis* [92]. Clinical data are limited, but with its similar mechanism to linezolid, it would appear that tedizolid will be a valuable tool against daptomycin-nonsusceptible enterococci.

The lone lipoglycopeptide with anti-VRE activity is oritavancin. Oritavancin possesses activity against VRE harboring both *vanA* and *vanB* genes, although MIC values in *E. faecalis* with *vanA* are 16–32-fold higher than in non-*vanA* carriers [131]. In another study of 403 enterococcal isolates from Canada, 98.7 % of *E. faecalis* isolates were susceptible to oritavancin, and all vancomycin-resistant *E. faecium* isolates were inhibited by oritavancin concentrations ≤0.5 μg/mL [132]. Data among strains resistant to daptomycin are scarce, however, and clinical data have not yet emerged to demonstrate the efficacy of oritavancin in systemic infections. The one-time dosing regimen, while great for skin infections, also leads to troublesome pharmacokinetic issues with prolonged dosing regimens such as those used in deep-seated infections. Still, oritavancin is an interesting agent that warrants further study against VRE.

Although there are a few agents that possess activity against daptomycin-nonsusceptible enterococci, options are limited. Other than the agents listed above, tigecycline and quinupristin-dalfopristin possess in vitro activity against enterococci. Tigecycline, however, possesses little to no clinical utility as a single agent, and quinupristin-dalfopristin is limited by tolerability issues and inherent resistance among *E. faecalis*. Owing to the limited data and options available to us against daptomycin-nonsusceptible enterococci, further study is imperative to guide future clinical decision-making.

6 Conclusions

Daptomycin is an important piece of the current therapeutic armament against multidrug-resistant bacteria. Although currently rare, the clinical emergence of daptomycin nonsusceptibility is concerning and warrants an understanding of its mechanisms and possible methods of combating isolates with this resistant phenotype. Going forward, it is imperative that daptomycin is employed as effectively as possible in the clinical setting based on the available data if we are to preserve it as an effective option.

References

1. Tally FP, DeBruin MF. Development of daptomycin for gram-positive infections. J Antimicrob Chemother. 2000;46:523–6.
2. Robbel L, Marahiel MA. Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery. J Biol Chem. 2010;285:27501–8.

3. Muraih JK, Pearson A, Silverman J, et al. Oligomerization of daptomycin on membranes. Biochim Biophys Acta. 1808;2011:1154–60.
4. Jung D, Rozek A, Okon M, et al. Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. Chem Biol. 2004;11:949–57.
5. Alborn Jr WE, Allen NE, Preston DA. Daptomycin disrupts membrane potential in growing Staphylococcus aureus. Antimicrob Agents Chemother. 1991;35:2282–7.
6. Silverman JA, Perlmutter NG, Shapiro HM. Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus. Antimicrob Agents Chemother. 2003;47:2538–44.
7. Pogliano J, Pogliano N, Silverman JA. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol. 2012;194:4494–504.
8. Safdar N, Andes D, Craig WA. In vivo pharmacodynamic activity of daptomycin. Antimicrob Agents Chemother. 2004;48:63–8.
9. Werth BJ, Steed ME, Ireland CE, et al. Defining daptomycin resistance prevention exposures in vancomycin-resistant Enterococcus faecium and E. faecalis. Antimicrob Agents Chemother. 2014;58:5253–61.
10. Arbeit RD, Maki D, Tally FP, et al. The safety and efficacy of daptomycin for the treatment of complicated skin and skin-structure infections. Clin Infect Dis. 2004;38:1673–81.
11. Fowler Jr VG, Boucher HW, Corey GR, et al. Daptomycin versus standard therapy for bacteremia and endocarditis caused by Staphylococcus aureus. N Engl J Med. 2006;355:653–65.
12. Liang SY, Khair HN, McDonald JR, et al. Daptomycin versus vancomycin for osteoarticular infections due to methicillin-resistant Staphylococcus aureus (MRSA): a nested case-control study. Eur J Clin Microbiol Infect Dis. 2014;33:659–64.
13. Ramaswamy DP, Amodio-Groton M, Scholand SJ. Use of daptomycin in the treatment of vancomycin-resistant enterococcal urinary tract infections: a short case series. BMC Urol. 2013;13:33.
14. Munita JM, Murray BE, Arias CA. Daptomycin for the treatment of bacteraemia due to vancomycin-resistant enterococci. Int J Antimicrob Agents. 2014;44:387–95.
15. Pertel PE, Bernardo P, Fogarty C, et al. Effects of prior effective therapy on the efficacy of daptomycin and ceftriaxone for the treatment of community-acquired pneumonia. Clin Infect Dis. 2008;46:1142–51.
16. Silverman JA, Mortin LI, Vanpraagh AD, et al. Inhibition of daptomycin by pulmonary surfactant: in vitro modeling and clinical impact. J Infect Dis. 2005;191:2149–52.
17. Sharma M, Riederer K, Chase P, et al. High rate of decreasing daptomycin susceptibility during the treatment of persistent Staphylococcus aureus bacteremia. Eur J Clin Microbiol Infect Dis. 2008;27:433–7.
18. Sakoulas G, Rose W, Rybak MJ, et al. Evaluation of endocarditis caused by methicillin-susceptible Staphylococcus aureus developing nonsusceptibility to daptomycin. J Clin Microbiol. 2008;46:220–4.
19. Humphries RM, Kelesidis T, Tewhey R, et al. Genotypic and phenotypic evaluation of the evolution of high-level daptomycin nonsusceptibility in vancomycin-resistant Enterococcus faecium. Antimicrob Agents Chemother. 2012;56:6051–3.
20. Munita JM, Mishra NN, Alvarez D, et al. Failure of high-dose daptomycin for bacteremia caused by daptomycin-susceptible Enterococcus faecium harboring LiaSR substitutions. Clin Infect Dis. 2014;59:1277–80.
21. Sader HS, Farrell DJ, Jones RN. Antimicrobial activity of daptomycin tested against gram-positive strains collected in European hospitals: results from 7 years of resistance surveillance (2003–2009). J Chemother. 2011;23:200–6.
22. Sader HS, Moet GJ, Farrell DJ, et al. Antimicrobial susceptibility of daptomycin and comparator agents tested against methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci: trend analysis of a 6-year period in US medical centers (2005–2010). Diagn Microbiol Infect Dis. 2011;70:412–6.
23. Sader HS, Farrell DJ, Flamm RK, et al. Daptomycin activity tested against 164457 bacterial isolates from hospitalised patients: summary of 8 years of a Worldwide Surveillance Programme (2005–2012). Int J Antimicrob Agents. 2014;43:465–9.
24. Friedman L, Alder JD, Silverman JA. Genetic changes that correlate with reduced susceptibility to daptomycin in Staphylococcus aureus. Antimicrob Agents Chemother. 2006;50:2137–45.
25. Patel D, Husain M, Vidaillac C, et al. Mechanisms of in-vitro-selected daptomycin-non-susceptibility in Staphylococcus aureus. Int J Antimicrob Agents. 2011;38:442–6.
26. Murthy MH, Olson ME, Wickert RW, et al. Daptomycin non-susceptible meticillin-resistant Staphylococcus aureus USA 300 isolate. J Med Microbiol. 2008;57:1036–8.
27. Yang SJ, Xiong YQ, Dunman PM, et al. Regulation of mprF in daptomycin-nonsusceptible Staphylococcus aureus strains. Antimicrob Agents Chemother. 2009;53:2636–7.
28. Rubio A, Conrad M, Haselbeck RJ, et al. Regulation of mprF by antisense RNA restores daptomycin susceptibility to daptomycin-resistant isolates of Staphylococcus aureus. Antimicrob Agents Chemother. 2011;55:364–7.
29. Oku Y, Kurokawa K, Ichihashi N, et al. Characterization of the Staphylococcus aureus mprF gene, involved in lysinylation of phosphatidylglycerol. Microbiology. 2004;150:45–51.
30. Staubitz P, Neumann H, Schneider T, et al. MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance. FEMS Microbiol Lett. 2004;231:67–71.
31. Ernst CM, Staubitz P, Mishra NN, et al. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog. 2009;5, e1000660.
32. Mishra NN, Yang SJ, Chen L, et al. Emergence of daptomycin resistance in daptomycin-naive rabbits with methicillin-resistant Staphylococcus aureus prosthetic joint infection is associated with resistance to host defense cationic peptides and mprF polymorphisms. PLoS One. 2013;8, e71151.
33. Jones T, Yeaman MR, Sakoulas G, et al. Failures in clinical treatment of Staphylococcus aureus infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrob Agents Chemother. 2008;52:269–78.
34. Kaatz GW, Lundstrom TS, Seo SM. Mechanisms of daptomycin resistance in Staphylococcus aureus. Int J Antimicrob Agents. 2006;28:280–7.
35. Yang SJ, Kreiswirth BN, Sakoulas G, et al. Enhanced expression of dltABCD is associated with the development of daptomycin nonsusceptibility in a clinical endocarditis isolate of Staphylococcus aureus. J Infect Dis. 2009;200:1916–20.
36. Mishra NN, Bayer AS, Weidenmaier C, et al. Phenotypic and genotypic characterization of daptomycin-resistant methicillin-resistant Staphylococcus aureus strains: relative roles of mprF and dlt operons. PLoS One. 2014;9, e107426.
37. Mishra NN, Rubio A, Nast CC, et al. Differential adaptations of methicillin-resistant Staphylococcus aureus to serial in vitro passage in daptomycin: evolution of daptomycin resistance and role of membrane carotenoid content and fluidity. Int J Microbiol. 2012;2012:683450.
38. Mishra NN, Yang SJ, Sawa A, et al. Analysis of cell membrane characteristics of in vitro-selected daptomycin-resistant strains of methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2009;53:2312–8.

39. Mishra NN, Liu GY, Yeaman MR, et al. Carotenoid-related alteration of cell membrane fluidity impacts Staphylococcus aureus susceptibility to host defense peptides. Antimicrob Agents Chemother. 2011;55:526–31.
40. Clausen VA, Bae W, Throup J, et al. Biochemical characterization of the first essential two-component signal transduction system from Staphylococcus aureus and Streptococcus pneumoniae. J Mol Microbiol Biotechnol. 2003;5:252–60.
41. Bayer AS, Schneider T, Sahl HG. Mechanisms of daptomycin resistance in Staphylococcus aureus: role of the cell membrane and cell wall. Ann N Y Acad Sci. 2013;1277:139–58.
42. Bertsche U, Weidenmaier C, Kuehner D, et al. Correlation of daptomycin resistance in a clinical Staphylococcus aureus strain with increased cell wall teichoic acid production and D-alanylation. Antimicrob Agents Chemother. 2011;55:3922–8.
43. Bertsche U, Yang SJ, Kuehner D, et al. Increased cell wall teichoic acid production and D-alanylation are common phenotypes among daptomycin-resistant methicillin-resistant Staphylococcus aureus (MRSA) clinical isolates. PLoS One. 2013;8, e67398.
44. Cafiso V, Bertuccio T, Purrello S, et al. dltA overexpression: a strain-independent keystone of daptomycin resistance in methicillin-resistant Staphylococcus aureus. Int J Antimicrob Agents. 2014;43:26–31.
45. Kuroda M, Kuroda H, Oshima T, et al. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. Mol Microbiol. 2003;49:807–21.
46. Kuroda M, Ohta T, Uchiyama I, et al. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet. 2001;357:1225–40.
47. Dengler V, Meier PS, Heusser R, et al. Induction kinetics of the Staphylococcus aureus cell wall stress stimulon in response to different cell wall active antibiotics. BMC Microbiol. 2011;11:16.
48. Camargo IL, Neoh HM, Cui L, et al. Serial daptomycin selection generates daptomycin-nonsusceptible Staphylococcus aureus strains with a heterogeneous vancomycin-intermediate phenotype. Antimicrob Agents Chemother. 2008;52:4289–99.
49. Yin S, Daum RS, Boyle-Vavra S. VraSR two-component regulatory system and its role in induction of pbp2 and vraSR expression by cell wall antimicrobials in Staphylococcus aureus. Antimicrob Agents Chemother. 2006;50:336–43.
50. Gardete S, Wu SW, Gill S, et al. Role of VraSR in antibiotic resistance and antibiotic-induced stress response in Staphylococcus aureus. Antimicrob Agents Chemother. 2006;50:3424–34.
51. Muthaiyan A, Silverman JA, Jayaswal RK, et al. Transcriptional profiling reveals that daptomycin induces the Staphylococcus aureus cell wall stress stimulon and genes responsive to membrane depolarization. Antimicrob Agents Chemother. 2008;52:980–90.
52. Mehta S, Cuirolo AX, Plata KB, et al. VraSR two-component regulatory system contributes to mprF-mediated decreased susceptibility to daptomycin in in vivo-selected clinical strains of methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2012;56:92–102.
53. Mwangi MM, Wu SW, Zhou Y, et al. Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. Proc Natl Acad Sci U S A. 2007;104:9451–6.
54. Cui L, Tominaga E, Neoh HM, et al. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate Staphylococcus aureus. Antimicrob Agents Chemother. 2006;50:1079–82.
55. Patel JB, Jevitt LA, Hageman J, et al. An association between reduced susceptibility to daptomycin and reduced susceptibility to vancomycin in Staphylococcus aureus. Clin Infect Dis. 2006;42:1652–3.
56. Sakoulas G, Alder J, Thauvin-Eliopoulos C, et al. Induction of daptomycin heterogeneous susceptibility in Staphylococcus aureus by exposure to vancomycin. Antimicrob Agents Chemother. 2006;50:1581–5.
57. Pillai SK, Gold HS, Sakoulas G, et al. Daptomycin nonsusceptibility in Staphylococcus aureus with reduced vancomycin susceptibility is independent of alterations in MprF. Antimicrob Agents Chemother. 2007;51:2223–5.
58. Ruzin A, Severin A, Moghazeh SL, et al. Inactivation of mprF affects vancomycin susceptibility in Staphylococcus aureus. Biochim Biophys Acta. 1621;2003:117–21.
59. Rose WE, Leonard SN, Sakoulas G, et al. Daptomycin activity against Staphylococcus aureus following vancomycin exposure in an in vitro pharmacodynamic model with simulated endocardial vegetations. Antimicrob Agents Chemother. 2008;52:831–6.
60. Julian K, Kosowska-Shick K, Whitener C, et al. Characterization of a daptomycin-nonsusceptible vancomycin-intermediate Staphylococcus aureus strain in a patient with endocarditis. Antimicrob Agents Chemother. 2007;51:3445–8.
61. Vidaillac C, Gardete S, Tewhey R, et al. Alternative mutational pathways to intermediate resistance to vancomycin in methicillin-resistant Staphylococcus aureus. J Infect Dis. 2013;208:67–74.
62. Trier DA, Gank KD, Kupferwasser D, et al. Platelet antistaphylococcal responses occur through P2X1 and P2Y12 receptor-induced activation and kinocidin release. Infect Immun. 2008;76:5706–13.
63. Xiong YQ, Mukhopadhyay K, Yeaman MR, et al. Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of Staphylococcus aureus. Antimicrob Agents Chemother. 2005;49:3114–21.
64. Bayer AS, Mishra NN, Sakoulas G, et al. Heterogeneity of mprF sequences in methicillin-resistant Staphylococcus aureus clinical isolates: role in cross-resistance between daptomycin and host defense antimicrobial peptides. Antimicrob Agents Chemother. 2014;58:7462–7.
65. Cheung AL, Bayer AS, Yeaman MR, et al. Site-specific mutation of the sensor kinase GraS in Staphylococcus aureus alters the adaptive response to distinct cationic antimicrobial peptides. Infect Immun. 2014;82:5336–45.
66. Rose WE, Leonard SN, Rybak MJ. Evaluation of daptomycin pharmacodynamics and resistance at various dosage regimens against Staphylococcus aureus isolates with reduced susceptibilities to daptomycin in an in vitro pharmacodynamic model with simulated endocardial vegetations. Antimicrob Agents Chemother. 2008;52:3061–7.
67. Ortwine JK, Werth BJ, Sakoulas G, et al. Reduced glycopeptide and lipopeptide susceptibility in Staphylococcus aureus and the "seesaw effect": taking advantage of the back door left open? Drug Resist Updat. 2013;16:73–9.
68. Sieradzki K, Tomasz A. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of Staphylococcus aureus. J Bacteriol. 1999;181:7566–70.
69. Werth BJ, Vidaillac C, Murray KP, et al. Novel combinations of vancomycin plus ceftaroline or oxacillin against methicillin-resistant vancomycin-intermediate Staphylococcus aureus (VISA) and heterogeneous VISA. Antimicrob Agents Chemother. 2013;57:2376–9.
70. Leonard SN. Synergy between vancomycin and nafcillin against Staphylococcus aureus in an in vitro pharmacokinetic/pharmacodynamic model. PLoS One. 2012;7, e42103.
71. Leonard SN, Rolek KM. Evaluation of the combination of daptomycin and nafcillin against vancomycin-intermediate Staphylococcus aureus. J Antimicrob Chemother. 2013;68:644–7.
72. Fox PM, Lampen RJ, Stumpf KS, et al. Successful therapy of experimental endocarditis caused by vancomycin-resistant

Staphylococcus aureus with a combination of vancomycin and beta-lactam antibiotics. Antimicrob Agents Chemother. 2006;50:2951–6.
73. Climo MW, Patron RL, Archer GL. Combinations of vancomycin and beta-lactams are synergistic against staphylococci with reduced susceptibilities to vancomycin. Antimicrob Agents Chemother. 1999;43:1747–53.
74. Yang SJ, Xiong YQ, Boyle-Vavra S, et al. Daptomycin-oxacillin combinations in treatment of experimental endocarditis caused by daptomycin-nonsusceptible strains of methicillin-resistant Staphylococcus aureus with evolving oxacillin susceptibility (the "seesaw effect"). Antimicrob Agents Chemother. 2010;54:3161–9.
75. Dhand A, Bayer AS, Pogliano J, et al. Use of antistaphylococcal beta-lactams to increase daptomycin activity in eradicating persistent bacteremia due to methicillin-resistant Staphylococcus aureus: role of enhanced daptomycin binding. Clin Infect Dis. 2011;53:158–63.
76. Berti AD, Sakoulas G, Nizet V, et al. beta-Lactam antibiotics targeting PBP1 selectively enhance daptomycin activity against methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2013;57:5005–12.
77. Barber KE, Werth BJ, Ireland CE, et al. Potent synergy of ceftobiprole plus daptomycin against multiple strains of Staphylococcus aureus with various resistance phenotypes. J Antimicrob Chemother. 2014;69:3006–10.
78. Werth BJ, Sakoulas G, Rose WE, et al. Ceftaroline increases membrane binding and enhances the activity of daptomycin against daptomycin-nonsusceptible vancomycin-intermediate Staphylococcus aureus in a pharmacokinetic/pharmacodynamic model. Antimicrob Agents Chemother. 2013;57:66–73.
79. Rose WE, Schulz LT, Andes D, et al. Addition of ceftaroline to daptomycin after emergence of daptomycin-nonsusceptible Staphylococcus aureus during therapy improves antibacterial activity. Antimicrob Agents Chemother. 2012;56:5296–302.
80. Werth BJ, Barber KE, Ireland CE, et al. Evaluation of ceftaroline, vancomycin, daptomycin, or ceftaroline plus daptomycin against daptomycin-nonsusceptible methicillin-resistant Staphylococcus aureus in an in vitro pharmacokinetic/pharmacodynamic model of simulated endocardial vegetations. Antimicrob Agents Chemother. 2014;58:3177–81.
81. Sakoulas G, Moise PA, Casapao AM, et al. Antimicrobial salvage therapy for persistent staphylococcal bacteremia using daptomycin plus ceftaroline. Clin Ther. 2014;36:1317–33.
82. Steed ME, Vidaillac C, Rybak MJ. Novel daptomycin combinations against daptomycin-nonsusceptible methicillin-resistant Staphylococcus aureus in an in vitro model of simulated endocardial vegetations. Antimicrob Agents Chemother. 2010;54:5187–92.
83. Steed ME, Werth BJ, Ireland CE, et al. Evaluation of the novel combination of high-dose daptomycin plus trimethoprim-sulfamethoxazole against daptomycin-nonsusceptible methicillin-resistant Staphylococcus aureus using an in vitro pharmacokinetic/pharmacodynamic model of simulated endocardial vegetations. Antimicrob Agents Chemother. 2012;56:5709–14.
84. Avery LM, Steed ME, Woodruff AE, et al. Daptomycin-nonsusceptible vancomycin-intermediate staphylococcus aureus vertebral osteomyelitis cases complicated by bacteremia treated with high-dose daptomycin and trimethoprim-sulfamethoxazole. Antimicrob Agents Chemother. 2012;56:5990–3.
85. Claeys KC, Smith JR, Casapao AM, Mynatt RP, Avery L, Shroff A, Yamamura D, Davis SL, Rybak MJ. Combination daptomycin and trimethoprim/sulfamethoxazole on clinical outcomes in methicillin-resistant S. aureus Infections. Antimicrob Agents Chemother. 2015;59(4):1969–76.
86. Rybak JM, Barber KE, Rybak MJ. Current and prospective treatments for multidrug-resistant gram-positive infections. Expert Opin Pharmacother. 2013;14:1919–32.
87. Barber KE, Ireland CE, Bukavyn N, et al. Observation of "seesaw effect" with vancomycin, teicoplanin, daptomycin and ceftaroline in 150 unique MRSA strains. Infect Dis Ther. 2014;3:35–43.
88. Sader HS, Flamm RK, Jones RN. Antimicrobial activity of ceftaroline tested against staphylococci with reduced susceptibility to linezolid, daptomycin, or vancomycin from U.S. hospitals, 2008 to 2011. Antimicrob Agents Chemother. 2013;57:3178–81.
89. Casapao AM, Davis SL, Barr VO, et al. Large retrospective evaluation of the effectiveness and safety of ceftaroline fosamil therapy. Antimicrob Agents Chemother. 2014;58:2541–6.
90. Rybak JM, Marx K, Martin CA. Early experience with tedizolid: clinical efficacy, pharmacodynamics, and resistance. Pharmacotherapy. 2014;34:1198–208.
91. Flamm RK, Mendes RE, Hogan PA, et al. In vitro activity of linezolid as assessed through the 2013 LEADER surveillance program. Diagn Microbiol Infect Dis. 2015;81(4):283–9.
92. Sahm DF, Deane J, Bien PA, et al. Results of the surveillance of Tedizolid activity and resistance program: in vitro susceptibility of gram-positive pathogens collected in 2011 and 2012 from the United States and Europe. Diagn Microbiol Infect Dis. 2015;81:112–8.
93. (CLSI) CaLSI. Performance standards for antimicrobial susceptibility testing; 24th Informational Supplement. CLSI M100-S24. Clinical and Laboratory Science Institute (CLSI), Wayne, PA, 2014.
94. Mendes RE, Sader HS, Flamm RK, et al. Telavancin in vitro activity against a collection of methicillin-resistant Staphylococcus aureus isolates, including resistant subsets, from the United States. Antimicrob Agents Chemother. 2015;59:1811–4.
95. Steed ME, Vidaillac C, Rybak MJ. Evaluation of telavancin activity versus daptomycin and vancomycin against daptomycin-nonsusceptible Staphylococcus aureus in an in vitro pharmacokinetic/pharmacodynamic model. Antimicrob Agents Chemother. 2012;56:955–9.
96. Xiong YQ, Hady WA, Bayer AS, et al. Telavancin in therapy of experimental aortic valve endocarditis in rabbits due to daptomycin-nonsusceptible methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2012;56:5528–33.
97. Joson J, Grover C, Downer C, et al. Successful treatment of methicillin-resistant Staphylococcus aureus mitral valve endocarditis with sequential linezolid and telavancin monotherapy following daptomycin failure. J Antimicrob Chemother. 2011;66:2186–8.
98. Vidaillac C, Parra-Ruiz J, Rybak MJ. In vitro time-kill analysis of oritavancin against clinical isolates of methicillin-resistant Staphylococcus aureus with reduced susceptibility to daptomycin. Diagn Microbiol Infect Dis. 2011;71:470–3.
99. McKay GA, Beaulieu S, Arhin FF, et al. Time-kill kinetics of oritavancin and comparator agents against Staphylococcus aureus, Enterococcus faecalis and Enterococcus faecium. J Antimicrob Chemother. 2009;63:1191–9.
100. Mendes RE, Sader HS, Flamm RK, et al. Oritavancin activity against Staphylococcus aureus causing invasive infections in U.S. and European hospitals: a 5-year international surveillance program. Antimicrob Agents Chemother. 2014;58:2921–4.
101. Jones RN, Sader HS, Flamm RK. Update of dalbavancin spectrum and potency in the USA: report from the SENTRY Antimicrobial Surveillance Program (2011). Diagn Microbiol Infect Dis. 2013;75:304–7.
102. Lewis 2nd JS, Owens A, Cadena J, et al. Emergence of daptomycin resistance in Enterococcus faecium during daptomycin therapy. Antimicrob Agents Chemother. 2005;49:1664–5.
103. Munoz-Price LS, Lolans K, Quinn JP. Emergence of resistance to daptomycin during treatment of vancomycin-resistant Enterococcus faecalis infection. Clin Infect Dis. 2005;41:565–6.

104. Jordan S, Junker A, Helmann JD, et al. Regulation of LiaRS-dependent gene expression in bacillus subtilis: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. J Bacteriol. 2006;188:5153–66.
105. Wolf D, Kalamorz F, Wecke T, et al. In-depth profiling of the LiaR response of Bacillus subtilis. J Bacteriol. 2010;192:4680–93.
106. Mascher T, Zimmer SL, Smith TA, et al. Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of Bacillus subtilis. Antimicrob Agents Chemother. 2004;48:2888–96.
107. Larson TJ, Ehrmann M, Boos W. Periplasmic glycerophosphodiester phosphodiesterase of Escherichia coli, a new enzyme of the glp regulon. J Biol Chem. 1983;258:5428–32.
108. Mileykovskaya E, Dowhan W. Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim Biophys Acta. 1788;2009:2084–91.
109. Tropp BE. Cardiolipin synthase from Escherichia coli. Biochim Biophys Acta. 1997;1348:192–200.
110. Zhang YM, Rock CO. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol. 2008;6:222–33.
111. Arias CA, Panesso D, McGrath DM, et al. Genetic basis for in vivo daptomycin resistance in enterococci. N Engl J Med. 2011;365:892–900.
112. Tran TT, Panesso D, Gao H, et al. Whole-genome analysis of a daptomycin-susceptible enterococcus faecium strain and its daptomycin-resistant variant arising during therapy. Antimicrob Agents Chemother. 2013;57:261–8.
113. Munita JM, Tran TT, Diaz L, et al. A liaF codon deletion abolishes daptomycin bactericidal activity against vancomycin-resistant Enterococcus faecalis. Antimicrob Agents Chemother. 2013;57:2831–3.
114. Reyes J, Panesso D, Tran TT, et al. A liaR deletion restores susceptibility to daptomycin and antimicrobial peptides in multidrug-resistant Enterococcus faecalis. J Infect Dis. 2015;211(8):1317–25.
115. Palmer KL, Daniel A, Hardy C, et al. Genetic basis for daptomycin resistance in enterococci. Antimicrob Agents Chemother. 2011;55:3345–56.
116. Tran TT, Panesso D, Mishra NN, et al. Daptomycin-resistant Enterococcus faecalis diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. mBio. 2013;4:e00281–313.
117. Diaz L, Tran TT, Munita JM, et al. Whole-genome analyses of Enterococcus faecium isolates with diverse daptomycin MICs. Antimicrob Agents Chemother. 2014;58:4527–34.
118. Steed ME, Vidaillac C, Rose WE, et al. Characterizing vancomycin-resistant Enterococcus strains with various mechanisms of daptomycin resistance developed in an in vitro pharmacokinetic/pharmacodynamic model. Antimicrob Agents Chemother. 2011;55:4748–54.
119. Hall Snyder A, Werth BJ, Barber KE, et al. Evaluation of the novel combination of daptomycin plus ceftriaxone against vancomycin-resistant enterococci in an in vitro pharmacokinetic/pharmacodynamic simulated endocardial vegetation model. J Antimicrob Chemother. 2014;69:2148–54.
120. Munita JM, Panesso D, Diaz L, et al. Correlation between mutations in liaFSR of Enterococcus faecium and MIC of daptomycin: revisiting daptomycin breakpoints. Antimicrob Agents Chemother. 2012;56:4354–9.
121. Sakoulas G, Nonejuie P, Nizet V, et al. Treatment of high-level gentamicin-resistant Enterococcus faecalis endocarditis with daptomycin plus ceftaroline. Antimicrob Agents Chemother. 2013;57:4042–5.
122. Sakoulas G, Bayer AS, Pogliano J, et al. Ampicillin enhances daptomycin- and cationic host defense peptide-mediated killing of ampicillin- and vancomycin-resistant Enterococcus faecium. Antimicrob Agents Chemother. 2012;56:838–44.
123. Smith JR, Barber KE, Raut A, Aboutaleb M, Sakoulas G, Rybak MJ. Beta-lactam combinations with daptomycin provide synergy against vancomycin-resistant Enterococcus faecalis and Enterococcus faecium. J Antimicrob Chemother. 2015;70(6):1738–43.
124. Sakoulas G, Rose W, Nonejuie P, et al. Ceftaroline restores daptomycin activity against daptomycin-nonsusceptible vancomycin-resistant Enterococcus faecium. Antimicrob Agents Chemother. 2014;58:1494–500.
125. Werth BJ, Barber KE, Tran KN, et al. Ceftobiprole and ampicillin increase daptomycin susceptibility of daptomycin-susceptible and -resistant VRE. J Antimicrob Chemother. 2015;70(2):489–93.
126. Henry X, Verlaine O, Amoroso A, et al. Activity of ceftaroline against Enterococcus faecium PBP5. Antimicrob Agents Chemother. 2013;57:6358–60.
127. Zhanel GG, Calic D, Schweizer F, et al. New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. Drugs. 2010;70:859–86.
128. Crank CW, Scheetz MH, Brielmaier B, et al. Comparison of outcomes from daptomycin or linezolid treatment for vancomycin-resistant enterococcal bloodstream infection: a retrospective, multicenter, cohort study. Clin Ther. 2010;32:1713–9.
129. Whang DW, Miller LG, Partain NM, et al. Systematic review and meta-analysis of linezolid and daptomycin for treatment of vancomycin-resistant enterococcal bloodstream infections. Antimicrob Agents Chemother. 2013;57:5013–8.
130. Balli EP, Venetis CA, Miyakis S. Systematic review and meta-analysis of linezolid versus daptomycin for treatment of vancomycin-resistant enterococcal bacteremia. Antimicrob Agents Chemother. 2014;58:734–9.
131. Mendes RE, Woosley LN, Farrell DJ, et al. Oritavancin activity against vancomycin-susceptible and vancomycin-resistant Enterococci with molecularly characterized glycopeptide resistance genes recovered from bacteremic patients, 2009–2010. Antimicrob Agents Chemother. 2012;56:1639–42.
132. Karlowsky JA, Walkty AJ, Baxter MR, et al. In vitro activity of oritavancin against Gram-positive pathogens isolated in Canadian hospital laboratories from 2011 to 2013. Diagn Microbiol Infect Dis. 2014;80:311–5.

Resistance to Linezolid

22

Eleni Ntokou and Birte Vester

1 Introduction

Linezolid is an antimicrobial agent that binds to the bacterial ribosome and thereby inhibits protein synthesis. Soon after its release as a clinical drug, it became clear that bacteria could become resistant to linezolid. The resistance mechanisms are mainly causing alteration of the drug target site, but probably efflux might also play a role. The resistance is still rare in surveillance studies, but outbreaks of resistant clones from hospitals have been observed. So far the main mechanisms of resistance are occurrence of mutations in ribosomal genes or obtaining plasmids with a gene coding for a methyltransferase providing resistance. The most obvious way to avoid resistance may be development of derivatives of linezolid overcoming the known resistance mechanisms.

2 Linezolid and Its Derivatives

Linezolid belongs to the oxazolidinones, a synthetic drug class, and is one of few new drugs on the market for antibiotics in many years. The history of the discovery of linezolid has already been extensively reviewed [1–4]. Oxazolidinones were primarily identified and patented by E. I. du Pont de Nemours & Company (DuPont) in 1978 [5]. DUP-105 and DUP-721 were developed as first lead compounds of oxazolidinone antibacterials and showed activity against Gram-positive bacteria, but the project was terminated due to lethal toxicity in animal models [4, 6]. Later, scientists at Upjohn Laboratories started a project in order to modify the original compound and produce new oxazolidinones, with better antibacterial activities and higher safety levels. Among a series of oxazolidinones, PNU-100766 (Linezolid) and PNU-100592 (Eperezolid) showed oral efficacy, good water solubility, and good activity against Gram-positive bacteria. Both of them were further evaluated by phase 1 clinical trials but only linezolid proceeded to phase 2 clinical trials due to its superior bioavailability. Linezolid was approved by FDA in 2000 and marketed as Zyvox™ [4, 7]. Linezolid has been employed for treating diseases caused by Gram-positive bacteria [8, 9], which include streptococci, vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), some Gram-negative anaerobic species, and *Mycobacterium tuberculosis* [10–12].

Linezolid (Fig. 22.1a) is proven to be a highly effective drug and a good alternative for the treatment of difficult infections being able to be administered either intravenously or orally. However, it does have some liabilities and can cause adverse effects such as interaction with serotonergic agents that could lead to serotonin syndrome in patients with depression, and production of reversible thrombocytopenia and bone marrow suppression when given for prolonged periods of time [13, 14]. The biggest issue raised by the use of linezolid in clinical practice, soon after it was available on the market, was the appearance of linezolid-resistant strains of *S. aureus* and enterococci [15, 16]. The mechanisms that confer this resistance will be described in following sections of this chapter. However, development of derivatives of linezolid to overcome this issue is currently underway (Fig. 22.1) [17].

The most important linezolid derivative is currently tedizolid (Fig. 22.1b) (formerly torezolid), which was under clinical development by Cubist pharmaceuticals for the treatment of serious Gram-positive infections. Tedizolid phosphate (TR-701) is an inactive prodrug that is chemically converted by serum phosphatases to the active form tedizolid (TR-700) [18]. Tedizolid phosphate was approved by the FDA (20/06/2014) with the commercial name Sivextro™. Sivextro is indicated for the treatment of acute bacterial skin and skin structure infections (ABSSSI). It is active against Gram-positive organisms, including staphylococci, enterococci, streptococci, and certain anaerobes [19, 20].

E. Ntokou, Ph.D. • B. Vester, Ph.D. (✉)
Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark
e-mail: elenin@bmb.sdu.dk; b.vester@bmb.sdu.dk

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_22

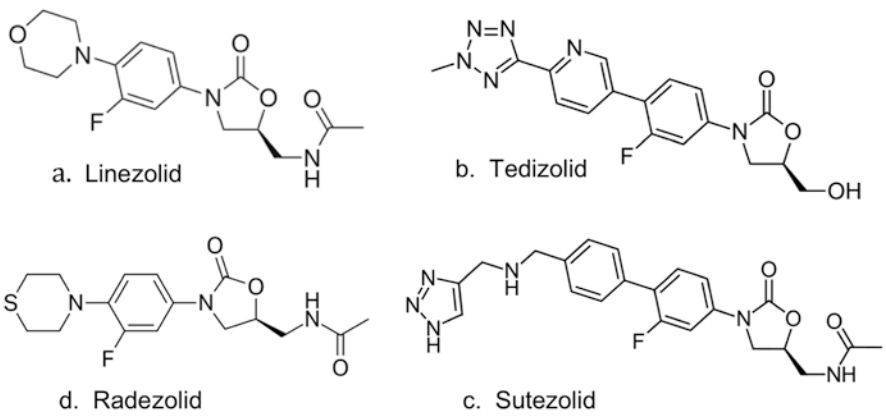


Fig. 22.1 Chemical structural formula for linezolid (**a**) and three derivatives (**b**)–(**d**)

Tedizolid demonstrates a greater potency than linezolid, at least fourfold for all bacteria tested [20]. Of particular interest, were the tested linezolid-resistant *S. aureus* strains, which possess mutations in chromosomal genes encoding ribosomal rRNA and proteins, or carrying the horizontally transferable *cfr* gene. Methylation of A2503 of 23S rRNA by the Cfr methyltransferase confers resistance to linezolid but not to tedizolid because of structural differences between the two drugs [21]. Initial studies have also shown that tedizolid may not have the negative effects on serotonergic agents and thrombocytopenia as linezolid show [22, 23].

Other derivatives under investigation are radezolid and sutezolid (Fig. 22.1c, d). Radezolid is a unique oxazolidinone because it has activity against fastidious Gram-negative bacteria like *H. influenzae* and *M. catarrhalis,* as well as against Gram-positive bacteria, including MRSA, linezolid-resistant staphylococci and enterococci [24]. Radezolid has completed two phase 2 clinical trials to date: the first in community-acquired pneumonia (CAP) and the second trial in complicated skin and skin structure infections (cSSSI) [2]. To date, phase III trials have not been initiated [25]. It is unclear at this point, based upon published literature, whether radezolid has any advantages over linezolid. Sutezolid is a linezolid derivative with superior bactericidal activity against *M. tuberculosis* as demonstrated by a Phase 2 clinical study [26].

Because linezolid resistance has started to arise by various mechanisms, in various bacteria, the development of new derivatives seems to be the next step in the battle against isolates resistant to this class. The derivatives mentioned earlier in this section demonstrate higher potency and lower resistance rates compared to linezolid. Due to their properties, they could potentially compensate at occasions where linezolid-resistant isolates arise. They will probably not yet replace linezolid in clinical use, as it is still a widely used antibiotic with relatively low incidence of resistance.

3 Mechanism of Action of Linezolid

Early studies of the effect of oxazolidinones pointed to inhibition of protein synthesis in growing bacteria [27] and suggested an effect on synthesis initiation, which was also supported by later studies [28, 29]. Studies of the effect on peptidyl transferase using puromycin reactions reported contradicting results that might be due to the relative unnatural conditions of these assays. Other studies demonstrated frame-shifting and nonsense suppression [30] and effect on fMet-tRNA binding and translocation [31].

The fact that linezolid binds to the peptidyl transferase center (PTC) of the bacterial ribosome (illustrated in Fig. 22.2) was first indicated by mutations in 23S ribosomal RNA conferring resistance [32], 23S mutagenesis studies, and cross-linking studies [33, 34]. The site was finally confirmed and defined in 2008 by crystal structures of linezolid bound to the 50S ribosomal subunit from the archaeon *Haloarcula marismortui* [35] and from the bacterium *Deinococcus radiodurans* [36]. The site is in the bottom of the cleft of the 50S ribosomal subunit where the 3′-ends of aminoacyl-tRNA and peptidyl-tRNA are positioned for peptide transfer (Fig. 22.2b), and is highly conserved in all bacteria. The same site in the ribosome binds other antibiotics such as chloramphenicol, clindamycin, tiamulin, and streptogramin A, several of which are characterized as peptidyl transferase inhibitors. It seems like the size and the environment of the PTC facilitates binding of a range of antibiotics, which at binding interfere with the peptide transfer process. They can either disturb the positioning of aminoacyl-tRNA or peptidyl-tRNA for peptide transfer or directly block some movements required during peptide transfer. How the effect will show up in various assays to elucidate the specific mechanism will also depend on their exact competition with the components of the peptide synthesis apparatus. A very

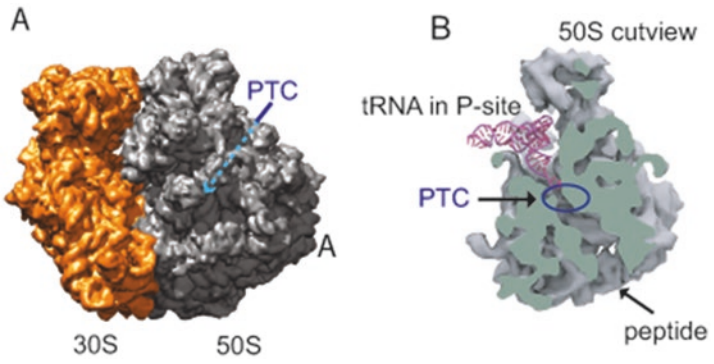


Fig. 22.2 (**a**) A model of the two ribosomal subunits in bacteria (based on PDB: 4YBB). The *arrow* points to the peptidyl transferase center in the middle of 50S where the amino acids are added together and where linezolid binds. (**b**) A cut-view of the 50S subunit (based on PDB: 2 J00, 2 J01, 2 J02, 2 J03), again showing the PTC area in the *blue circle*

recent study of ribosome function in a linezolid-resistant *Staphylococcus epidermidis* mutant showed a functional and structural adaptation of ribosomes. The study reported an increased peptidyl transferase activity, as measured by puromycin reactivity, as well as an enhanced growth rate in the presence of linezolid [37]. Even though the very exact step of inhibition has not been determined for oxazolidinones and maybe will never be completely elucidated, as more than one step might be involved, it can be concluded that the general effect of linezolid is inhibition of protein synthesis by binding to the peptidyl transferase center of the bacterial ribosome and affecting some step directly related to the peptidyl transferase reaction.

4 Mechanisms of Resistance

Several ways of resistance to linezolid have been published. The very well investigated and proven ones are mutations in 23S rRNA in the peptidyl transferase area of the ribosome, and methylation of 23S rRNA nucleotide A2503. The less proven but highly indicative ones are mutations in the ribosomal protein L3 and efflux. In addition, mutations in ribosomal protein L4 have been connected with reduced linezolid susceptibility but the extent of this correlation remains to be elucidated. Finally, fitness cost in relation to resistance seems to be an issue. The following section will review the present knowledge of this field.

4.1 Resistance Caused by 23S rRNA Mutations

Although early laboratory investigations suggested that resistance to linezolid might be slow to emerge [32, 38], as almost all bacteria have multiple copies of the 23S rRNA gene, linezolid-resistant strains soon appeared [15, 39]. The first linezolid-resistant strains were associated with mutations in domain V of the 23S rRNA genes, mainly G2576U transversion. Over time various mutations have been identified in domain V of 23S rRNA (Fig. 22.3) and they remain the predominant mutations conferring linezolid resistance [55]. The G2576U transversion is the most prevalent mutation in linezolid-resistant clinical isolates, including *S. aureus*, coagulase negative staphylococci (CoNS), viridans group streptococci, *Enterococcus faecium*, and *Enterococcus faecalis* [56, 57]. The first reported linezolid-resistant enterococcal isolates were obtained from patients treated with linezolid as part of the *Linezolid Compassionate Use Program* (1999). They had the G2576U mutation in multiple operons of the 23S rRNA genes and with MICs correlating to the number of mutated operons [58].

The first clinical isolate of linezolid-resistant *S. aureus*, with a G2576U mutation, was reported in 2001 [15]. Later, this isolate was found to contain five copies of the 23S rRNA gene, all of which were mutated at position 2576 [59] and again a clear correlation between the number of mutated rRNA operons and the linezolid MIC was established [40, 60, 61]. Most reports of the G2576U mutation in clinical isolates is associated with some form of increased or prolonged linezolid treatment, and it has been shown that the duration of linezolid exposure and dose can affect the number of mutated rRNA operons and thus linezolid resistance [62]. Mutant gene-dosage effects have also been seen in laboratory-derived linezolid-resistant *S. aureus* mutants and in clinical isolates of linezolid-resistant enterococci [40, 41]. A report from 2011 demonstrated that the G2576U mutation was retained in a *Staphylococcus haemolyticus* isolate even after 30 serial passages in antibiotic-free medium [42], although some studies have documented reversion of the G2576U mutation in the absence of linezolid pressure [41, 63]. Therefore prolonged linezolid usage should be judicious and minimized in clinical settings.

The linezolid-binding site at the PTC comprises conserved nucleotides (G2061, A2451, C2452, A2503, U2504, G2505, U2506, and U2585), which interact directly with linezolid, see Fig. 22.3 [35, 36]. Laboratory derived strains selected for linezolid resistance show mutations in either nucleotides at the proximity of the binding pocket (2061, 2452, 2503, 2504, and 2505) or at nucleotides further away from it (2032, 2062, 2192 2447, 2453, 2499, 2500, 2576, 2571, 2572, 2608, and 2612) [32, 36, 38, 43–48, 64–67]. The degree of linezolid resistance is not a simple function of the

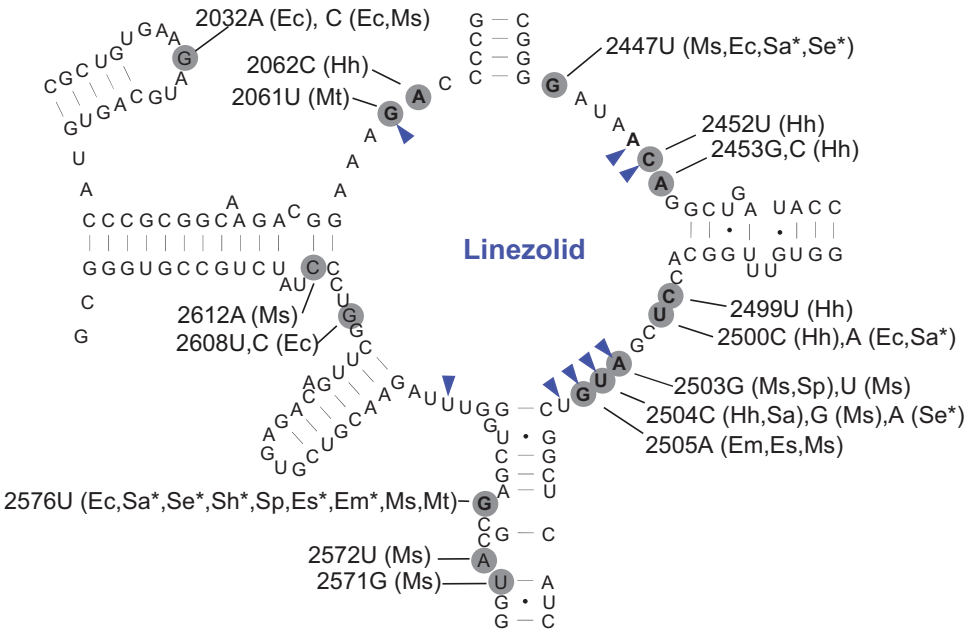


Fig. 22.3 A secondary structure model of the peptidyl transferase loop of domain V of 23S rRNA (*E. coli* sequence and numbering). *Blue triangles*: nucleotides that form the linezolid-binding pocket, *gray circles*: mutations that confer linezolid resistance with bold type for nucleotides where mutations have a considerable effect on linezolid MIC and regular type for mutations with a small to moderate effect. Organisms: *E. coli* (Ec), *S. aureus* (Sa), *S. epidermis* (Se), *S. haemolyticus* (Sh), *S. pneumoniae* (Sp), *E. faecalis* (Es), *E. faecium* (Em), *Mycobacterium smegmatis* (Ms), *M. tuberculosis* (Mt), and *Halobacterium halobium* (Hh) [32, 38, 40–54]. Asterisks indicate mutations found in clinical isolates

nucleotide-linezolid distance and distal nucleotides that do not interact with linezolid directly, as G2576U and G2447U can confer significantly high resistance [3].

Acquired resistance to linezolid has been observed in various clinical isolates of Gram-positive cocci. A methicillin-resistant *S. aureus* (MRSA) bloodstream isolate, derived from a patient exposed to a prolonged course of linezolid, developed resistance and had a U2500A mutation in the 23S rRNA and a loss of a single copy of the gene in the most resistant isolates [41]. Various clinical strains of *S. aureus, S. epidermidis, E. faecium, E. faecalis* that are highly resistant to linezolid show a variety of 23S rRNA mutations including G2447U [52], A2503G [45], U2504C [45], U2504A [51], and G2505A [68], despite of evidence of fitness cost associated with some of these mutations [60]. Some additional mutations of the 23S rRNA operons have been reported at positions G2603U [69–71] and C2534U [51, 52] but direct relationship between these mutations and linezolid resistance is not yet established.

Up to date, G2576U is the most common mutation found in clinical isolates [72]. In addition, the U2500A and G2447U mutations have been reported in linezolid-resistant clinical isolates of staphylococci and these mutations have also been shown to confer linezolid resistance in in vitro selected mutants of *E. coli* and *Mycobacterium smegmatis* [38, 46].

4.2 Resistance Caused by Alterations in 23S rRNA Modification

Ribosomal RNA is intrinsically modified with methyl groups and pseudouridine residues, and these modifications are clustered at functional centers on the ribosome. Methylations can also be an acquired trait, and it is well established that RNA modifications placed at or near an antibiotic-binding site can affect drug binding to the ribosome [73]. Resistance generally occurs either by the inactivation of an indigenous methyltransferase or the acquisition of an antibiotic resistance methyltransferase.

Some housekeeping modifications at the PTC are shown to affect linezolid susceptibility. The pseudouridylation of 23S rRNA nucleotide 2504 confers reduced susceptibility to linezolid, clindamycin, and tiamulin, suggesting that this modification may have evolved as an intrinsic resistance mechanism to protect bacteria from PTC-binding antibiotics [74]. Inactivation of the methyltransferase targeting G2445 in 23S rRNA results in decreased susceptibility to linezolid in *Streptococcus pneumoniae* [43, 75]. Likewise, a mutation inactivating the methyltransferase RlmN that methylates 23S rRNA at the C2 position of A2503 also results in slightly lowered linezolid susceptibility in *S. aureus* [76, 77]. None of these mechanisms of linezolid resistance or reduced susceptibility has yet been shown to be of clinical importance, either because of nonoccurrence or not being revealed yet. This is in contrast to the only known transferable form of linezolid resistance conferred by the multi-resistance gene *cfr* that has been found in many clinical strains, especially in *Staphylococcus*. Cfr encodes an rRNA methyltransferase [78] that adds a methyl group at the C8 position of the 23S rRNA nucleotide A2503 [79], a position interacting directly with linezolid and where mutations have shown to result in resistance (see Fig. 22.3). The methylation confers some resistance to linezolid as well as resistance to five other classes of antibiotics that bind at overlapping nonidentical sites at the PTC [80, 81]. A direct interference of the methylation with drug binding is supported by the X-ray

structures of linezolid bound to the *Deinococcus radiodurans* and *H. marismortui* 50S subunits [35, 36].

The *cfr* gene was originally discovered on multiresistance plasmids isolated during surveillance studies of florfenicol resistance in *Staphylococcus* spp. of animal origin [82, 83]. In 2005, the first *cfr*-positive clinical strain of a methicillin-resistant *S. aureus* was reported from a patient briefly treated with linezolid [84]. The strain had *cfr* on the chromosome together with the *ermB* gene on a transposable genetic element and the co-expression of these two rRNA methyltransferase genes conferred resistance to all clinically relevant antibiotics that target the large ribosomal subunit [81]. Since then a large number of staphylococcal clinical isolates containing *cfr* in different genetic contexts have been found around the world [85–90]. In some instances, a connection between the resistant isolates and prior linezolid treatment can be documented (i.e., see section on clinical linezolid-resistant strains below). The *cfr* gene has also been identified in other pathogenic bacteria, both Gram-positive and Gram-negative, often from animals and with no relation to linezolid treatment. The presence of *cfr* on mobile genetic elements such as plasmids and transposons in different geographical locations strongly suggests that it can be disseminated within the microbial community and spread among pathogenic bacteria, thus conferring resistance to linezolid without prior exposure to the drug.

4.3 Linezolid Resistance and a Conceivable Relationship to Mutations in Ribosomal Proteins L3

Mutations in the ribosomal L3 protein have recently received attention as a linezolid resistance determinant. The main part of ribosomal protein L3 is positioned on the surface of the large ribosomal subunit, but a loop extends into the PTC near the linezolid-binding site. Bacterial L3 mutations have been associated with resistance to linezolid, tiamulin/valnemulin, and anisomycin, that all bind to overlapping sites at the PTC [3]. The first L3 resistance mutation in bacteria was detected by selection with tiamulin, and its role in resistance was verified by transfection and plasmid-coded mutant L3 expression [91]. Since then, a number of studies have associated L3 mutations with linezolid resistance in various staphylococci and few other clinical relevant pathogens. A selection of some of these is displayed in Table 22.1. As evident in the table, most of the L3 mutations are present together with one or two other resistance determinants, namely 23S rRNA mutations and the *cfr* gene. Unfortunately, most of the studies presenting L3 mutations do not provide evidence that the L3 mutations are the direct cause of resistance. Seemingly, only Cfr and the 23S rRNA mutations give a medium to high resistance and it might be that the appearance of the L3 mutations are merely a selection to adopt to changes in the 23S rRNA (see section discussing fitness cost below). Nevertheless, the positions of most of the L3 mutations are relatively close to the linezolid binding in the ribosome with the closest being at a distance of approximately 7 Å [3]. Also, the relation between decreased susceptibility to the pleuromutilins retapamulin and tiamulin and L3 mutations in the same region [46, 98, 105, 106] supports the relation between L3 mutations and linezolid resistance, as pleuromutilins and linezolid bind at overlapping sites in the PTC but are otherwise very different [80]. There are also reports about L3 mutations that have been detected in linezolid susceptible strains and are therefore not considered relevant to linezolid resistance (e.g., L101V that is positioned far from the PTC [100]). At the moment, it is difficult to establish exactly which L3 mutations do have a relation to reduced linezolid susceptibility, although the circumstantial evidence point to the part of the L3 protein nearest to PTC with some variations between species. One study of in vitro development of linezolid resistance in *M. tuberculosis*, as well as findings in clinical isolates, does provide strong evidence for the involvement of an L3 C154R mutation in linezolid resistance [103]. This is also supported by another finding concerning the same L3 mutation plus a neighboring mutation in clinical samples of *M. tuberculosis* [104].

4.4 Other Aspects of Linezolid Resistance: Fitness Cost, Cross-resistance, and Enhancement of Growth

In addition to reports about L3 mutations there are also reports about L4 mutations related to linezolid resistance [3]. Part of the ribosomal protein L4 is also placed relatively close to the PTC, but in the tunnel through which nascent peptides exit the ribosome [3]. Again, most studies do not prove a relationship between L4 mutations and resistance effects, except for a surveillance study of *S. pneumoniae* with a six-nucleotide deletion in the L4 gene (ΔW65-R66) in one strain and a neighboring six-nucleotide deletion (ΔK68-G69) in another strain [107]. These deletions caused a slightly reduced susceptibility to linezolid, as evident by transformations, and were associated with a fitness cost [107]. The amino acid deletions are located in the same region as mutations known to be involved in macrolide resistance [108], and as macrolide antibiotics bind to a site neighboring, but not directly overlapping, the linezolid-binding site, we imagine the effect of these deletions is probably caused by an allosteric mechanism. In general, the L4 mutations presented in relation to linezolid resistance do not present a

Table 22.1 A selection of mutations in L3 that have been associated with linezolid resistance in staphylococci and *Mycobacterium tuberculosis*

L3 mutations	Organism	Remarks[a]	Reference
ΔF127-H146	*S. aureus*	In vitro selected mutant	[92]
Q136H/H146Δ	*S. aureus*	L4-G69A/T70P/G71S	[93]
G137A/L94V[b]	*S. epidermidis*	2576 T	[55]
G139R	*S. aureus*	T, 2576 T	[94]
G139R/M156T	*S. hominis*	T, 2576 T	[95]
ΔS145	*S. aureus*		[96]
ΔS145/H146Y	*S. aureus*	*cfr*	[97]
H146R/M156T/L101V[b]	*S. epidermidis*	T, 2215A, 2576 T, L4-ins70G	[98]
H146Q/V154L/A157R/L101V[b]	*S. epidermidis*	T, L4- ins70G/[c]	[98]
H146Q/L94V[b]	*S. epidermidis*	L4-$_{71}$GGR$_{72}$/[c]	[55]
H146Q/V154L	*S. epidermidis*	2319U, L4-$_{71}$GGR$_{72}$	[93]
H146Q/V154L/A157R	*S. epidermidis*	C2534T, L4-$_{71}$GGR$_{72}$	[99]
F147L	*S. epidermidis*	*cfr*	[93]
F147L/L94V[b]	*S. epidermidis*	L4[c]	[55]
F147L/L94V[b]	*S. epidermidis*	*cfr*, L4-G71D/[c]	[55]
F147I/L101V[b]	*S. epidermidis*	T, 2576 T	[98]
F147I	*S. hominis*	T, 2576 T	[95]
F147L/A157R/L101V[b]	*S. epidermidis*	*cfr*, L4[c]	[100]
F147L/A157R/L101V[b]	*S. epidermidis*	L4-K68R/[c]	[100]
G152D	*S. aureus*	In vitro selected mutant, 2447 T	[92]
G152D	*S. aureus*	T	[94]
G152D	*S. haemolyticus*	*cfr*	[87]
G152D/D159Y/L101V[b]	*S. epidermidis*	T, 2504A/2534 T	[51]
G152D	*S. epidermidis*	90 % T, +/− cfr, +/− 2576 T	[90]
G152D/D159E/A160P/L94V[b]	*S. epidermidis*	T, 2504A, 2530A, 2631U	[101]
G155R	*S. aureus*	In vitro selected mutant	[92]
G155R/M169L	*S. aureus*	In vitro selected mutant	[92]
M156T	*S. haemolyticus*	T, *cfr*, 2576 T	[88]
A157R	*S. epidermidis*	2447 T	[96]
S158Y/D159Y/L101V[b]	*S. epidermidis*	*cfr*	[102]
S158F/D159Y	*S. cohnii*	*cfr*, L4-N20S/A133T/V155I	[102]
Y158F	*S. cohnii*	*cfr*	[87]
ΔM169-G174	*S. aureus*	*cfr*	[97]
C154R	*M. tuberculosis*	In vitro selected mutant	[103]
C154R	*M. tuberculosis*	+/− 2061 T	[104]
H155R	*M. tuberculosis*		[104]

All isolates are clinical except from the ones depicted as "In vitro selected mutant". Information about treatment with linezolid was omitted for the strains from reference [55], because of inadequate data. The L3 positions are according to the various organisms and can thus correspond to similar positions although they have different numbering

[a]Selected additional information: treatment with linezolid (T), contain *cfr* gene (*cfr*), potential additional resistance determinants (xxxxN refers to 23S rRNA positions corresponding to *E. coli* 23S rRNA, L4-…. indicate additional mutations)

[b]L3 mutations that are considered strain markers and not relevant for antibiotic resistance are only included when found together with other mutations

[c]L4-N158S, which is not expected to influence linezolid resistance

consistent pattern and it is not definitively established which changes, if any, contribute directly to linezolid resistance.

Another potentially important resistance determinant is the presence of efflux pumps. Linezolid is not well suited for fighting Gram-negative pathogenic bacteria because they are intrinsically resistant due to efflux pumps that force linezolid out of the cell faster than it can accumulate [109, 110]. For example, a remarkably high linezolid MIC at 256 μg/mL (a 102-fold increase) was seen after cloning of a putative multidrug efflux pump from a *Vibrio cholerae* to a plasmid in a hypersensitive *E. coli* [111]. It is thus not surprising that changes in efflux in Gram-positive bacteria may influence the effect of linezolid. It has been shown that *S. aureus* possesses a gene for a major facilitator superfamily type multidrug efflux pump named LmrS that is capable of extruding linezolid [112]. Linezolid resistance caused by mutations

increasing the expression of ATP-binding cassette (ABC) transporter genes has been observed in *S. pneumoniae* [43, 75]. The mutations were found by genome sequencing of a linezolid-resistant strain and the effect was analyzed by gene disruption experiments [43]. A follow-up study involving stepwise increase of resistance by genome transformation supported the role of a specific mutation that increased expression of an ABC transporter as a resistance determinant [75]. However, not surprisingly, such changes may come with a cost in growth rate. Future experiments might reveal if efflux is a significant factor in linezolid resistance or not. As a general lesson from research on antibiotic resistance, starting to look might greatly enhance the insight.

It is one thing for bacteria to obtain a resistance determinant but another thing to sustain it and to avoid being outgrown by nonresistant neighbors. The maintenance and spread of resistance genes is related to their fitness cost. Expression of the linezolid resistance determinant Cfr in a laboratory strain had only a small effect on growth rate [113]. Such low fitness cost is troublesome as it suggests that cells can maintain a gene even in the absence of antibiotic selection. Competition experiments showed that cells with an inactivated *rlmN* gene (i.e., showing slightly lowered linezolid susceptibility, as mentioned above) outcompeted *S. aureus* wild-type cells under linezolid selection [77]. The fitness cost of resistance mutations varies, and is also dependent on the specific organism. A decrease in growth rates for 23S rRNA mutations at the PTC is expected because many of the nucleotides are phylogenetically conserved and are considered functionally important. For example, the single mutations in the PTC area of 23S rRNA in *M. smegmatis* that have the most significant effects on linezolid resistance show either a moderate (A2503G/U and G2447U) or a large (U2504G and G2576U) decrease in growth rate, where the G2576U mutation with the largest resistance effect results in a threefold slower growth [48, 50, 66]. This is consistent with the fact that although both the G2447U and G2576U mutations lead to 32-fold increases in linezolid MIC values, only the G2447U mutation was isolated by selection in the presence of linezolid [38, 48]. The G2576U mutation has also been studied extensively in *S. aureus*, where a progressive decrease in growth rate is observed with each additional 23S rRNA gene copy harboring the mutation [60]. However, the ability of the mutation to persist in one copy in the absence of antibiotic selection and the rapid reemergence of multiple mutated copies upon reexposure to linezolid suggests that a single copy has a minimal fitness cost [114]. Such a resistance mutation may be accompanied by other mutations that compensate for deleterious effects or act synergistically to enhance resistance. An example is the stepwise genome transformation study mentioned above [75], where linezolid resistance by G2576U in 23S rRNA comes with a fitness cost that can be counteracted by an L3 mutation at position Y137H in *S. pneumoniae*. The study shows that the L3 mutation alone does not confer reduced susceptibility to linezolid. The mutation corresponds to the L3 F147L mutation in *S. epidermis* that has been related to linezolid resistance in several studies (Table 22.1). It remains to be established how many of the mutations in Table 22.1 are true resistance determinants and how many are compensatory "fitness cost" mutations or just random mutations without any phenotypic effect. A possibly related matter has recently been published concerning linezolid-resistant *S. epidermis* strains that grow better in the presence of linezolid than in the absence, and which contained mutations at positions U2504A and C2534U in 23S rRNA together with L3 mutations G152D and D159Y [51]. Also, a synergistic effect of linezolid resistance determinants has been verified in *S. epidermis* with *cfr* plus C2534U in 23S rRNA (in two of six alleles) plus mutations in L3 and L4 [99]. Possible synergistic effects have also been reported for other PTC antibiotics in other bacteria such as *M. smegmatis* [48] and *Brachyspira* spp. [115, 116], indicating interplay between multiple mutations in relation to resistance, accommodation of mutations, and fitness cost. More specific information about the effects of the single and combined mutations is needed to elucidate their detailed interactions.

It was anticipated that purely synthetic compounds like linezolid would not show cross-resistance, but maybe cross-resistance is more a matter of sharing binding sites than being chemically similar. The efflux pumps that expel linezolid also work on other compounds [110, 112]. The methylation performed by Cfr provides linezolid resistance as well as resistance to five other classes of antibiotics [80, 81]. Examples of cross-resistance between PTC antibiotics resulting from 23S rRNA mutations have been observed [48, 66, 116, 117], although no straightforward relationship between overlapping binding sites and cross-resistance was found. There is a correlation between linezolid and chloramphenicol resistance for the single G2447U, A2503G, U2504G, G2505A, and G2576U mutations in *M. smegmatis* [48, 66]. However, this correlation does not apply for G2032A-U2504G and C2055A-U2504G double mutations and no relationship between linezolid, clindamycin, and valnemulin resistance could be observed [48, 66]. In addition, cross-resistance between linezolid and tiamulin has been documented for the G2447U and U2500A mutations in *E. coli* and the G2576U mutation in *E. coli* and *S. aureus* [46]. The different sets of specific bacteria, mutations and antibiotics reported in the literature preclude simple and common conclusion, and more information is needed.

5 Linezolid Resistance Among Clinical Isolates

As already mentioned, linezolid has a broad spectrum of activity against various Gram-positive clinical strains including *S. aureus,* CoNS, *E. faecalis, E. faecium, S. pneumoniae,* viridans group and other streptococci, β-hemolytic streptococci and other rarely isolated Gram-positive human pathogens [118]. It is also widely used to treat infections from multidrug-resistant (MDR) clinical isolates such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [119–125].

Clinical isolates with resistance to linezolid were first documented in 1999 and included two isolates from 2/169 patients (1.2 %) receiving linezolid treatment for enterococcal infections [14]. Both of the patients received linezolid for a long period of time in order to treat bacteraemia associated with intravascular devices. The first report of a clinical isolate of methicillin-resistant *S. aureus* with linezolid resistance was reported in 2001 and was isolated from an 85-year-old man who had received prior linezolid treatment [15]. The resistance was due to G2576U mutations in the V domain of the 23S rRNA [15]. The first report of *cfr* as a resistance determinant in a clinical staphylococcal isolate was in 2008 from the USA through the surveillance program LEADER [86].

Documented resistance to linezolid appears to be sporadic and can occur in outbreaks [118, 126–131]. In most cases of sporadic clinical isolates exhibiting resistance to linezolid, the resistance was associated with prior linezolid therapy [39, 63, 132–134] although there have been reports of rapid emergence of resistance after short-term treatment [135], or resistance not related to prior treatment with linezolid [136, 137].

Due to the widespread use of linezolid for treating nosocomial infections by MDR staphylococcal and enterococcal clinical isolates, a need immerged to monitor the spectrum and potency of linezolid and for that two surveillance programs have been established. The original surveillance program for linezolid was ZAPS (Zyvox Activity and Potency Surveillance) [129, 138–140] and was renamed ZAAPS, enrolling medical centers in Latin America (LATAM), Asia Pacific (APAC), and Europe [127, 130, 131, 141, 142]. The second surveillance program is the LEADER surveillance program and it has monitored linezolid activity, spectrum, and resistance rates in the USA since 2004 [121, 143–147]. The most recent results from the LEADER surveillance program are from 2011, and monitored 7303 Gram-positive clinical isolates from 60 medical centers. It shows that resistance to linezolid is particularly rare in clinical MRSA (≤0.2 %) and CoNS (≤1.2 %) [148]. Linezolid was one of the most active agents among 1160 enterococcal strains (66 % *E. faecalis*, 30.6 % *E. faecium*) with a susceptibility rate of 99.7 %. The most important finding in this surveillance program was a nonsusceptible viridans group streptococcus, *Streptococcus sanguinis* (MIC >8 μg/mL), that was encountered for the first time in this program [148]. In the same manner, the latest ZAAPS Program report tested linezolid and comparators against 7972 Gram-positive clinical isolates from 73 medical centers (33 countries) from five continents, in order to summarize its activity and spectrum. Resistance to linezolid occurred in ≤0.1 % of strains of *S. aureus*, ≤0.9 % of CoNS, and ≤0.3 % of enterococcal strains [93]. Although the results from the two surveillance programs appear to be encouraging, concerns are lately raised by the appearance of linezolid-resistant clinical isolates in multiple studies around the world. Enterococcal clinical isolates resistant to linezolid due to L3 mutations and *S. cohnii* clinical isolates resistant to linezolid harboring the *cfr* and the 23S rRNA mutation G2576U were documented from a multicenter study in China [149]. A study conducted on clinical isolates of CoNS from two hospitals in China reports the emergence of *cfr*-harboring CoNS [150]. Emergence of linezolid-resistant *S. aureus* from cystic fibrosis (CF) patients was documented in Ohio with isolates having L3 mutations or the 23S rRNA mutation G2576U, raising serious concerns for CF patients [94]. Linezolid-resistant clinical isolates of *E. faecium* were isolated in Ontario, Canada, from 2010 to 2012 in a study that documents the first appearance of *cfr* in a clinical isolate of *E. faecium* [151]. A linezolid-resistant *S. pneumoniae* isolate with a linezolid MIC at 4 μg/mL was encountered for the first time in the LEADER Program results for 2010, and molecular characterization indicated that this strain had wild-type 23S rRNA and L22 ribosomal protein DNA sequences but had mutations in the ribosomal protein L4: Q67K and G69V [152].

Concerns also rise by studies that document the dissemination of the *cfr* gene among linezolid-resistant clinical isolates of various species [87, 150, 151, 153–155]. In a recent study from China, linezolid-resistant staphylococcal clinical isolates had the *cfr* gene located on a plasmid segment identical to a sequenced 14 kb *cfr*-carrying segment, from the plasmid pSS-02 [87]. This plasmid was originally identified in staphylococci isolated from pigs. This finding indicates that closely related—if not identical—plasmids carrying the *cfr* gene can be exchanged between CoNS from animals and methicillin-resistant CoNS (MRCoNS) from humans and that these MRCoNS can be involved in severe infections in humans [87].

6 Clinical Significance of Linezolid Resistance and Concluding Remarks

Linezolid remains highly active against most staphylococci, and its value in treating serious infections caused by MRSA has been well documented. Its availability as an oral formulation makes it desirable for outpatient treatment [128]. However, up to a quarter of patients prescribed the oral formulation of linezolid are non-adherent with therapy [156].

Among patients treated with linezolid for extended periods, resistance rates may be significantly elevated as compared with data reported in surveillance studies. Clinicians should remain aware that linezolid resistance may arise following prolonged treatment with linezolid and of the possibility of linezolid-resistant staphylococci (LRS) in patients that have not been previously treated with linezolid, given the high incidence of LRSA carrying *cfr* [128]. As an example, cystic fibrosis patients with respiratory tract infections caused by *S. aureus* have LRSA rates of up to 11 %, related to the number and length of linezolid treatments [94]. In addition, linezolid resistance may be underreported based on technical complications in the interpretation of both MIC and disc diffusion results [157]. Compared with the Clinical and Laboratory Standards Institute broth microdilution reference method, one study demonstrated 8/15 (53.3 %) LRS were falsely reported susceptible by disc diffusion and 6/15 (40.0 %) by Etest [157].

Treatment options for linezolid-resistant isolates are limited, so susceptibility testing for linezolid resistance should be considered prior to using linezolid for serious infections. In addition, judicious use of linezolid, accurate identification of resistance, and application of strict infection control measures are essential to the preservation of linezolid as a therapeutic agent. Also, it is very important to clearly identify all linezolid resistance determinants. It is obvious that linezolid resistance may occur both as transmissible element (*cfr* gene) and as acquired ribosomal mutations and probably as efflux changes caused by mutations. It is possible that development of derivatives of linezolid can overcome some of the resistance determinant and there seems to be steps in this direction.

References

1. Mukhtar TA, Wright GD. Streptogramins, oxazolidinones, and other inhibitors of bacterial protein synthesis. Chem Rev. 2005;105(2):529–42. doi:10.1021/cr030110z.
2. Shaw KJ, Barbachyn MR. The oxazolidinones: past, present, and future. Ann N Y Acad Sci. 2011;1241:48–70. doi:10.1111/j.1749-6632.2011.06330.x.
3. Long KS, Vester B. Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob Agents Chemother. 2012;56(2):603–12. doi:10.1128/aac.05702-11.
4. Shinabarger D, Eliopoulos G. Resistance to linezolid. In: Mayers D, editor. Antimicrobial drug resistance. Infectious disease. Totowa: Humana Press; 2009. p. 247–57. doi:10.1007/978-1-59745-180-2_22.
5. Fugitt RB, Luckenbaugh RW. Bactericide, fungicide, plant diseases. Google Patents; 1978.
6. Slee AM, Wuonola MA, McRipley RJ, Zajac I, Zawada MJ, Bartholomew PT, Gregory WA, Forbes M. Oxazolidinones, a new class of synthetic antibacterial agents: in vitro and in vivo activities of DuP 105 and DuP 721. Antimicrob Agents Chemother. 1987;31(11):1791–7.
7. Brickner SJ, Barbachyn MR, Hutchinson DK, Manninen PR. Linezolid (ZYVOX), the first member of a completely new class of antibacterial agents for treatment of serious Gram-positive infections. J Med Chem. 2008;51(7):1981–90. doi:10.1021/jm800038g.
8. Muller-Serieys C. Ketolides and oxazolidinones. Mechanisms of action and antibacterial spectrum. Presse Medicale (Paris, France: 1983). 2000;29(37):2061–4.
9. Abb J. In vitro activity of linezolid, quinupristin-dalfopristin, vancomycin, teicoplanin, moxifloxacin and mupirocin against methicillin-resistant *Staphylococcus aureus*: comparative evaluation by the E-test and a broth microdilution method. Diagn MicrobiolInfectDis.2002;43(4):319–21.doi:S0732889302004078 [pii].
10. Norrby R. Linezolid—a review of the first oxazolidinone. Expert Opin Pharmacother. 2001;2(2):293–302. doi:10.1517/14656566.2.2.293.
11. Behra-Miellet J, Calvet L, Dubreuil L. Activity of linezolid against anaerobic bacteria. Int J Antimicrob Agents. 2003;22(1):28–34.
12. Sotgiu G, Centis R, D'Ambrosio L, Alffenaar JW, Anger HA, Caminero JA, Castiglia P, De Lorenzo S, Ferrara G, Koh WJ, Schecter GF, Shim TS, Singla R, Skrahina A, Spanevello A, Udwadia ZF, Villar M, Zampogna E, Zellweger JP, Zumla A, Migliori GB. Efficacy, safety and tolerability of linezolid containing regimens in treating MDR-TB and XDR-TB: systematic review and meta-analysis. Eur Respir J. 2012;40(6):1430–42. doi:10.1183/09031936.00022912.
13. Paladino JA. Linezolid: an oxazolidinone antimicrobial agent. Am J Health Syst Pharm. 2002;59(24):2413–25.
14. Zurenko GE, Yagi BH, Schaadt RD, Allison JW, Kilburn JO, Glickman SE, Hutchinson DK, Barbachyn MR, Brickner SJ. In vitro activities of U-100592 and U-100766, novel oxazolidinone antibacterial agents. Antimicrob Agents Chemother. 1996;40(4):839–45.
15. Tsiodras S, Gold HS, Sakoulas G, Eliopoulos GM, Wennersten C, Venkataraman L, Moellering RC, Ferraro MJ. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. Lancet. 2001;358(9277):207–8. doi:10.1016/S0140-6736(01)05410-1. S0140-6736(01)05410-1 [pii].
16. Sanchez Garcia M, De la Torre MA, Morales G, Pelaez B, Tolon MJ, Domingo S, Candel FJ, Andrade R, Arribi A, Garcia N, Martinez Sagasti F, Fereres J, Picazo J. Clinical outbreak of linezolid-resistant *Staphylococcus aureus* in an intensive care unit. JAMA. 2010;303(22):2260–4. doi:10.1001/jama.2010.757. 303/22/2260 [pii].
17. Fortuna CG, Bonaccorso C, Bulbarelli A, Caltabiano G, Rizzi L, Goracci L, Musumarra G, Pace A, Palumbo Piccionello A, Guarcello A, Pierro P, Cocuzza CE, Musumeci R. New linezolid-like 1,2,4-oxadiazoles active against Gram-positive multiresistant pathogens. Eur J Med Chem. 2013;65:533–45. doi:10.1016/j.ejmech.2013.03.069.
18. Kisgen JJ, Mansour H, Unger NR, Childs LM. Tedizolid: a new oxazolidinone antimicrobial. Am J Health Syst Pharm. 2014;71(8):621–33. doi:10.2146/ajhp130482.

19. Moellering RC. Tedizolid: a novel oxazolidinone for gram-positive infections. Clin Infect Dis. 2014;58 Suppl 1:S1–3. doi:10.1093/cid/cit658.
20. Schaadt R, Sweeney D, Shinabarger D, Zurenko G. In vitro activity of TR-700, the active ingredient of the antibacterial prodrug TR-701, a novel oxazolidinone antibacterial agent. Antimicrob Agents Chemother. 2009;53(8):3236–9. doi:10.1128/AAC.00228-09.
21. Locke JB, Rahawi S, Lamarre J, Mankin AS, Shaw KJ. Genetic environment and stability of *cfr* in methicillin-resistant *Staphylococcus aureus* CM05. Antimicrob Agents Chemother. 2012;56(1):332–40. doi:10.1128/aac.05420-11.
22. Prokocimer P, Bien P, Surber J, Mehra P, DeAnda C, Bulitta JB, Corey GR. Phase 2, randomized, double-blind, dose-ranging study evaluating the safety, tolerability, population pharmacokinetics, and efficacy of oral torezolid phosphate in patients with complicated skin and skin structure infections. Antimicrob Agents Chemother. 2011;55(2):583–92. doi:10.1128/aac.00076-10.
23. Flanagan S, Bartizal K, Minassian SL, Fang E, Prokocimer P. In vitro, in vivo, and clinical studies of tedizolid to assess the potential for peripheral or central monoamine oxidase interactions. Antimicrob Agents Chemother. 2013;57(7):3060–6. doi:10.1128/aac.00431-13.
24. Sutcliffe JA. Antibiotics in development targeting protein synthesis. Ann N Y Acad Sci. 2011;1241:122–52. doi:10.1111/j.1749-6632.2011.06323.x.
25. Bassetti M, Merelli M, Temperoni C, Astilean A. New antibiotics for bad bugs: where are we? Ann Clin Microbiol Antimicrob. 2013;12:22. doi:10.1186/1476-0711-12-22.
26. Wallis RS, Dawson R, Friedrich SO, Venter A, Paige D, Zhu T, Silvia A, Gobey J, Ellery C, Zhang Y, Eisenach K, Miller P, Diacon AH. Mycobactericidal activity of sutezolid (PNU-100480) in sputum (EBA) and blood (WBA) of patients with pulmonary tuberculosis. PLoS One. 2014;9(4), e94462. doi:10.1371/journal.pone.0094462.
27. Eustice DC, Feldman PA, Slee AM. The mechanism of action of DuP 721, a new antibacterial agent: effects on macromolecular synthesis. Biochem Biophys Res Commun. 1988;150(3):965–71.
28. Shinabarger DL, Marotti KR, Murray RW, Lin AH, Melchior EP, Swaney SM, Dunyak DS, Demyan WF, Buysse JM. Mechanism of action of oxazolidinones: effects of linezolid and eperezolid on translation reactions. Antimicrob Agents Chemother. 1997;41(10):2132–6.
29. Swaney SM, Aoki H, Ganoza MC, Shinabarger DL. The oxazolidinone linezolid inhibits initiation of protein synthesis in bacteria. Antimicrob Agents Chemother. 1998;42(12):3251–5.
30. Thompson J, O'Connor M, Mills JA, Dahlberg AE. The protein synthesis inhibitors, oxazolidinones and chloramphenicol, cause extensive translational inaccuracy in vivo. J Mol Biol. 2002;322(2):273–9.
31. Aoki H, Ke L, Poppe SM, Poel TJ, Weaver EA, Gadwood RC, Thomas RC, Shinabarger DL, Ganoza MC. Oxazolidinone antibiotics target the P site on *Escherichia coli* ribosomes. Antimicrob Agents Chemother. 2002;46(4):1080–5.
32. Kloss P, Xiong L, Shinabarger DL, Mankin AS. Resistance mutations in 23 S rRNA identify the site of action of the protein synthesis inhibitor linezolid in the ribosomal peptidyl transferase center. J Mol Biol. 1999;294(1):93–101. doi:10.1006/jmbi.1999.3247.
33. Colca JR, McDonald WG, Waldon DJ, Thomasco LM, Gadwood RC, Lund ET, Cavey GS, Mathews WR, Adams LD, Cecil ET, Pearson JD, Bock JH, Mott JE, Shinabarger DL, Xiong L, Mankin AS. Cross-linking in the living cell locates the site of action of oxazolidinone antibiotics. J Biol Chem. 2003;278(24):21972–9. doi:10.1074/jbc.M302109200. M302109200 [pii].
34. Leach KL, Swaney SM, Colca JR, McDonald WG, Blinn JR, Thomasco LM, Gadwood RC, Shinabarger D, Xiong L, Mankin AS. The site of action of oxazolidinone antibiotics in living bacteria and in human mitochondria. Mol Cell. 2007;26(3):393–402. doi:10.1016/j.molcel.2007.04.005. S1097-2765(07)00221-3 [pii].
35. Ippolito JA, Kanyo ZF, Wang D, Franceschi FJ, Moore PB, Steitz TA, Duffy EM. Crystal structure of the oxazolidinone antibiotic linezolid bound to the 50S ribosomal subunit. J Med Chem. 2008;51(12):3353–6. doi:10.1021/jm800379d.
36. Wilson DN, Schluenzen F, Harms JM, Starosta AL, Connell SR, Fucini P. The oxazolidinone antibiotics perturb the ribosomal peptidyl-transferase center and effect tRNA positioning. Proc Natl Acad Sci U S A. 2008;105(36):13339–44. doi:10.1073/pnas.0804276105.
37. Kokkori S, Apostolidi M, Tsakris A, Pournaras S, Stathopoulos C, Dinos G. Linezolid-dependent function and structure adaptation of ribosomes in a *Staphylococcus epidermidis* strain exhibiting linezolid dependence. Antimicrob Agents Chemother. 2014;58(8):4651–6. doi:10.1128/aac.02835-14.
38. Sander P, Belova L, Kidan YG, Pfister P, Mankin AS, Bottger EC. Ribosomal and non-ribosomal resistance to oxazolidinones: species-specific idiosyncrasy of ribosomal alterations. Mol Microbiol. 2002;46(5):1295–304.
39. Gonzales RD, Schreckenberger PC, Graham MB, Kelkar S, DenBesten K, Quinn JP. Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. Lancet. 2001;357(9263):1179. doi:10.1016/s0140-6736(00)04376-2.
40. Marshall SH, Donskey CJ, Hutton-Thomas R, Salata RA, Rice LB. Gene dosage and linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis*. Antimicrob Agents Chemother. 2002;46(10):3334–6.
41. Meka VG, Pillai SK, Sakoulas G, Wennersten C, Venkataraman L, DeGirolami PC, Eliopoulos GM, Moellering Jr RC, Gold HS. Linezolid resistance in sequential *Staphylococcus aureus* isolates associated with a T2500A mutation in the 23S rRNA gene and loss of a single copy of rRNA. J Infect Dis. 2004;190(2):311–7. doi:10.1086/421471.
42. Mazzariol A, Lo Cascio G, Kocsis E, Maccacaro L, Fontana R, Cornaglia G. Outbreak of linezolid-resistant *Staphylococcus haemolyticus* in an Italian intensive care unit. Eur J Clin Microbiol. 2012;31(4):523–7. doi:10.1007/s10096-011-1343-6.
43. Feng J, Lupien A, Gingras H, Wasserscheid J, Dewar K, Legare D, Ouellette M. Genome sequencing of linezolid-resistant *Streptococcus pneumoniae* mutants reveals novel mechanisms of resistance. Genome Res. 2009;19(7):1214–23. doi:10.1101/gr.089342.108. gr.089342.108 [pii].
44. Hillemann D, Rusch-Gerdes S, Richter E. In vitro-selected linezolid-resistant *Mycobacterium tuberculosis* mutants. Antimicrob Agents Chemother. 2008;52(2):800–1. doi:10.1128/aac.01189-07.
45. Livermore DM, Warner M, Mushtaq S, North S, Woodford N. In vitro activity of the oxazolidinone RWJ-416457 against linezolid-resistant and -susceptible staphylococci and enterococci. Antimicrob Agents Chemother. 2007;51(3):1112–4. doi:10.1128/aac.01347-06.
46. Miller K, Dunsmore CJ, Fishwick CW, Chopra I. Linezolid and tiamulin cross-resistance in *Staphylococcus aureus* mediated by point mutations in the peptidyl transferase center. Antimicrob Agents Chemother. 2008;52(5):1737–42. doi:10.1128/AAC.01015-07. AAC.01015-07 [pii].
47. Prystowsky J, Siddiqui F, Chosay J, Shinabarger DL, Millichap J, Peterson LR, Noskin GA. Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. Antimicrob Agents Chemother. 2001;45(7):2154–6. doi:10.1128/AAC.45.7.2154-2156.2001.
48. Long KS, Munck C, Andersen TM, Schaub MA, Hobbie SN, Bottger EC, Vester B. Mutations in 23S rRNA at the peptidyl

transferase center and their relationship to linezolid binding and cross-resistance. Antimicrob Agents Chemother. 2010;54(11): 4705–13. doi:10.1128/aac.00644-10.
49. Bourgeois-Nicolaos N, Massias L, Couson B, Butel MJ, Andremont A, Doucet-Populaire F. Dose dependence of emergence of resistance to linezolid in *Enterococcus faecalis* in vivo. J Infect Dis. 2007;195(10):1480–8. doi:10.1086/513876. JID37546 [pii].
50. Li BB, Wu CM, Wang Y, Shen JZ. Single and dual mutations at positions 2058, 2503 and 2504 of 23S rRNA and their relationship to resistance to antibiotics that target the large ribosomal subunit. J Antimicrob Chemother. 2011;66(9):1983–6. doi:10.1093/jac/dkr268.
51. Pournaras S, Ntokou E, Zarkotou O, Ranellou K, Themeli-Digalaki K, Stathopoulos C, Tsakris A. Linezolid dependence in *Staphylococcus epidermidis* bloodstream isolates. Emerg Infect Dis. 2013;19(1):129–32. doi:10.3201/eid1901.111527.
52. Wong A, Reddy SP, Smyth DS, Aguero-Rosenfeld ME, Sakoulas G, Robinson DA. Polyphyletic emergence of linezolid-resistant staphylococci in the United States. Antimicrob Agents Chemother. 2010;54(2):742–8. doi:10.1128/aac.00621-09.
53. Xiong L, Kloss P, Douthwaite S, Andersen NM, Swaney S, Shinabarger DL, Mankin AS. Oxazolidinone resistance mutations in 23S rRNA of *Escherichia coli* reveal the central region of domain V as the primary site of drug action. J Bacteriol. 2000; 182(19):5325–31.
54. Xu J, Golshani A, Aoki H, Remme J, Chosay J, Shinabarger DL, Ganoza MC. Protected nucleotide G2608 in 23S rRNA confers resistance to oxazolidinones in *E. coli*. Biochem Biophys Res Commun. 2005;328(2):471–6. doi:10.1016/j.bbrc.2004.12.189.
55. Campanile F, Mongelli G, Bongiorno D, Adembri C, Ballardini M, Falcone M, Menichetti F, Repetto A, Sabia C, Sartor A, Scarparo C, Tascini C, Venditti M, Zoppi F, Stefani S. Worrisome trend of new multiple mechanisms of linezolid resistance in staphylococcal clones diffused in Italy. J Clin Microbiol. 2013; 51(4):1256–9. doi:10.1128/jcm.00098-13.
56. Hill RL, Kearns AM, Nash J, North SE, Pike R, Newson T, Woodford N, Calver R, Livermore DM. Linezolid-resistant ST36 methicillin-resistant *Staphylococcus aureus* associated with prolonged linezolid treatment in two paediatric cystic fibrosis patients. J Antimicrob Chemother. 2010;65(3):442–5. doi:10.1093/jac/dkp494. dkp494 [pii].
57. Vardakas KZ, Kioumis I, Falagas ME. Association of pharmacokinetic and pharmacodynamic aspects of linezolid with infection outcome. Curr Drug Metab. 2009;10(1):2–12.
58. Zurenko GE TW, Hafkin B, et al Development of linezolid-resistant *Enterococcus faecium* in two compassionate use program patients treated with linezolid [abstract 848]. In: Program and abstracts of the 39th interscience conference on antimicrobial agents and chemotherapy (San Francisco). Washington, DC: American Society for Microbiology; 1999. p. 118.
59. Pillai SK, Sakoulas G, Wennersten C, Eliopoulos GM, Moellering Jr RC, Ferraro MJ, Gold HS. Linezolid resistance in *Staphylococcus aureus*: characterization and stability of resistant phenotype. J Infect Dis. 2002;186(11):1603–7. doi:10.1086/345368. JID020606 [pii].
60. Besier S, Ludwig A, Zander J, Brade V, Wichelhaus TA. Linezolid resistance in *Staphylococcus aureus*: gene dosage effect, stability, fitness costs, and cross-resistances. Antimicrob Agents Chemother. 2008;52(4):1570–2. doi:10.1128/AAC.01098-07. AAC.01098-07 [pii].
61. Ikeda-Dantsuji Y, Hanaki H, Nakae T, Takesue Y, Tomono K, Honda J, Yanagihara K, Mikamo H, Fukuchi K, Kaku M, Kohno S, Niki Y. Emergence of linezolid-resistant mutants in the susceptible cell population of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2011;55(5):2466–8. doi:10.1128/AAC.01548-10. AAC.01548-10 [pii].
62. Arias CA, Vallejo M, Reyes J, Panesso D, Moreno J, Castaneda E, Villegas MV, Murray BE, Quinn JP. Clinical and microbiological aspects of linezolid resistance mediated by the *cfr* gene encoding a 23S rRNA methyltransferase. J Clin Microbiol. 2008;46(3):892–6. doi:10.1128/JCM.01886-07. JCM.01886-07 [pii].
63. Swoboda S, Fritz S, Martignoni ME, Feldhues RA, Hoppe-Tichy T, Buchler MW, Geiss HK. Varying linezolid susceptibility of vancomycin-resistant *Enterococcus faecium* isolates during therapy: a case report. J Antimicrob Chemother. 2005;56(4):787–9. doi:10.1093/jac/dki318.
64. Carsenti-Dellamonica H, Galimand M, Vandenbos F, Pradier C, Roger PM, Dunais B, Sabah M, Mancini G, Dellamonica P. In vitro selection of mutants of *Streptococcus pneumoniae* resistant to macrolides and linezolid: relationship with susceptibility to penicillin G or macrolides. J Antimicrob Chemother. 2005; 56(4):633–42. doi:10.1093/jac/dki301.
65. Lobritz M, Hutton-Thomas R, Marshall S, Rice LB. Recombination proficiency influences frequency and locus of mutational resistance to linezolid in *Enterococcus faecalis*. Antimicrob Agents Chemother. 2003;47(10):3318–20.
66. Long KS, Poehlsgaard J, Hansen LH, Hobbie SN, Bottger EC, Vester B. Single 23S rRNA mutations at the ribosomal peptidyl transferase centre confer resistance to valnemulin and other antibiotics in *Mycobacterium smegmatis* by perturbation of the drug binding pocket. Mol Microbiol. 2009;71(5):1218–27. doi:10.1111/j.1365-2958.2009.06596.x.
67. Howe RA, Wootton M, Noel AR, Bowker KE, Walsh TR, MacGowan AP. Activity of AZD2563, a novel oxazolidinone, against *Staphylococcus aureus* strains with reduced susceptibility to vancomycin or linezolid. Antimicrob Agents Chemother. 2003;47(11):3651–2.
68. North SE, Ellington MJ, Johnson AP, Livermore DM, Woodford N. Novel pyrosequencing assays to detect T2500A and other mutations conferring linezolid resistance in *Staphylococcus aureus* (abstract C2-272). In: Program and abstracts of the 45th interscience conference on antimicrobial agents and chemotherapy, Washington Convention Center. Washington, DC: American Society for Microbiology; 2005. p. 102.
69. Lincopan N, de Almeida LM, Elmor de Araujo MR, Mamizuka EM. Linezolid resistance in *Staphylococcus epidermidis* associated with a G2603T mutation in the 23S rRNA gene. Int J Antimicrob Agents. 2009;34(3):281–2. doi:10.1016/j.ijantimicag.2009.02.023. S0924-8579(09)00112-5 [pii].
70. Seral C, Saenz Y, Algarate S, Duran E, Luque P, Torres C, Castillo FJ. Nosocomial outbreak of methicillin- and linezolid-resistant *Staphylococcus epidermidis* associated with catheter-related infections in intensive care unit patients. Int J Med Microbiol. 2011;301(4):354–8. doi:10.1016/j.ijmm.2010.11.001. S1438-4221(10)00144-X [pii].
71. Sorlozano A, Gutierrez J, Martinez T, Yuste ME, Perez-Lopez JA, Vindel A, Guillen J, Boquete T. Detection of new mutations conferring resistance to linezolid in glycopeptide-intermediate susceptibility *Staphylococcus hominis* subspecies *hominis* circulating in an intensive care unit. Eur J Clin Microbiol Infect Dis. 2010;29(1):73–80. doi:10.1007/s10096-009-0823-4.
72. Stefani S, Bongiorno D, Mongelli G, Campanile F. Linezolid resistance in Staphylococci. Pharmaceuticals. 2010;3(7): 1988–2006.
73. Long KS, Vester B. Antibiotic resistance mechanisms, with an emphasis on those related to the ribosome. In: Lovett ST, editor. EcoSal—Escherichia coli and Salmonella: cellular and molecular biology. Washington, DC: ASM Press; 2008. doi:10.1128/ecosalplus.2.5.7.

74. Toh SM, Mankin AS. An indigenous posttranscriptional modification in the ribosomal peptidyl transferase center confers resistance to an array of protein synthesis inhibitors. J Mol Biol. 2008;380(4):593–7. doi:10.1016/j.jmb.2008.05.027.
75. Billal DS, Feng J, Leprohon P, Legare D, Ouellette M. Whole genome analysis of linezolid resistance in *Streptococcus pneumoniae* reveals resistance and compensatory mutations. BMC Genomics. 2011;12:512. doi:10.1186/1471-2164-12-512.
76. Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE, Rhee HW, Hong JI, Hartland EL, Stinear TP, Howden BP. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog. 2010;6(6), e1000944. doi:10.1371/journal.ppat.1000944.
77. LaMarre JM, Howden BP, Mankin AS. Inactivation of the indigenous methyltransferase RlmN in *Staphylococcus aureus* increases linezolid resistance. Antimicrob Agents Chemother. 2011;55(6):2989–91. doi:10.1128/aac.00183-11.
78. Kehrenberg C, Schwarz S, Jacobsen L, Hansen LH, Vester B. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Mol Microbiol. 2005;57(4):1064–73. doi:10.1111/j.1365-2958.2005.04754.x.
79. Giessing AM, Jensen SS, Rasmussen A, Hansen LH, Gondela A, Long K, Vester B, Kirpekar F. Identification of 8-methyladenosine as the modification catalyzed by the radical SAM methyltransferase Cfr that confers antibiotic resistance in bacteria. RNA (New York, NY). 2009;15(2):327–36. doi:10.1261/rna.1371409.
80. Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. Antimicrob Agents Chemother. 2006;50(7):2500–5. doi:10.1128/aac.00131-06.
81. Smith LK, Mankin AS. Transcriptional and translational control of the *mlr* operon, which confers resistance to seven classes of protein synthesis inhibitors. Antimicrob Agents Chemother. 2008;52(5):1703–12. doi:10.1128/AAC.01583-07.
82. Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. Antimicrob Agents Chemother. 2000;44(9):2530–3.
83. Kehrenberg C, Schwarz S. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. Antimicrob Agents Chemother. 2006;50(4):1156–63. doi:10.1128/aac.50.4.1156-1163.2006.
84. Toh SM, Xiong L, Arias CA, Villegas MV, Lolans K, Quinn J, Mankin AS. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol. 2007;64(6):1506–14. doi:10.1111/j.1365-2958.2007.05744.x.
85. Cai JC, Hu YY, Zhou HW, Chen GX, Zhang R. Dissemination of the same *cfr*-carrying plasmid among methicillin-resistant *Staphylococcus aureus* and coagulase-negative staphylococci isolates in China. Antimicrob Agents Chemother. 2015. doi:10.1128/aac.04580-14.
86. Mendes RE, Deshpande LM, Castanheira M, DiPersio J, Saubolle MA, Jones RN. First report of *cfr*-mediated resistance to linezolid in human staphylococcal clinical isolates recovered in the United States. Antimicrob Agents Chemother. 2008;52(6):2244–6. doi:10.1128/AAC.00231-08. AAC.00231-08 [pii].
87. Cui L, Wang Y, Li Y, He T, Schwarz S, Ding Y, Shen J, Lv Y. Cfr-mediated linezolid-resistance among methicillin-resistant coagulase-negative staphylococci from infections of humans. PLoS One. 2013;8(2), e57096. doi:10.1371/journal.pone.0057096.
88. Rajan V, Kumar VG, Gopal S. A *cfr*-positive clinical staphylococcal isolate from India with multiple mechanisms of linezolid-resistance. Indian J Med Res. 2014;139(3):463–7.
89. Bender J, Strommenger B, Steglich M, Zimmermann O, Fenner I, Lensing C, Dagwadordsch U, Kekule AS, Werner G, Layer F. Linezolid resistance in clinical isolates of *Staphylococcus epidermidis* from German hospitals and characterization of two *cfr*-carrying plasmids. J Antimicrob Chemother. 2015;70(6):1630–8. doi:10.1093/jac/dkv025.
90. Baos E, Candel FJ, Merino P, Pena I, Picazo JJ. Characterization and monitoring of linezolid-resistant clinical isolates of *Staphylococcus epidermidis* in an intensive care unit 4 years after an outbreak of infection by *cfr*-mediated linezolid-resistant *Staphylococcus aureus*. Diagn Microbiol Infect Dis. 2013;76(3):325–9. doi:10.1016/j.diagmicrobio.2013.04.002.
91. Bosling J, Poulsen SM, Vester B, Long KS. Resistance to the peptidyl transferase inhibitor tiamulin caused by mutation of ribosomal protein l3. Antimicrob Agents Chemother. 2003;47(9):2892–6.
92. Locke JB, Hilgers M, Shaw KJ. Novel ribosomal mutations in *Staphylococcus aureus* strains identified through selection with the oxazolidinones linezolid and torezolid (TR-700). Antimicrob Agents Chemother. 2009;53(12):5265–74. doi:10.1128/AAC.00871-09. AAC.00871-09 [pii].
93. Mendes RE, Hogan PA, Streit JM, Jones RN, Flamm RK. Zyvox(R) Annual appraisal of potency and spectrum (ZAAPS) program: report of linezolid activity over 9 years (2004–12). J Antimicrob Chemother. 2014;69(6):1582–8. doi:10.1093/jac/dkt541.
94. Endimiani A, Blackford M, Dasenbrook EC, Reed MD, Bajaksouszian S, Hujer AM, Rudin SD, Hujer KM, Perreten V, Rice LB, Jacobs MR, Konstan MW, Bonomo RA. Emergence of linezolid-resistant *Staphylococcus aureus* after prolonged treatment of cystic fibrosis patients in Cleveland, Ohio. Antimicrob Agents Chemother. 2011;55(4):1684–92. doi:10.1128/AAC.01308-10. AAC.01308-10 [pii].
95. de Almeida LM, de Araujo MR, Sacramento AG, Pavez M, de Souza AG, Rodrigues F, Gales AC, Lincopan N, Sampaio JL, Mamizuka EM. Linezolid resistance in Brazilian *Staphylococcus hominis* strains is associated with L3 and 23S rRNA ribosomal mutations. Antimicrob Agents Chemother. 2013;57(8):4082–3. doi:10.1128/aac.00437-13.
96. Locke JB, Hilgers M, Shaw KJ. Mutations in ribosomal protein L3 are associated with oxazolidinone resistance in staphylococci of clinical origin. Antimicrob Agents Chemother. 2009;53(12):5275–8. doi:10.1128/aac.01032-09.
97. Locke JB, Morales G, Hilgers M, G CK, Rahawi S, JosePicazo J, Shaw KJ, Stein JL. Elevated linezolid resistance in clinical *cfr*-positive *Staphylococcus aureus* isolates is associated with co-occurring mutations in ribosomal protein L3. Antimicrob Agents Chemother. 2010;54(12):5352–5. doi:10.1128/AAC.00714-10. AAC.00714-10 [pii].
98. Kosowska-Shick K, Julian KG, McGhee PL, Appelbaum PC, Whitener CJ. Molecular and epidemiologic characteristics of linezolid-resistant coagulase-negative staphylococci at a tertiary care hospital. Diagn Microbiol Infect Dis. 2010;68(1):34–9. doi:10.1016/j.diagmicrobio.2010.05.007.
99. LaMarre J, Mendes RE, Szal T, Schwarz S, Jones RN, Mankin AS. The genetic environment of the *cfr* gene and the presence of other mechanisms account for the very high linezolid resistance of *Staphylococcus epidermidis* isolate 426-3147L. Antimicrob Agents Chemother. 2013;57(3):1173–9. doi:10.1128/aac.02047-12.
100. Mendes RE, Deshpande LM, Farrell DJ, Spanu T, Fadda G, Jones RN. Assessment of linezolid resistance mechanisms among *Staphylococcus epidermidis* causing bacteraemia in Rome, Italy. J Antimicrob Chemother. 2010;65(11):2329–35. doi:10.1093/jac/dkq331. dkq331 [pii].
101. Barros M, Branquinho R, Grosso F, Peixe L, Novais C. Linezolid-Resistant *Staphylococcus epidermidis*, Portugal, 2012. Emerg Infect Dis. 2014;20(5):903–5. doi:10.3201/eid2005.130783.

102. Mendes RE, Deshpande L, Rodriguez-Noriega E, Ross JE, Jones RN, Morfin-Otero R. First report of Staphylococcal clinical isolates in Mexico with linezolid resistance caused by *cfr*: evidence of in vivo *cfr* mobilization. J Clin Microbiol. 2010;48(8):3041–3. doi:10.1128/JCM.00880-10. JCM.00880-10 [pii].
103. Beckert P, Hillemann D, Kohl TA, Kalinowski J, Richter E, Niemann S, Feuerriegel S. *rplC* T460C identified as a dominant mutation in linezolid-resistant *Mycobacterium tuberculosis* strains. Antimicrob Agents Chemother. 2012;56(5):2743–5. doi:10.1128/aac.06227-11.
104. Zhang Z, Pang Y, Wang Y, Liu C, Zhao Y. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with linezolid resistance in multidrug-resistant and extensively drug-resistant tuberculosis in China. Int J Antimicrob Agents. 2014;43(3):231–5. doi:10.1016/j.ijantimicag.2013.12.007.
105. Gentry DR, Rittenhouse SF, McCloskey L, Holmes DJ. Stepwise exposure of *Staphylococcus aureus* to pleuromutilins is associated with stepwise acquisition of mutations in *rplC* and minimally affects susceptibility to retapamulin. Antimicrob Agents Chemother. 2007;51(6):2048–52. doi:10.1128/aac.01066-06.
106. Kosowska-Shick K, Clark C, Credito K, McGhee P, Dewasse B, Bogdanovich T, Appelbaum PC. Single- and multistep resistance selection studies on the activity of retapamulin compared to other agents against *Staphylococcus aureus* and *Streptococcus pyogenes*. Antimicrob Agents Chemother. 2006;50(2):765–9. doi:10.1128/aac.50.2.765-769.2006.
107. Wolter N, Smith AM, Farrell DJ, Schaffner W, Moore M, Whitney CG, Jorgensen JH, Klugman KP. Novel mechanism of resistance to oxazolidinones, macrolides, and chloramphenicol in ribosomal protein L4 of the *pneumococcus*. Antimicrob Agents Chemother. 2005;49(8):3554–7. doi:10.1128/aac.49.8.3554-3557.2005.
108. Gregory ST, Dahlberg AE. Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23 S ribosomal RNA. J Mol Biol. 1999;289(4):827–34. doi:10.1006/jmbi.1999.2839.
109. Barbachyn MR, Ford CW. Oxazolidinone structure-activity relationships leading to linezolid. Angew Chem Int Ed Engl. 2003;42(18):2010–23. doi:10.1002/anie.200200528.
110. Schumacher A, Trittler R, Bohnert JA, Kummerer K, Pages JM, Kern WV. Intracellular accumulation of linezolid in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes*: role of enhanced efflux pump activity and inactivation. J Antimicrob Chemother. 2007;59(6):1261–4. doi:10.1093/jac/dkl380.
111. Smith KP, Kumar S, Varela MF. Identification, cloning, and functional characterization of EmrD-3, a putative multidrug efflux pump of the major facilitator superfamily from *Vibrio cholerae* O395. Arch Microbiol. 2009;191(12):903–11. doi:10.1007/s00203-009-0521-8.
112. Floyd JL, Smith KP, Kumar SH, Floyd JT, Varela MF. LmrS is a multidrug efflux pump of the major facilitator superfamily from *Staphylococcus aureus*. Antimicrob Agents Chemother. 2010;54(12):5406–12. doi:10.1128/aac.00580-10.
113. LaMarre JM, Locke JB, Shaw KJ, Mankin AS. Low fitness cost of the multidrug resistance gene *cfr*. Antimicrob Agents Chemother. 2011;55(8):3714–9. doi:10.1128/aac.00153-11.
114. Tsakris A, Pillai SK, Gold HS, Thauvin-Eliopoulos C, Venkataraman L, Wennersten C, Moellering Jr RC, Eliopoulos GM. Persistence of rRNA operon mutated copies and rapid re-emergence of linezolid resistance in *Staphylococcus aureus*. J Antimicrob Chemother. 2007;60(3):649–1. doi:10.1093/jac/dkm246. dkm246 [pii].
115. Hidalgo A, Carvajal A, Vester B, Pringle M, Naharro G, Rubio P. Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hyodysenteriae* in Spain. Antimicrob Agents Chemother. 2011;55(7):3330–7. doi:10.1128/aac.01749-10.
116. Pringle M, Poehlsgaard J, Vester B, Long KS. Mutations in ribosomal protein L3 and 23S ribosomal RNA at the peptidyl transferase centre are associated with reduced susceptibility to tiamulin in *Brachyspira* spp. isolates. Mol Microbiol. 2004;54(5):1295–306. doi:10.1111/j.1365-2958.2004.04373.x.
117. Vester B, Douthwaite S. Macrolide resistance conferred by base substitutions in 23S rRNA. Antimicrob Agents Chemother. 2001;45(1):1–12.
118. Flamm RK, Farrell DJ, Mendes RE, Ross JE, Sader HS, Jones RN. ZAAPS Program results for 2010: an activity and spectrum analysis of linezolid using clinical isolates from 75 medical centres in 24 countries. J Chemother (Florence, Italy). 2012;24(6):328–37. doi:10.1179/1973947812y.0000000039.
119. Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat Rev Microbiol. 2012;10(4):266–78. doi:10.1038/nrmicro2761.
120. Boucher HW, Corey GR. Epidemiology of methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis. 2008;46 Suppl 5:S344–9. doi:10.1086/533590.
121. Farrell DJ, Mendes RE, Ross JE, Sader HS, Jones RN. LEADER Program results for 2009: an activity and spectrum analysis of linezolid using 6,414 clinical isolates from 56 medical centers in the United States. Antimicrob Agents Chemother. 2011;55(8):3684–90. doi:10.1128/aac.01729-10.
122. Kock R, Becker K, Cookson B, van Gemert-Pijnen JE, Harbarth S, Kluytmans J, Mielke M, Peters G, Skov RL, Struelens MJ, Tacconelli E, Navarro Torne A, Witte W, Friedrich AW. Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. Euro Surveill. 2010;15(41):19688.
123. Rivera AM, Boucher HW. Current concepts in antimicrobial therapy against select Gram-positive organisms: methicillin-resistant *Staphylococcus aureus*, penicillin-resistant pneumococci, and vancomycin-resistant enterococci. Mayo Clin Proc. 2011;86(12):1230–43. doi:10.4065/mcp.2011.0514.
124. Kollef MH, Shorr A, Tabak YP, Gupta V, Liu LZ, Johannes RS. Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia. Chest. 2005;128(6):3854–62. doi:10.1378/chest.128.6.3854.
125. Shorr AF. Epidemiology of staphylococcal resistance. Clin Infect Dis. 2007;45 Suppl 3:S171–6. doi:10.1086/519473.
126. Anderegg TR, Sader HS, Fritsche TR, Ross JE, Jones RN. Trends in linezolid susceptibility patterns: report from the 2002–2003 worldwide Zyvox Annual Appraisal of Potency and Spectrum (ZAAPS) Program. Int J Antimicrob Agents. 2005;26(1):13–21. doi:10.1016/j.ijantimicag.2005.02.019.
127. Biedenbach DJ, Farrell DJ, Mendes RE, Ross JE, Jones RN. Stability of linezolid activity in an era of mobile oxazolidinone resistance determinants: results from the 2009 Zyvox(R) Annual Appraisal of Potency and Spectrum program. Diagn Microbiol Infect Dis. 2010;68(4):459–67. doi:10.1016/j.diagmicrobio.2010.09.018.
128. Gu B, Kelesidis T, Tsiodras S, Hindler J, Humphries RM. The emerging problem of linezolid-resistant *Staphylococcus*. J Antimicrob Chemother. 2013;68(1):4–11. doi:10.1093/jac/dks354.
129. Jones RN, Ballow CH, Biedenbach DJ. Multi-laboratory assessment of the linezolid spectrum of activity using the Kirby-Bauer disk diffusion method: report of the Zyvox Antimicrobial Potency Study (ZAPS) in the United States. Diagn Microbiol Infect Dis. 2001;40(1–2):59–66.
130. Jones RN, Fritsche TR, Sader HS, Ross JE. Zyvox annual appraisal of potency and spectrum program results for 2006: an activity and spectrum analysis of linezolid using clinical isolates from 16 countries. Diagn Microbiol Infect Dis. 2007;59(2):199–209. doi:10.1016/j.diagmicrobio.2007.06.001.

131. Jones RN, Kohno S, Ono Y, Ross JE, Yanagihara K. ZAAPS International Surveillance Program (2007) for linezolid resistance: results from 5591 Gram-positive clinical isolates in 23 countries. Diagn Microbiol Infect Dis. 2009;64(2):191–201. doi:10.1016/j.diagmicrobio.2009.03.001.
132. Johnson AP, Tysall L, Stockdale MV, Woodford N, Kaufmann ME, Warner M, Livermore DM, Asboth F, Allerberger FJ. Emerging linezolid-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from two Austrian patients in the same intensive care unit. Eur J Clin Microbiol Infect Dis. 2002;21(10):751–4. doi:10.1007/s10096-002-0807-0.
133. Auckland C, Teare L, Cooke F, Kaufmann ME, Warner M, Jones G, Bamford K, Ayles H, Johnson AP. Linezolid-resistant enterococci: report of the first isolates in the United Kingdom. J Antimicrob Chemother. 2002;50(5):743–6.
134. Bassetti M, Farrel PA, Callan DA, Topal JE, Dembry LM. Emergence of linezolid-resistant *Enterococcus faecium* during treatment of enterococcal infections. Int J Antimicrob Agents. 2003;21(6):593–4.
135. Seedat J, Zick G, Klare I, Konstabel C, Weiler N, Sahly H. Rapid emergence of resistance to linezolid during linezolid therapy of an *Enterococcus faecium* infection. Antimicrob Agents Chemother. 2006;50(12):4217–9. doi:10.1128/aac.00518-06.
136. Rahim S, Pillai SK, Gold HS, Venkataraman L, Inglima K, Press RA. Linezolid-resistant, vancomycin-resistant *Enterococcus faecium* infection in patients without prior exposure to linezolid. Clin Infect Dis. 2003;36(11):E146–8. doi:10.1086/374929.
137. Ntokou E, Stathopoulos C, Kristo I, Dimitroulia E, Labrou M, Vasdeki A, Makris D, Zakynthinos E, Tsakris A, Pournaras S. Intensive care unit dissemination of multiple clones of linezolid-resistant *Enterococcus faecalis* and *Enterococcus faecium*. J Antimicrob Chemother. 2012;67(8):1819–23. doi:10.1093/jac/dks146.
138. Ballow CH, Jones RN, Biedenbach DJ. A multicenter evaluation of linezolid antimicrobial activity in North America. Diagn Microbiol Infect Dis. 2002;43(1):75–83.
139. Bell JM, Turnidge JD, Ballow CH, Jones RN. Multicentre evaluation of the in vitro activity of linezolid in the Western Pacific. J Antimicrob Chemother. 2003;51(2):339–45.
140. Bolmstrom A, Ballow CH, Qwarnstrom A, Biedenbach DJ, Jones RN. Multicentre assessment of linezolid antimicrobial activity and spectrum in Europe: report from the Zyvox antimicrobial potency study (ZAPS-Europe). Clin Microbiol Infect. 2002;8(12):791–800.
141. Flamm RK, Mendes RE, Ross JE, Sader HS, Jones RN. An international activity and spectrum analysis of linezolid: ZAAPS Program results for 2011. Diagn Microbiol Infect Dis. 2013;76(2):206–13. doi:10.1016/j.diagmicrobio.2013.01.025.
142. Ross JE, Fritsche TR, Sader HS, Jones RN. Oxazolidinone susceptibility patterns for 2005: International Report from the Zyvox Annual Appraisal of Potency and Spectrum Study. Int J Antimicrob Agents. 2007;29(3):295–301. doi:10.1016/j.ijantimicag.2006.09.025.
143. Draghi DC, Sheehan DJ, Hogan P, Sahm DF. In vitro activity of linezolid against key Gram-positive organisms isolated in the united states: results of the LEADER 2004 surveillance program. Antimicrob Agents Chemother. 2005;49(12):5024–32. doi:10.1128/aac.49.12.5024-5032.2005.
144. Draghi DC, Sheehan DF, Hogan P, Sahm DF. Current antimicrobial resistance profiles among methicillin-resistant *Staphylococcus aureus* encountered in the outpatient setting. Diagn Microbiol Infect Dis. 2006;55(2):129–33. doi:10.1016/j.diagmicrobio.2006.01.003.
145. Farrell DJ, Mendes RE, Ross JE, Jones RN. Linezolid surveillance program results for 2008 (LEADER Program for 2008). Diagn Microbiol Infect Dis. 2009;65(4):392–403. doi:10.1016/j.diagmicrobio.2009.10.011.
146. Jones RN, Fritsche TR, Sader HS, Ross JE. LEADER surveillance program results for 2006: an activity and spectrum analysis of linezolid using clinical isolates from the United States (50 medical centers). Diagn Microbiol Infect Dis. 2007;59(3):309–17. doi:10.1016/j.diagmicrobio.2007.06.004.
147. Jones RN, Ross JE, Castanheira M, Mendes RE. United States resistance surveillance results for linezolid (LEADER Program for 2007). Diagn Microbiol Infect Dis. 2008;62(4):416–26. doi:10.1016/j.diagmicrobio.2008.10.010.
148. Flamm RK, Mendes RE, Ross JE, Sader HS, Jones RN. Linezolid surveillance results for the United States: LEADER surveillance program 2011. Antimicrob Agents Chemother. 2013;57(2):1077–81. doi:10.1128/aac.02112-12.
149. Chen H, Wu W, Ni M, Liu Y, Zhang J, Xia F, He W, Wang Q, Wang Z, Cao B, Wang H. Linezolid-resistant clinical isolates of enterococci and *Staphylococcus cohnii* from a multicentre study in China: molecular epidemiology and resistance mechanisms. Int J Antimicrob Agents. 2013;42(4):317–21. doi:10.1016/j.ijantimicag.2013.06.008.
150. Yang XJ, Chen Y, Yang Q, Qu TT, Liu LL, Wang HP, Yu YS. Emergence of *cfr*-harbouring coagulase-negative staphylococci among patients receiving linezolid therapy in two hospitals in China. J Med Microbiol. 2013;62(Pt 6):845–50. doi:10.1099/jmm.0.051003-0.
151. Patel SN, Memari N, Shahinas D, Toye B, Jamieson FB, Farrell DJ. Linezolid resistance in *Enterococcus faecium* isolated in Ontario, Canada. Diagn Microbiol Infect Dis. 2013;77(4):350–3. doi:10.1016/j.diagmicrobio.2013.08.012.
152. Flamm RK, Farrell DJ, Mendes RE, Ross JE, Sader HS, Jones RN. LEADER surveillance program results for 2010: an activity and spectrum analysis of linezolid using 6801 clinical isolates from the United States (61 medical centers). Diagn Microbiol Infect Dis. 2012;74(1):54–61. doi:10.1016/j.diagmicrobio.2012.05.012.
153. Diaz L, Kiratisin P, Mendes RE, Panesso D, Singh KV, Arias CA. Transferable plasmid-mediated resistance to linezolid due to *cfr* in a human clinical isolate of *Enterococcus faecalis*. Antimicrob Agents Chemother. 2012;56(7):3917–22. doi:10.1128/aac.00419-12.
154. Morales G, Picazo JJ, Baos E, Candel FJ, Arribi A, Pelaez B, Andrade R, de la Torre MA, Fereres J, Sanchez-Garcia M. Resistance to linezolid is mediated by the *cfr* gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. Clin Infect Dis. 2010;50(6):821–5. doi:10.1086/650574.
155. Bonilla H, Huband MD, Seidel J, Schmidt H, Lescoe M, McCurdy SP, Lemmon MM, Brennan LA, Tait-Kamradt A, Puzniak L, Quinn JP. Multicity outbreak of linezolid-resistant *Staphylococcus epidermidis* associated with clonal spread of a *cfr*-containing strain. Clin Infect Dis. 2010;51(7):796–800. doi:10.1086/656281.
156. Ball AT, Xu Y, Sanchez RJ, Shelbaya A, Deminski MC, Nau DP. Nonadherence to oral linezolid after hospitalization: a retrospective claims analysis of the incidence and consequence of claim reversals. Clin Ther. 2010;32(13):2246–55. doi:10.1016/s0149-2918(10)80027-x.
157. Tenover FC, Williams PP, Stocker S, Thompson A, Clark LA, Limbago B, Carey RB, Poppe SM, Shinabarger D, McGowan Jr JE. Accuracy of six antimicrobial susceptibility methods for testing linezolid against staphylococci and enterococci. J Clin Microbiol. 2007;45(9):2917–22. doi:10.1128/jcm.00913-07.

Mechanism of the Antibacterial Activity and Resistance of Polymyxins

23

Matthew D. Johnson, Roger L. Nation, and Jian Li

1 Introduction

Discovered in the 1940s, polymyxins are antimicrobial peptides produced by the Gram-positive soil bacterium, *Paenibacillus polymyxa,* which biosynthesizes polymyxins using non-ribosomal peptide synthetase enzymes [1–3]. Polymyxin B and E (polymyxin E was originally named colistin but was determined to have an identical structure) were used clinically in the late 1950s against Gram-negative bacterial infections [4, 5]. However, nephrotoxic and neurotoxic effects of polymyxin treatment became evident, causing a decline in the use of the polymyxins in the 1970s [6]. Soon, newer antibiotics, such as the aminoglycosides, replaced polymyxins in the clinic. However, since the early 2000s the emergence of multidrug-resistant (MDR) Gram-negative organisms, combined with a lack of novel antimicrobial agents, has led to the resurgence of interest in polymyxins as a last-line treatment ([7–10]; Nation and Li 2009).

The use of polymyxins has led to an increase in polymyxin resistance that threatens ongoing clinical application of these important antibiotics [11, 12]. The current EUCAST breakpoints for colistin resistance are >2, >2, and >4 mg/L for *Acinetobacter* species*, Enterobacteriaceae*, and *Pseudomonas* species, respectively [13]. It should be recognized that minimum inhibitory concentration (MIC) measurements do not reveal the presence of small subpopulations of polymyxin-resistant cells in susceptible isolates, a phenomenon referred to as polymyxin heteroresistance [8, 9]. The resistant subpopulations are a form of phenotypic heterogeneity and may be considered as a population-based strategy beneficial for bacterial survival [14]. Generally, resistance rates to polymyxins are relatively low in most parts of the world, as indicated by the SENTRY program [15]. However, there is growing concern in some regions about the increase in polymyxin resistance. For example, a recent study by Monaco *et al*. reported that 43 % of all *Klebsiella pneumoniae* isolates collected from 21 hospitals across Italy were colistin resistant [16]. Resistance to polymyxins has been characterized in various Gram-negative bacteria including *Salmonella* species, *Pseudomonas aeruginosa*, *K. pneumoniae*, *Acinetobacter baumannii*, and *Escherichia coli* [17–20]. In order to develop more potent polymyxin derivatives and tackle the emergence of polymyxin resistance, the mechanism of polymyxin action and resistance must be fully understood. The current understanding of the antimicrobial action and mechanisms of microbial resistance to polymyxins are reviewed in this chapter.

2 Lipopolysaccharide and the Bacterial Outer Membrane

To understand the mechanisms of antibacterial activity and resistance of polymyxins, it is crucial to note the structure of Gram-negative bacterial outer membrane. Polymyxins are bactericidal against Gram-negative bacteria; they target components of the outer membrane (OM) that are usually essential to bacterial viability and pathogenicity [21]. The OM is the defining characteristic of Gram-negative bacteria. Unlike the inner membrane (IM), the OM is an asymmetric bilayer that comprises an outer leaflet containing a very large proportion of lipopolysaccharide (LPS) and a phospholipid inner leaflet. The bacterial OM functions as a selectively permeable barrier, capable of protecting the cell from toxic compounds including many antibiotics, while allowing the import of essential nutrients [22, 23]. Membrane proteins constitute about 50 % of OM mass, and many OM proteins, such as porins (OmpA), ABC transporters, and export systems, are responsible for the selectivity of the OM permeability [24]. In addition to its role as a barrier, the OM is important for infection and pathogenicity of Gram-negative

M.D. Johnson • R.L. Nation, Ph.D. (✉) • J. Li, Ph.D. (✉)
Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia
e-mail: Roger.Nation@monash.edu; Colistin.Polymyxin@gmail.com

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_23

bacteria [23]. Many proteins, such as type III secretion systems, pili, and autotransporters (protein export systems that create a pore in the OM and secrete their own passenger peptide, e.g., Pet), are found in the OM [24]. Moreover, the LPS component of the OM is a causative agent of sepsis [23, 24].

The primary target of polymyxins is LPS, the most prominent component in the outer leaflet of the OM. LPS is comprised of the amphiphilic lipid A (endotoxin), core oligosaccharide, and a repeating distal polysaccharide termed *o*-antigen [23]. Lipid A is comprised of two D-gluco-configured pyranosidic sugars that are covalently linked as β(1→6) dimers [25]. Each sugar has a phosphate group and a number of acyl chains that confer hydrophobicity. The disaccharide sugar backbone of lipid A is linked to an oligosaccharide molecule, normally comprised of heptose and keto-deoxyoctulosonate (KDO), and this molecule is often referred to as the core oligosaccharide. The hydrophobic acyl chains of the core oligosaccharide function as the membrane, and the oligosaccharide backbone supports additional *O*-antigen glycan polymers [25]. The LPS molecules are bound together largely by the charge-based interactions between Mg^{2+} and Ca^{2+} ions and the negatively charged phosphate component of lipid A [26, 27]. The acyl chains of lipid A bind by hydrophobic interactions, and the oligosaccharide components of lipid A form intermolecular hydrogen bonds [25]. Together, these interactions make LPS a stable permeability barrier of which lipid A is an essential component. Consequently, antimicrobials that target LPS, such as polymyxins, are highly sought after.

3 Polymyxins

Polymyxins are amphiphilic lipopeptides; the *N*-terminal fatty acyl chain and D-Phenylalanine-L-Leucine form two hydrophobic domains that are separated by polar L-Threonine and cationic L-alpha-gamma-diaminobutyric acid (Dab) residues [28]. The bactericidal activity of polymyxins is initiated by an electrostatic interaction with lipid A in the bacterial OM. As described above, the stability of the OM is mediated by charge-based interactions between divalent inorganic cations and the anionic phosphate groups of lipid A. The cationic polymyxins bind lipid A with greater affinity than other cations [21, 29]. As a consequence, polymyxins disrupt the electrostatic interactions between lipid A molecules and the inorganic cations, causing a reduction in OM integrity [21]. In addition, the initial electrostatic interactions between lipid A and the cationic peptide of polymyxins results in the lipophilic fatty acyl chains and hydrophobic amino acids of polymyxins to be proximal to the acyl chains of lipid A [28]. Consequently, the hydrophobic domains of polymyxins insert between the acyl chains of lipid A, further destabilizing lipid A interactions [30, 31]. The antibacterial action of polymyxins is dependent on both of the aforementioned chemical interactions. This is exemplified by the fact that polymyxin B nonapeptide, which lacks a hydrophobic fatty acyl chain, and colistin methanesulfonate, in which the cationic residues are masked, are inactive against Gram-negative bacteria (Bergen et al. 2006; [29]).

As described above, polymyxins require both electrostatic and hydrophobic interactions for antibacterial activity [32, 33]. However, the mechanism which leads from these interactions to killing is poorly understood at a molecular level. The popular model for polymyxin-induced killing proposes that the increased permeability of the OM caused by the insertion of polymyxins into the LPS layer of the cell results in the "self-promoted" uptake of polymyxins into the periplasm and subsequent insertion of polymyxins into the IM [31, 33–35]. It is assumed that the interaction of polymyxins with the IM causes transient pore formation and membrane thinning. The increased permeability of the IM may result in an inability to sustain controlled import and export across this barrier, thus eradicating electrochemical gradients, making respiration impossible [21, 31]. Alternatively, the insertion of polymyxins may induce vesicle-mediated mixing between the IM and OM and cause a loss of phospholipid composition and overall membrane disruption, potentially leading to an osmotic imbalance and cell lysis [36]. Another proposed mechanism of killing is reactive oxygen-mediated killing. The Fenton reaction produces hydroxyl radicals that are thought to induce DNA damage and cell death. Recently, increased hydroxyl radical production was measured in strains of *A. baumannii* after treatment with polymyxins [37]. In addition, the antimicrobial action of polymyxins was shown to be inhibited by chemicals that scavenged free hydroxyl radicals (thiourea) or inhibited the Fenton cycle (dipyridyl) [37]. However, killing by free radicals is controversial and the results could not be reproduced for polymyxins by our laboratory (unpublished data); several other studies have shown that antimicrobial killing by kanamycin is independent of reactive oxygen species [38, 39]. Another mechanism of killing by polymyxins, which involves protein function inhibition, has also been described. In 2014, Deris *et al.* proved that the type-II NADH-quinone oxidoreductase (NDH-2) is inhibited by polymyxin B (Fig. 23.1). Results indicated that ubiquinone binding was competitively inhibited, and NADH was non-competitively inhibited by polymyxin B, and a similar observation has been reported in *Mycobacterium tuberculosis* ([40–42], Fig. 23.1). Although the exact details regarding polymyxin-mediated bacterial killing remain unknown, the initial interaction of polymyxins with lipid A is pivotal to the killing process; this is exemplified in the current known resistance mechanisms.

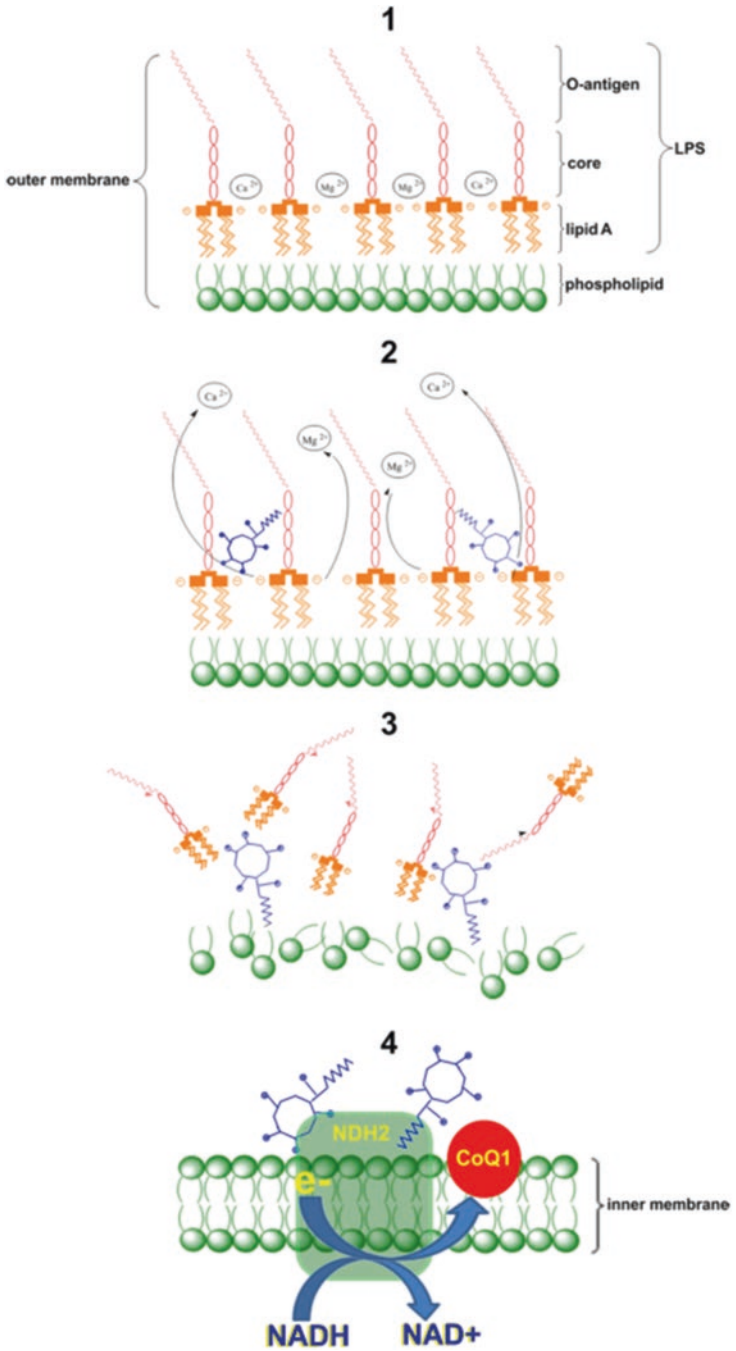


Fig. 23.1 Diagrammatic representation of a possible mode of action of polymyxins at the levels of the outer and inner membranes of the Gram-negative bacterial cell. *Step* 1: Polymyxins target the outer membrane of Gram-negative bacteria. *Step* 2: The positively charged polymyxins displace divalent cations that bridge adjacent LPS molecules. *Step* 3: The electrostatic interaction weakens the stability of the outer membrane and the hydrophobic insertion destabilizes the outer membrane through hydrophobic expansion producing damage to the outer membrane. *Step* 4: Polymyxins penetrate into the inner membrane and inhibit the respiratory enzyme NDH-2. Figure is adapted from Deris *et al.* [40, 41] with permission from *Bioconjugate Chemistry*

4 Mechanisms of Polymyxin Resistance in Gram-Negative Bacteria

The molecular details of the interaction of polymyxins with the OM, as described above, indicate that polymyxin-mediated killing is initiated by the specific electrostatic interaction of polymyxins with lipid A. Therefore, it is not unexpected that the majority of polymyxin resistance mechanisms involve LPS and lipid A. The known lipid A-based resistance mechanisms include lipid A modification by the addition of 4-amino-L-arabinose (L-Ara4N), phosphoethanolamine (PEtn), or galactosamine (GalN), and complete LPS loss [43–46]. Each mechanism has a distinct impact on the chemistry and constitution of the LPS layer, activity of polymyxins, and the fitness of the bacteria.

4.1 Lipid A Modifications

The affinity of polymyxins for the OM of the Gram-negative bacterial cell is dependent on the electrostatic interaction between the cationic residues of polymyxins and the anionic phosphate groups of lipid A. Disrupting this interaction, whilst maintaining outer leaflet integrity, is crucial for polymyxin-resistant bacteria. Three major lipid A modifications that have been reported for polymyxin resistance are addition of 4-amino-L-arabinose, phosphoethanolamine, and galactosamine (Fig. 23.2). In each case the target of the modification is the phosphate groups of lipid A, and each modification functions to mask the negative charge presented by the phosphate groups of native lipid A.

The addition of 4-amino-L-arabinose (L-Ara4N) is the most ubiquitous of all lipid A modifications that confer polymyxin resistance. L-Ara4N modification requires the substitution of one or more phosphate groups of lipid A with L-Ara4N [46, 48] (Fig. 23.2). As a result, there is no anionic domain for the polymyxin molecule to bind. Without this initial polar interaction, the affinity of polymyxins for lipid A is dramatically reduced [29]. Consequently, MICs of strains with this modification increased. *Salmonella enterica*, *P. aeruginosa*, and *E. coli* have been shown to have MICs of up to 64, 520, and 32 mg/L, respectively [49–52]. In *Burkholderia* species very high levels of L-Ara4N modification occur and, as a result, mutants deficient in L-Ara4N modification are 16,000-fold more susceptible to polymyxins measured by viable colony counts at 10 mg/L [53]. The control of this L-Ara4N modification is crucial for the development of L-Ara4N-mediated polymyxin resistance. L-Ara4N is synthesized by the bacterial cell, and the synthesis and modification is a multistep process that requires genes coded in the *Pmr/Arn* locus.

L-Ara4N modification occurs in the periplasm and is catalyzed by a glycosyltransferase (PmrK/ArnT). However, regulation of L-Ara4N modification begins in the cytoplasm and with L-Ara4N synthesis. In the first step of L-Ara4N synthesis, UDP-glucuronic acid is made from UDP-glucose by the UDP-glucose dehydrogenase PmrE/Ugd [23, 54]. UDP-glucuronic acid is converted to UDP-4-keto-pyranose by oxidative carboxylation catalyzed by the formyltransferase PmrI/ArnA [54, 55]. The UDP-L-Ara4O C-4″ transaminase, PmrH/ArnB, then catalyzes the synthesis of UDP-L-Ara4N from UDP-4-keto-pyranose [54, 56]. A second round of formylation by PmrI/ArnA generates UDP-beta-L-Ara4FN before the next phase of L-Ara4N synthesis, which requires the action of IM protein PmrF/ArnC. Binding of UDP-beta-L-Ara4FN to PmrF/ArnC acts to localize the molecule to the IM where PmrJ/ArnD deformylates the molecule generating L-Ara4N. Upon binding to an IM flippase, comprised of PmrL/ArnE and PmrM/ArnF, L-Ara4N is translocated across the IM into the periplasm [57]. Finally, the glycosyltransferase, PrmK/ArnT, transfers the L-Ara4N molecule onto lipid A.

Polymyxin resistance by phenolethanolamine (PEtn) modification of lipid A is mainly found in *S. enterica* and *A. baumannii*. PEtn modification occurs by the addition of PEtn at the anionic phosphate domain of lipid A [43, 58] (Fig. 23.2). As with L-Ara4N, the consequence of this modification is to disrupt the electrostatic interactions that polymyxins require for their initial interaction with lipid A. The addition of PEtn to lipid A is catalyzed by PmrC, a PEtn transferase also known as EptA or PagB [57]. PmrC is an IM protein that has a large periplasmic *C*-terminal PEtn transferase domain. This topology allows PmrC to be proximal to both PEtn and lipid A. PmrC is encoded in the first gene in a polycistronic operon that also contains PmrA and PmrB (a two-component system that regulates the Pmr/Arn locus, see below). Although the *pmrC* gene is widely found in other species, there has been no evidence that the locus is important for polymyxin resistance. Interestingly, when the *E. coli* PmrC ortholog was deleted, resistance to polymyxins increased [19]. In addition to the varied phenotypic roles of PmrC, the genetic organization also varies between species; polymyxin-resistant clinical isolates were shown to have multiple active PmrC paralogs [59]. Genomic analysis revealed that 20 % of sequenced strains had two PmrC genes,

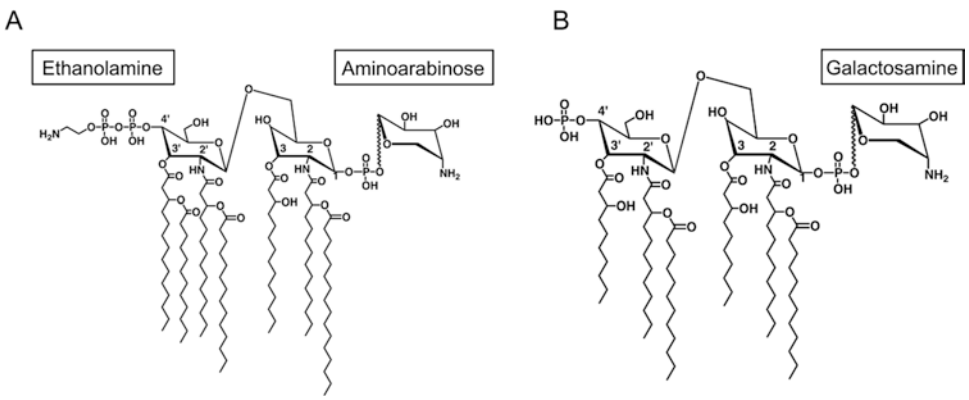


Fig. 23.2 LPS with modified phosphate groups. (**a**) Ethanolamine (*left*) and aminoarabinose (*right*) modifications. (**b**) Galactosamine (*right*) modification and unmodified phosphate group (*left*). Figure adapted from Pelletier *et al.* [47] *Antimicrobial Agents and Chemotherapy*

4 % contained three or more. In strains where PEtn has been shown to confer resistance to polymyxins, the resistance was robust; when PEtn modification was disabled, polymyxin B resistance was reduced by three- to five-fold and >100-fold in *S. enterica* and *A. baumannii*, respectively [17]. Overall, there is a disconnection between PEtn modification, the action of PmrC, and polymyxin resistance, which is exemplified by the mixed phenotypes of various species with PmrC [17, 19, 59]. Such variation is suggestive of a mechanism that requires additional factors to provide resistance.

Polymyxin resistance by the addition of galactosamine (GalN) to lipid A has recently been reported [47]. Again, the site of modification is the anionic phosphate domain of lipid A (Fig. 23.2). Galactosamine is structurally very similar to L-Ara4N, which suggests that the effect of this modification would conceal the negative phosphate groups. Of the three lipid A modification resistance mechanisms, galactosamine modification has been observed the least. Currently there is only one report documenting four polymyxin-resistant strains with this modification. *A. baumannii* strain MAC204 was selected in vitro for polymyxin resistance by iterative selection in 1 mg/L colistin, after which the GalN modification was discovered in MAC204 by tandem MS-MS [47]. The modification was later discovered in three clinical *A. baumannii* isolates. The MIC of strains harboring the GalN-modified lipid A was up to 400 times that of the parent strain without galactosamine modification [47]. However, galactosamine modification has not been shown to confer polymyxin resistance in isolation, as all GalN modified lipid A molecules were also PEtn modified [47]. The prevalence of this modification in polymyxin-resistant isolates in different bacterial species remains to be seen.

4.2 Regulation of Lipid A Modification Mechanisms

4.2.1 Regulation of L-Ara4N Modification of Lipid A

Lipid A modification by the addition of L-Ara4N is regulated by two-component systems in response to environmental signals. Although the genes involved in lipid A modification are conserved, their regulation varies between species; the best characterized regulatory systems are those of *S. enterica*, *E. coli*, and *P. aeruginosa*.

In *S. enterica* the two-component system PmrAB acts to control the L-Ara4N modification operon (*pmrHFIJKLM*), and PmrAB directly activates these operons in response to environmental signals. PmrB is a histidine kinase that senses low pH and high concentrations of Al^{3+} or Fe^{3+} [60]. Upon activation by autophosphorylation, PmrB activates the response regulator PmrA by phosphorylation. Activated PmrA directly binds promoters within its regulon, including *pmrHFIJKLM*, increasing expression of L-Ara4N synthesis and transfer genes [61, 62]. The PhoPQ two-component system also activates the *pmrHFIJKLM* locus in *S. enterica*. However, the activation is via a local regulator *pmrD*. PhoPQ responds to low Mg^{2+} concentrations, low pH, cationic peptides, or extracellular DNA [63, 64]. PmrD functions by inhibiting the dephosphorylation of the PmrA response regulator [65]. As a result the amount of active PmrA is increased and subsequently the activation of the *pmrHFIJKLM* locus is also increased. This indirect regulation by PhoPQ is unique to *Salmonella*, in *E. coli* the PhoPQ two-component system does not cross-talk to PmrA; as a consequence lipid A modification by L-Ara4N addition is not induced in conditions of low Mg^{2+} concentrations [52].

As in *Salmonella* and *E. coli*, PmrAB is the primary two-component system involved in controlling L-Ara4N addition to lipid A in *P. aeruginosa*. However, in *P. aeruginosa* four two-component systems have been reported to activate the *pmrHFIJKLME* operon; PmrAB, PhoPQ, ParRS, and CprRS [18, 66–68]. PmrAB works in a similar fashion directly activating the *pmrHFIJKLME* locus, but the PhoPQ two-component system, contrary to the PhoPQ network in *S. enterica*, activates the *pmr* locus directly [69]. As in *S. enterica* and *E. coli*, the action of both PhoP and PmrA at the *pmrHIJKLME* locus is to activate transcription. In *S. enterica*, PhoQ responds to the presence of antimicrobial [63]. However, in *P. aeruginosa* antimicrobial peptides are recognized by the two other two-component systems, ParRS and CprRS [18, 66]. The ParRS and CprRS systems each directly activate the *pmr* operon in response to antimicrobial peptides.

4.2.2 Regulation of PEtn Modification of Lipid A

As with the regulation of the L-Ara4N modification, the regulation of PEtn modification is controlled by the two-component system PmrAB, which responds to environmental signals [43, 70]. PEtn modification is found in *S. enterica*, *E. coli*, and *A. baumannii*; the transfer of PEtn to lipid A is catalyzed by PEtn transferase (PmrC) in these species [43]. PmrC is encoded in a polycistronic operon with PmrAB. As with the regulation of the L-Ara4N modification, the PmrB histidine kinase responds to environmental signals and the response regulator PmrA binds promoters to activate transcription. Upon phosphorylation PmrA activates *pmrC* transcription increasing the modification of lipid A by PEtn addition [70]. A reduction of PEtn modified lipid A was observed when *S. enterica* was grown in high Mg^{2+} concentrations, a condition that reduces PmrAB activation. A similar network is found in *A. baumannii*, showing that PmrAB is capable of activating polymyxin resistance by PEtn modification [59]. Polymyxin resistance was increased up to 128-fold when constitutively active PmrAB mutants were introduced into a wild-type strain [71]. Many of the PmrB

mutations were located within the predicted histidine kinase domain. In addition, the transcription of *pmrC,* and two *pmrC* paralogs (*eptA1* and *eptA2*) was increased in constitutive PmrB mutant backgrounds [17, 59, 71, 72].

Despite the aforementioned similarities, there are several key differences in the control of PEtn modification between species. In *A. baumannii,* PmrB was shown to be required for polymyxin resistance in low pH conditions but not for Fe^{3+}-induced resistance; this suggests that other two-component systems are involved in controlling polymyxin resistance [72]. In addition, in *S. enterica* there is an additional level of complexity conferred by LpxT, which is a phosphate transferase. LpxT competes with PmrC for the phosphate group on lipid A [19]. Upon activation PmrAB activates a small regulator PmrR which represses the expression of LpxT. As a result, PmrAB increases PEtn modification of lipid A by direct PmrC activation and indirect LpxT repression [19].

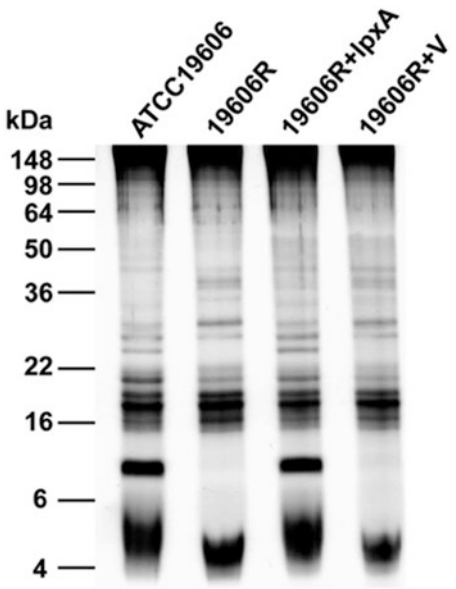


Fig. 23.3 Colistin-resistant *A. baumannii* strains do not produce LPS. PAGE separation and carbohydrate-specific silver staining of purified LPS of colistin-susceptible parent strain ATCC 19606, colistin-resistant variant 19606R, 19606R complemented with *lpxA* (19606R + lpxA), or 19606R containing vector only (19606R + V). The positions of standard molecular mass markers are shown on the left. Adapted from Moffatt *et al*. [44], *Antimicrobial Agents and Chemotherapy*

4.3 Polymyxin Resistance by Loss of Lipid A

An alternative to lipid A modification is the complete loss of lipid A biosynthesis. As previously mentioned, lipid A is an important component of the outer leaflet of the OM. The interactions between lipid A molecules are required for membrane stability and the chemistry between membrane lipids and membrane proteins is critical for membrane function [73]. Therefore, it is reasonable to assume that lipid A is essential, and in most cases this is true. However, lipid A-deficient strains of *A. baumannii* have been extensively characterized [74]. The lipid A-deficient strains have a complete loss of LPS and therefore the initial target of polymyxins is removed [44] (Fig. 23.3). This OM restructuring conferred a high level of resistance, and lipid A-deficient strains were found to have an MIC > 256 mg/L. Although many LPS mutants have been characterized in *A. baumannii*, few other cases of polymyxin resistance via lipid A deficiency have been documented [44, 75].

The analysis of 13 individual derivatives of *A. baumannii* ATCC 19606 that had been selected for growth on colistin showed LPS deficiency [44]. Whole genome re-sequencing revealed that each strain harbored a unique mutation in the lipid A biosynthesis gene cluster. The mutations found in each strain comprised single-base missense and frameshift mutations, IS element insertions, and deletions. Lipid A biosynthesis is a complicated process requiring many proteins that are coded on the *lpx* cluster; each unique mutation was located in *lpxA*, *lpxC*, or *lpxD* (Fig. 23.4). The *lpxA* gene encodes a UDP-*N*-acetylglucosamine acyltransferase (LpxA) that is involved in the first step of lipid A biosynthesis, *lpxC* encodes a UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase that catalyzes the second step in lipid A biosynthesis, and *lpxD* encodes a UDP-3-*O*-(3-hydroxymyristoyl) glucosamine *N*-acetyltransferase, which is the third step in lipid A biosynthesis [44, 74]. Any mutation that disrupts the function of these enzymes will halt the production of lipid A. Interestingly, no mutations have been mapped in genes that encode enzymes which operate further down the pathway.

Despite the loss of lipid A biosynthesis, and therefore the complete loss of LPS, an OM was still observable by scanning electron microscopy [74]. It is unclear what alterations occur in *A. baumannii* that compensate for the loss of lipid A. Transcriptomic analysis of strains containing *lpxA* mutations showed a large number of genes were differentially regulated. Processes affected include: phospholipid transport, lipoprotein biosynthesis, and the synthesis of poly-β-1, 6-*N*-acetylglucosamine, a cell surface polysaccharide [77]. However, despite the viability of the cells, the OM permeability was greatly increased, resulting in increased susceptibility to many antibiotics including cefepime, teicoplanin, and azithromycin [74]. In addition, antibiograms comparing the susceptibility of paired resistant and susceptible *A. baumannii* isolates indicated that most antimicrobials tested were more effective against colistin-resistant strains [78]. The case for using polymyxins in combination therapy is therefore strengthened by the fact that polymyxin resistance comes at such a fitness cost [74, 78].

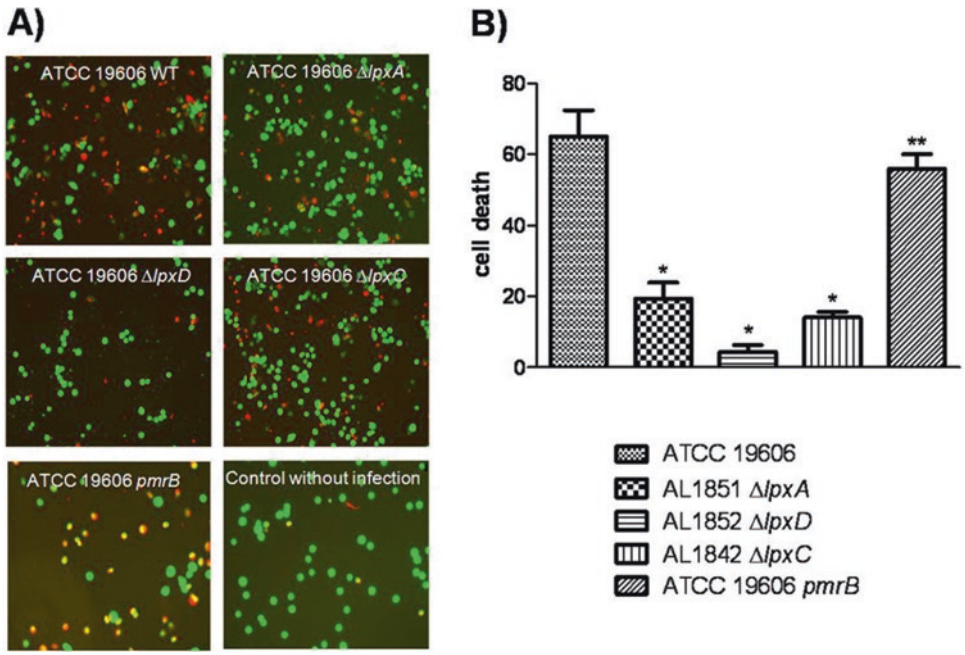


Fig. 23.4 Abilities of different *A. baumannii* strains to cause cell death of A549 alveolar cells. (**a**) Fluorescence microscopy images of human alveolar A549 cells infected with each of the *A. baumannii* strains and stained with the LIVE/DEAD Cellstain double-staining kit. Healthy cells with intact membranes are *stained green*, and dead cells with permeabilized membranes are *stained red*. A549 cells were incubated with the *A. baumannii* strains ATCC 19606 WT, AL1851Δ*lpxA*, Al1852Δ*lpxD*, AL1842Δ*lpxC*, and ATCC19606 *pmrB* for 20 h or left uninfected. (**b**) Quantification of A549 cell death caused *by A. baumannii* ATCC19606 WT and AL1851Δ*lpxA*, AL1852Δ*lpxD*, and AL1842Δ*lpxC* mutants and the *pmrB* mutant. The results of six independent experiments are shown as means and SD. **P*<0.01 between the parental strain and each of the designated mutants; **not statistically different. Adapted from Beceiro *et al*. [76], *Antimicrobial Agents and Chemotherapy*

4.4 Other Resistance Mechanisms

In several different bacteria, membrane proteins have been found to be involved in polymyxin resistance. Often the role in polymyxin resistance is inferred from phenotype observations of knock-out mutations; the direct role of each polymyxin resistance mechanism and the molecular details of each system are rarely characterized. In addition, the techniques used to infer susceptibility or resistance differ between experiments, making cross comparison difficult. Mechanisms of resistance to polymyxins, other than lipid A loss or modification, include active efflux and protection by capsule polysaccharide [20, 79].

In *Neisseria menigitidis*, a genome-wide transposon mutant library was screened for polymyxin susceptibility and several genes were identified including *lptA* (phosphoethanolamine transferase gene), *mtrCDE* (efflux pump operon), and *porB* (outer membrane porin). The wild-type *N. menigitidis* strain used in the study had a polymyxin B MIC of 512 mg/L; mutants in *mtrCDE*, *porB*, and *lptA* conferred polymyxin B MICs of 32, 32, and 2 mg/L, respectively [79]. The role of LptA in polymyxin resistance was predicted to be via lipid A modification, as described above. MtrCDE and PorB are thought to be involved in the active efflux of polymyxins from the cell. However, there is currently no direct evidence that MtrCDE and PorB function to actively pump polymyxins out of the cell [79].

Screens of *kpnGH* mutants for polymyxin resistance in *K. pneumoniae* revealed that these genes may have roles in polymyxin resistance [20]. The *kpnGH* operon codes for an efflux pump. Susceptibility was increased by two-fold in *kpnGH* mutant strains relative to wild type, measured by Kirby Bauer disc diffusion assay. However, the susceptibility of many other antimicrobials also changed in this mutant, which is suggestive of a more general defect caused by the mutation [20].

In *Vibrio cholerae*, ToxR was found to activate a putative OM porin, OmpU [80]. Deletions of *toxR* and *ompU* genes caused increased susceptibility to polymyxins; a 100-fold reduction in percentage survival after 45 min in 12 mg/L polymyxin B was discovered [80]. Currently, the role of OmpU in polymyxin resistance is not understood. More recent publications suggest that OmpU has a role as a sensor that regulates gene expression through the action of sigma factor E [81]. In addition, the role of OmpU varies at the species level. Levels of polymyxin resistance were only reduced twofold in *ompU* knockout strains of *V. splendidus* [82].

The two-component efflux pump, RosAB, has been implicated in polymyxin resistance in *Yersinia enterocolitica* [83]. Mutants lacking functional *rosAB* were more susceptible

to polymyxins; survival of *rosAB* mutants grown in 125 mg/L polymyxin B was reduced to 5 % compared to the wild type, which was reduced to 50 % [83]. In support of this, chemical perturbation of efflux pumps by the addition of 2-carbonyl cyanide m-chlorophenylhydrazone increased polymyxin susceptibility in wild-type cells. In addition, the mutant phenotype could be complemented by the expression of exogenous *rosAB* efflux pump genes, indicating that the deletion of these genes, and no other effects, caused the susceptible phenotype [83]. However, the role of RosAB in polymyxin resistance could be through a secondary mechanism as RosAB has been shown to regulate LPS biosynthesis in *Y. enterocolitica* [84]. A study on the *Y. enterocolitica* LPS biosynthesis pathway showed that mutations of LPS biosynthesis genes conferred polymyxin susceptibility. Therefore, RosAB may be required for the activation of LPS biosynthesis genes and consequently polymyxin resistance.

The presence and regulation of capsule polysaccharides in *K. pneumoniae* has been shown to be important for polymyxin resistance [85, 86]. A clinical isolate, which had been previously shown to produce a capsule, was used to study the resistance to polymyxins [85]. *K. pneumoniae cps* (capsule polysaccharide synthesis) knock-out mutants were susceptible to polymyxin B, the percentage relative survival at 4 units/mL was reduced from 70 to 12.5 % in a *cps* knock-out mutant strain [85]. In addition, a luciferase promoter-reporter system was used to measure the expression of *cps* during polymyxin B exposure. Polymyxin B exposure at 0.5 units/mL increased *cps* promoter activity 1.3-fold, and resistance to polymyxin B increased concomitantly [85]. The mechanism of resistance was initially proposed to be based on occlusion of the lipid A target by the capsule layer. However, it has also been reported that the capsule polysaccharide inhibits polymyxin B action by electrostatic charge-mediated trapping [86]. The proposed mechanism suggests that the anionic capsule polysaccharide (CPS) binds the cationic domains of polymyxin. The binding of CPS to polymyxin neutralizes the charged molecules on polymyxin, thereby preventing the initial charge-based interactions of polymyxin with lipid A [86].

5 Biological Consequences of Polymyxin Resistance

The evolution of polymyxin resistance by lipid A modification is poorly understood. Lipid A modifications may have evolved as part of an arms race with antimicrobial peptide producing bacteria such as *Pa. polymyxa*. Alternatively, the ability of the cell to modify OM chemistry by lipid A modification may have been required for survival in unique conditions such as during infection. In this case, resistance to polymyxins may have been a secondary adaptation. It is likely that lipid A modification evolved as a protective mechanism from environmental stresses encountered during infection and as a consequence of exposure to antimicrobial peptides [87].

Modification and loss of lipid A may cause considerable cost to fitness (Fig. 23.4). A comparison of *A. baumannii* strains in a mouse infection model showed that loss of lipid A and modification of lipid A reduced the competitive index of the *A. baumannii* to 0.09 and 0.35, respectively [76]. In addition, cell death of A549 lung epithelial cells induced by *A. baumannii* strains without lipid A was reduced three-fold. Interestingly, *A. baumannii* cells containing modified lipid A were capable of conferring similar levels of A549 cell death to that caused by wild-type *A. baumannii*. These results suggest that modification of lipid A is a less costly form or polymyxin resistance when measured in a biological context [76] (Fig. 23.4). The loss of lipid A is an unusual phenotype with only a few cases of lipid A-deficient bacteria currently known [44, 75, 88, 89]. Lipid A loss has been shown to have significant effects on the innate immune response to *A. baumannii* infection [90]. Lipid A-deficient strains were more susceptible to human antimicrobial peptide LL-37 [90]. In addition, lipid A-deficient strains of *A. baumannii* elicited a reduced immune response, two- to four-fold less NF-κB activation and TNF-α secretion compared to wild-type [90]. A transcriptomic comparison of wild-type and lipid A-deficient *A. baumannii* strains indicated that the genes involved in outer membrane homeostasis, such as *mla* (retrograde phospholipid biosynthesis) and *lol* (lipoprotein transport), are upregulated in the lipid A-deficient strains [77]. Furthermore, transmission electron microscopy indicated that the outer membrane is maintained in the lipid A-deficient strains. However, further investigation is required to understand how these outer membrane modifications are utilized by *A. baumannii* to compensate for the loss of lipid A [44, 77].

In *P. aeruginosa*, colistin resistance that developed in vivo was shown to be costly to *biological* fitness [45]. Each colistin-resistant isolate had mutation(s) in their PmrAB and PhoPQ regulatory systems that resulted in high levels of lipid A modification and colistin resistance (colistin MIC >512 mg/L) [45]. However, when the selection was removed (repeated passages without colistin), secondary suppressor mutations occurred causing the phenotype to revert to colistin sensitivity [45]. The nature of the suppressor mutations is unknown, but the high frequency of reversion is indicative of a substantial fitness cost caused by lipid A modification.

The effect of polymyxin resistance, either by lipid A loss or modification, has an impact on the fitness of the bacterium in the context of infection [45, 76, 77]. As a consequence, the fitness of polymyxin-resistant strains is only increased during polymyxin treatment and is lost when selection pressure is removed [45]. Understanding the adverse effects that

polymyxin resistance has on the viability and pathogenicity of the bacteria will aid the development of polymyxin combination therapies and treatment regimens.

6 Summary

The rise of multidrug-resistant bacteria and lack of novel antimicrobials have given new significance to polymyxins as a last-line therapeutic option. Polymyxins initially target lipid A, an essential component of Gram-negative bacteria. Currently, the complete molecular details of polymyxin-induced killing remain unknown. More research is required to fully elucidate the detailed mechanism of action. The effects of polymyxins binding to lipid A and subsequent permeation of the outer membrane of the organism facilitates entry into the cell of drugs that are normally occluded by the outer membrane. As a consequence, many compounds normally devoid of antimicrobial activity against Gram-negatives may exhibit antimicrobial activity in combination. Novel therapies using existing polymyxin B and colistin in combination with other approved drugs will increase our arsenal against Gram-negative infections. A greater understanding of polymyxin killing and emergence of resistance will aid the discovery of a new generation of antimicrobials that target polymyxin-resistant pathogens.

References

1. Ainsworth GC, Brown AM, Brownlee G. Aerosporin, an antibiotic produced by Bacillus aerosporus Greer. Nature. 1947;159 (4060):263.
2. Benedict RG, Langlykke AF. Antibiotic activity of *Bacillus polymyxa*. J Bacteriol. 1947;54(1):24.
3. Stansly PG, Shepherd RG, White HJ. Polymyxin: a new chemotherapeutic agent. Bull Johns Hopkins Hosp. 1947;81(1): 43–54.
4. Koyama Y, Kurosawa A, Tsuchiya A, Takakuta K. A new antibiotic "colistin" produced by spore-forming soil bacteria. J Antibiot (Tokyo). 1950;3:457–8.
5. Wilkinson S. Identity of colistin and polymyxin E. Lancet. 1963; 281(7287):922–3.
6. Wolinsky E, Hines JD. Neurotoxic and nephrotoxic effects of colistin in patients with renal disease. N Engl J Med. 1962; 266(15):759–62.
7. Evans ME, Feola DJ, Rapp RP. Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria. Ann Pharmacother. 1999;33(9):960–7.
8. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2006; 50:2946–50. doi:10.1128/AAC.00103-06.
9. Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect Dis. 2006;6:589–601. doi:10.1016/S1473-3099(06)70580-1.
10. Zavascki AP, Goldani LZ, Li J, Nation RL. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J Antimicrob Chemother. 2007;60:1206–15. doi:10.1093/jac/dkm357.
11. Cai Y, Chai D, Wang R, Liang B, Bai N. Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies. J Antimicrob Chemother. 2012;67:1607–15. doi:10.1093/jac/dks084.
12. Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Updat. 2010;13:132–8. doi:10.1016/j.drup.2010.05.002.
13. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Clinical breakpoints. European committee on antimicrobial susceptibility testing, EUCAST 2014. http://www.eucast.org/clinical_breakpoints; 2014.
14. Wang X, Kang Y, Lou C, Zhao T, Liu L, Jiang X, Fu R, An S, Chen J, Jiang N, Ren L, Wang Q, Baille JK, Gao Z, Yu J. Heteroresistance at the single-cell level: adapting to antibiotic stress through a population-based strategy and growth-controlled interphenotypic coordination. MBio. 2014;5:1–9. doi:10.1128/mBio.00942-13.Editor.
15. Gales AC, Castanheira M, Jones RN, Sader HS. Antimicrobial resistance among Gram-negative bacilli isolated from Latin America: results from SENTRY Antimicrobial Surveillance Program (Latin America, 2008–2010). Diagn Microbiol Infect Dis. 2012;73:354–60. doi:10.1016/j.diagmicrobio.2012.04.007.
16. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini G. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Eurosurveillance. 2014;19:20939. doi:10.2807/1560-7917.ES2014.19.42.20939.
17. Arroyo LA, Herrera CM, Fernandez L, Hankins JV, Trent MS, Hancock REW. The pmrCAB operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. Antimicrob Agents Chemother. 2011;55:3743–51. doi:10.1128/AAC.00256-11.
18. Fernández L, Gooderham WJ, Bains M, Mcphee JB, Wiegand I, Hancock REW. Adaptive resistance to the last hope antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS adaptive resistance to the "Last Hope" antibiotics polymyxin B and colistin in P. Antimicrob Agents Chemother. 2010;54:3372–82. doi:10.1128/AAC.00242-10.
19. Herrera CM, Hankins JV, Trent MS. Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol Microbiol. 2010;76:1444–60. doi:10.1111/j.1365-2958.2010.07150.x.
20. Srinivasan VB, Singh BB, Priyadarshi N, Chauhan NK, Rajamohan G. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. PLoS One. 2014;9, e96288. doi:10.1371/journal.pone.0096288.
21. Hancock RE. Peptide antibiotics. Lancet. 1997;349:418–22. doi:10.1016/S0140-6736(97)80051-7.
22. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67:549. doi:10.1128/MMBR.67.4.593.
23. Raetz CRH, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem. 2002;71:635–700. doi:10.1146/annurev.biochem.71.110601.135414.
24. Koebnik R, Locher KP, Van Gelder P. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol. 2000;37:239–53.
25. Silipo A, Molinaro A, Cescutti P, Bedini E, Rizzo R, Parrilli M, Lanzetta R. Complete structural characterization of the lipid A fraction of a clinical strain of *B. cepacia genomovar* I lipopolysaccharide. Glycobiology. 2005;15:561–70. doi:10.1093/glycob/cwi029.

26. Levy BYSB, Leive L. An equilibrium between two fractions of lipopolysaccharide in *Escherichia coli*. Proc Natl Acad Sci U S A. 1968;61(4):1435–9.
27. Schnaitman CA. Effect of Ethylenediaminetetraacetic Acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J Bacteriol. 1971;108:553–63.
28. Velkov T, Roberts K. Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. Future Microbiol. 2013;8:1–20. doi:10.2217/fmb.13.39.Pharmacology.
29. Velkov T, Thomson P, Li J. Structure-activity relationships of polymyxin antibiotics. J Med Chem. 2010;53:1898–916. doi:10.1021/jm900999h.Structure.
30. Meredith JJ, Dufour A, Bruch MD. Comparison of the structure and dynamics of the antibiotic peptide polymyxin B and the inactive nonapeptide in aqueous trifluoroethanol by NMR spectroscopy. J Phys Chem B. 2009;113:544–51. doi:10.1021/jp808379x.
31. Powers J-PS, Hancock REW. The relationship between peptide structure and antibacterial activity. Peptides. 2003;24:1681–91. doi:10.1016/j.peptides.2003.08.023.
32. Bruch MD, Cajal Y, Koh JT, Jain MK. Higher-order structure of polymyxin B: The functional significance of topological flexibility. J Am Chem Soc. 1999;121:11993–2004. doi:10.1021/ja992376m.
33. Kidric J. Solution structure of polymyxins B and E and effect of binding to lipopolysaccharide: an NMR and molecular modeling study. J Med Chem. 1999;42(22):4604–13.
34. Clausell A, Garcia-Subirats M, Pujol M, Busquets MA, Rabanal F, Cajal Y. Gram-negative outer and inner membrane models: insertion of cyclic cationic lipopeptides. J Phys Chem B. 2007;111:551–63. doi:10.1021/jp064757+.
35. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. Trends Biotechnol. 1998;16(2):82–8.
36. Dixon RA, Chopra I. Polymyxin B and polymyxin B nonapeptide alter cytoplasmic membrane permeability in *Escherichia coli*. J Antimicrob Chemother. 1986;18:557–63.
37. Sampson TR, Liu X, Schroeder MR, Kraft CS, Burd EM, Weiss DS. Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. Antimicrob Agents Chemother. 2012;56:5642–9. doi:10.1128/AAC.00756-12.
38. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. Killing by bactericidal antibiotics does not depend on reactive oxygen species. Science. 2013;339:1213–6. doi:10.1126/science.1232688.
39. Liu Y, Imlay JA. Cell death from antibiotics without the involvement of reactive oxygen species. Science. 2013;339:1210–3. doi:10.1126/science.1232751.
40. Deris ZZ, Akter J, Sivashangarie S, Roberts KD, Thompson PE, Nation RL, Li J, Velkov T. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. J Antibiot (Tokyo). 2014;67:147–51. doi:10.1038/ja.2013.111.A.
41. Deris ZZ, Swarbrick JD, Roberts KD, Azad MA, Akter J, Horne AS, Nation RL, Rogers KL, Thompson PE, Velkov T, Li J. Probing the penetration of antimicrobial polymyxin lipopeptides into gram-negative bacteria. Bioconjug Chem. 2014;25:750–60. doi:10.1021/bc500094d.
42. Mogi T, Murase Y, Mori M, Shiomi K, Omura S, Paranagama MP, Kita K. Polymyxin B identified as an inhibitor of alternative NADH dehydrogenase and malate: quinone oxidoreductase from the Gram-positive bacterium *Mycobacterium smegmatis*. J Biochem. 2009;146:491–9. doi:10.1093/jb/mvp096.
43. Lee H, Hsu F, Turk J, Eduardo A, Groisman EA. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. J Bacteriol. 2004;186(13):4124–33. doi:10.1128/JB.186.13.4124.
44. Moffatt JH, Harper M, Harrison P, Hale JDF, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother. 2010;54:4971–7. doi:10.1128/AAC.00834-10.
45. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Høiby N. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother. 2012;56:1019–30. doi:10.1128/AAC.05829-11.
46. Nummila K, Kilpelainen I, Zahringer U, Vaara M, Helander IM. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. Mol Microb. 1995;16(2):271–8.
47. Pelletier MR, Casella LG, Jones JW, Adams MD, Zurawski DV, Hazlett KRO, Doi Y, Ernst RK. Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2013;57:4831–40. doi:10.1128/AAC.00865-13.
48. Isshiki Y, Kawahara K, Za U. Isolation and characterisation of disodium (4-amino-4-deoxy-beta-L-arabinopyranosyl)-(1→8)-(D-glycero-alpha-D-talo-oct-2-ulopyranosylona te)-(2→4)-(methyl 3-deoxy-D-manno-oct-2-ulopyranosid)onate from the lipopolysaccharide of *Burkholderia cepacia*. Carbohydr Res. 1998;313:21–7.
49. Gooderham WJ, Gellatly SL, Sanschagrin F, McPhee JB, Bains M, Cosseau C, Levesque RC, Hancock REW. The sensor kinase PhoQ mediates virulence in *Pseudomonas aeruginosa*. Microbiology. 2009;155:699–711. doi:10.1099/mic.0.024554-0.
50. McPhee JB, Lewenza S, Hancock REW. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol Microbiol. 2003;50:205–17. doi:10.1046/j.1365-2958.2003.03673.x.
51. Tran AX, Lester ME, Stead CM, Raetz CRH, Maskell DJ, McGrath SC, Cotter RJ, Trent MS. Resistance to the antimicrobial peptide polymyxin requires myristoylation of *Escherichia coli* and *Salmonella typhimurium* lipid A. J Biol Chem. 2005;280:28186–94. doi:10.1074/jbc.M505020200.
52. Winfield MD, Groisman EA. Phenotypic differences between *Salmonella* and *Escherichia coli* resulting from the disparate regulation of homologous genes. Proc Natl Acad Sci U S A. 2004;101:17162–7. doi:10.1073/pnas.0406038101.
53. Hamad MA, Di Lorenzo F, Molinaro A, Valvano MA. Aminoarabinose is essential for lipopolysaccharide export and intrinsic antimicrobial peptide resistance in *Burkholderia cenocepacia*(†). Mol Microbiol. 2012;85:962–74. doi:10.1111/j.1365-2958.2012.08154.x.
54. Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SI. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol. 1998;27:1171–82.
55. Breazeale SD, Ribeiro AA, McClerren AL, Raetz CRH. A formyltransferase required for polymyxin resistance in *Escherichia coli* and the modification of lipid A with 4-Amino-4-deoxy-L--arabinose. Identification and function of UDP-4-deoxy-4-formamido-L-arabinose. J Biol Chem. 2005;280:14154–67. doi:10.1074/jbc.M414265200.
56. Trent MS, Ribeiro AA, Lin S, Cotter RJ, Raetz CR. An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked

donor. J Biol Chem. 2001;276:43122–31. doi:10.1074/jbc.M106961200.
57. Yan A, Guan Z, Raetz CRH. An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in Escherichia coli. J Biol Chem. 2007;282:36077–89. doi:10.1074/jbc.M706172200.
58. Zhou Z, Ribeiro AA, Lin S, Cotter RJ, Miller SI, Raetz CR. Lipid A modifications in polymyxin-resistant Salmonella typhimurium: PMRA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. J Biol Chem. 2001;276:43111–21. doi:10.1074/jbc.M106960200.
59. Lesho E, Yoon E-J, McGann P, Snesrud E, Kwak Y, Milillo M, Onmus-Leone F, Preston L, St Clair K, Nikolich M, Viscount H, Wortmann G, Zapor M, Grillot-Courvalin C, Courvalin P, Clifford R, Waterman PE. Emergence of colistin-resistance in extremely drug-resistant Acinetobacter baumannii containing a novel pmrCAB operon during colistin therapy of wound infections. J Infect Dis. 2013;208:1142–51. doi:10.1093/infdis/jit293.
60. Chen HD, Groisman EA. The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications. Annu Rev Microbiol. 2013;67:83–112. doi:10.1146/annurev-micro-092412-155751.
61. Gunn JS, Ernst RK, McCoy AJ, Miller SI. Constitutive mutations of the Salmonella enterica serovar Typhimurium transcriptional virulence regulator phoP. Infect Immun. 2000;68:3758–62.
62. Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of Salmonella enterica serovar typhimurium. Infect Immun. 2000;68:6139–46.
63. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, Xu W, Klevit RE, Le Moual H, Miller SI. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell. 2005;122:461–72. doi:10.1016/j.cell.2005.05.030.
64. Johnson L, Horsman SR, Charron-Mazenod L, Turnbull AL, Mulcahy H, Surette MG, Lewenza S. Extracellular DNA-induced antimicrobial peptide resistance in Salmonella enterica serovar Typhimurium. BMC Microbiol. 2013;13:115. doi:10.1186/1471-2180-13-115.
65. Kox LFF, Wo MMSM, Groisman EA. A small protein that mediates the activation of a two-component system by another two-component system. EMBO. 2000;19:1861–72.
66. Fernández L, Jenssen H, Bains M, Wiegand I, Gooderham WJ, Hancock REW. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in Pseudomonas aeruginosa independently of ParRS. Antimicrob Agents Chemother. 2012;56:6212–22. doi:10.1128/AAC.01530-12.
67. Moskowitz SM, Ernst RK, Miller SI. PmrAB, A two-component regulatory system of Pseudomonas aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A PmrAB. J Bacteriol. 2004;186(2):575. doi:10.1128/JB.186.2.575.
68. Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2011;55:1211–21. doi:10.1128/AAC.01252-10.
69. Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE. PhoP-PhoQ homologues in Pseudomonas aeruginosa regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol Microbiol. 1999;34:305–16.
70. Kato A, Chen HD, Latifi T, Groisman EA. Reciprocal control between a bacterium's regulatory system and the modification status of its lipopolysaccharide. Mol Cell. 2012;47:897–908. doi:10.1016/j.molcel.2012.07.017.
71. Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the pmrAB two-component regulatory system. Antimicrob Agents Chemother. 2011;55:3370–9. doi:10.1128/AAC.00079-11.
72. Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. Resistance to colistin in Acinetobacter baumannii associated with mutations in the PmrAB two-component system. Antimicrob Agents Chemother. 2009;53:3628–34. doi:10.1128/AAC.00284-09.
73. Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ, Robinson CV. Membrane proteins bind lipids selectively to modulate their structure and function. Nature. 2014;510:172–5. doi:10.1038/nature13419.
74. Moffatt JH, Harper M, Adler B, Nation RL, Li J, Boyce JD. Insertion sequence ISAba11 is involved in colistin resistance and loss of lipopolysaccharide in Acinetobacter baumannii. Antimicrob Agents Chemother. 2011;55:3022–4. doi:10.1128/AAC.01732-10.
75. García-Quintanilla M, Pulido MR, Moreno-Martínez P, Martín-Peña R, López-Rojas R, Pachón J, McConnell MJ. Activity of host antimicrobials against multidrug-resistant Acinetobacter baumannii acquiring colistin resistance through loss of lipopolysaccharide. Antimicrob Agents Chemother. 2014;58:2972–5. doi:10.1128/AAC.02642-13.
76. Beceiro A, Moreno A, Fernández N, Vallejo JA, Aranda J, Adler B, Harper M, Boyce JD, Bou G. Biological cost of different mechanisms of colistin resistance and their impact on virulence in Acinetobacter baumannii. Antimicrob Agents Chemother. 2014;58:518–26. doi:10.1128/AAC.01597-13.
77. Henry R, Vithanage N, Harrison P, Seemann T, Coutts S, Moffatt JH, Nation RL, Li J, Harper M, Adler B, Boyce JD. Colistin-resistant, lipopolysaccharide-deficient Acinetobacter baumannii responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-β-1,6-N-acetylglucosamine. Antimicrob Agents Chemother. 2012;56:59–69. doi:10.1128/AAC.05191-11.
78. Li J, Nation RL, Owen RJ, Wong S, Spelman D, Franklin C. Antibiograms of multidrug-resistant clinical Acinetobacter baumannii: promising therapeutic options for treatment of infection with colistin-resistant strains. Clin Infect Dis. 2007;45:594–8. doi:10.1086/520658.
79. Tzeng Y, Ambrose KD, Zughaier S, Miller YK, Shafer WM, David S, Zhou X, Stephens DS. Cationic antimicrobial peptide resistance in Neisseria meningitidis. J Bacteriol. 2005;187(15):5387. doi:10.1128/JB.187.15.5387.
80. Mathur J, Waldor MK. The vibrio cholerae ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. Mol Microbiol. 2004;72(6):3577–83. doi:10.1128/IAI.72.6.3577.
81. Mathur J, Davis BM, Waldor MK. Antimicrobial peptides activate the Vibrio cholerae sigmaE regulon through an OmpU-dependent signalling pathway. Mol Microbiol. 2007;63:848–58. doi:10.1111/j.1365-2958.2006.05544.x.
82. Duperthuy M, Binesse J, Le Roux F, Romestand B, Caro A, Got P, Givaudan A, Mazel D, Bachère E, Destoumieux-Garzón D. The major outer membrane protein OmpU of Vibrio splendidus contributes to host antimicrobial peptide resistance and is required for virulence in the oyster Crassostrea gigas. Environ Microbiol. 2010;12:951–63. doi:10.1111/j.1462-2920.2009.02138.x.
83. Bengoechea JA, Skurnik M. Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in Yersinia. Mol Microbiol. 2000;37:67–80.
84. Bengoechea JA, Zhang L, Toivanen P, Skurnik M. Regulatory network of lipopolysaccharide O-antigen biosynthesis in Yersinia

enterocolitica includes cell envelope-dependent signals. Mol Microbiol. 2002;44:1045–62.
85. Campos MA, Vargas MA, Regueiro V, Llompart CM, Albertí S, Bengoechea JA. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun. 2004;72:7107–14. doi:10.1128/IAI.72.12.7107-7114.2004.
86. Llobet E, Tomás JM, Bengoechea JA. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology. 2008;154:3877–86. doi:10.1099/mic.0.2008/022301-0.
87. Needham BD, Trent MS. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol. 2013;11:467–81. doi:10.1038/nrmicro3047.
88. Bos MP, Tommassen J. Viability of a capsule- and lipopolysaccharide-deficient mutant of Neisseria meningitidis. Infect Immun. 2005;73:6194–7. doi:10.1128/IAI.73.9.6194-6197.2005.
89. Keck M, Gisch N, Moll H, Vorhölter F-J, Gerth K, Kahmann U, Lissel M, Lindner B, Niehaus K, Holst O. Unusual outer membrane lipid composition of the gram-negative, lipopolysaccharide-lacking mycobacterium Sorangium cellulosum So ce56. J Biol Chem. 2011;286:12850–9. doi:10.1074/jbc.M110.194209.
90. Moffatt JH, Harper M, Mansell A, Crane B, Fitzsimons TC, Nation RL, Li J, Adler B, Boyce JD. Lipopolysaccharide-deficient Acinetobacter baumannii shows altered signaling through host Toll-like receptors and increased susceptibility to the host antimicrobial peptide LL-37. Infect Immun. 2013;81:684–9. doi:10.1128/IAI.01362-12.
91. Aguirre A, Lejona S, Véscovi EG, Soncini FC. Phosphorylated PmrA interacts with the promoter region of ugd in Salmonella enterica serovar typhimurium. J Bacteriol. 2000;182:3874–6.
92. Anisimov AP, Dentovskaya SV, Titareva GM, Bakhteeva IV, Shaikhutdinova RZ, Balakhonov SV, Lindner B, Kocharova NA, Senchenkova SN, Holst O, Pier GB, Knirel YA. Intraspecies and temperature-dependent variations in susceptibility of Yersinia pestis to the bactericidal action of serum and to polymyxin B. Infect Immun. 2005;73:7324–31. doi:10.1128/IAI.73.11.7324-7331.2005.
93. Chambers JR, Sauer K. The MerR-like regulator BrlR impairs Pseudomonas aeruginosa biofilm tolerance to colistin by repressing PhoPQ. J Bacteriol. 2013;195:4678–88. doi:10.1128/JB.00834-13.
94. Chantratita N, Tandhavanant S, Wikraiphat C, Trunck LA, Rholl DA, Thanwisai A, Saiprom N, Limmathurotsakul D, Korbsrisate S, Day NPJ, Schweizer HP, Peacock SJ. Proteomic analysis of colony morphology variants of Burkholderia pseudomallei defines a role for the arginine deaminase system in bacterial survival. J Proteomics. 2012;75:1031–42. doi:10.1016/j.jprot.2011.10.015.
95. Dentovskaya SV, Anisimov AP, Kondakova AN, Lindner B, Bystrova OV, Svetoch TE, Shaikhutdinova RZ, Ivanov SA, Bakhteeva IV, Titareva GM, Knirel AYA. Functional characterization and biological significance of Yersinia pestis lipopolysaccharide biosynthesis genes. Biochem Biokhimiia. 2011;76:808–22. doi:10.1134/S0006297911070121.
96. Fernández L, Alvarez-Ortega C, Wiegand I, Olivares J, Kocíncová D, Lam JS, Martínez JL, Hancock REW. Characterization of the polymyxin B resistome of Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2013;57:110–9. doi:10.1128/AAC.01583-12.
97. Fu W, Yang F, Kang X, Zhang X, Li Y, Xia B, Jin C. First structure of the polymyxin resistance proteins. Biochem Biophys Res Commun. 2007;361:1033–7. doi:10.1016/j.bbrc.2007.07.144.
98. Gunn JS, Hohmann EL, Miller SI. Transcriptional regulation of Salmonella virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. J Bacteriol. 1996;178:6369–73.
99. Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, Kaul RK, Johansen HK, Høiby N, Moskowitz SM. Polymyxin resistance of Pseudomonas aeruginosa phoQ mutants is dependent on additional two-component regulatory systems. Antimicrob Agents Chemother. 2013;57:2204–15. doi:10.1128/AAC.02353-12.
100. Ito-Kagawa M, Koyama Y. Selective cleavage of a peptide antibiotic, colisitin by colistinase. J Antibiot (Tokyo). 1980;33(12):1551–5.
101. Kim Y, Bae IK, Lee H, Jeong SH, Yong D, Lee K. In vivo emergence of colistin resistance in Acinetobacter baumannii clinical isolates of sequence type 357 during colistin treatment. Diagn Microbiol Infect Dis. 2014;79:362–6. doi:10.1016/j.diagmicrobio.2014.03.027.
102. López-Rojas R, McConnell MJ, Jiménez-Mejías ME, Domínguez-Herrera J, Fernández-Cuenca F, Pachón J. Colistin resistance in a clinical Acinetobacter baumannii strain appearing after colistin treatment: effect on virulence and bacterial fitness. Antimicrob Agents Chemother. 2013;57:4587–9. doi:10.1128/AAC.00543-13.
103. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Høiby N, Moskowitz SM. PhoQ mutations promote lipid A modification and polymyxin resistance of Pseudomonas aeruginosa found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother. 2011;55:5761–9. doi:10.1128/AAC.05391-11.
104. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms. PLoS Pathog. 2008;4, e1000213. doi:10.1371/journal.ppat.1000213.
105. Reinés M, Llobet E, Llompart CM, Moranta D, Pérez-Gutiérrez C, Bengoechea JA. Molecular basis of Yersinia enterocolitica temperature-dependent resistance to antimicrobial peptides. J Bacteriol. 2012;194:3173–88. doi:10.1128/JB.00308-12.
106. Rodriguez CH, Hernan RC, Bombicino K, Karina B, Granados G, Gabriela G, Nastro M, Marcela N, Vay C, Carlos V, Famiglietti A, Angela F. Selection of colistin-resistant Acinetobacter baumannii isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagn Microbiol Infect Dis. 2009;65:188–91. doi:10.1016/j.diagmicrobio.2009.05.019.
107. Snitkin ES, Zelazny AM, Gupta J, Palmore TN, Murray PR, Segre JA. Genomic insights into the fate of colistin resistance and Acinetobacter baumannii during patient treatment. Genome Res. 2013;23:1155–62. doi:10.1101/gr.154328.112.
108. Tan C, Li J, Nation RL. Activity of colistin against heteroresistant Acinetobacter baumannii and emergence of resistance in an. Antimicrob Agents Chemother. 2007;51:3413–5. doi:10.1128/AAC.01571-06.
109. Wang X, Ribeiro AA, Guan Z, Raetz CRH. Identification of undecaprenyl phosphate-beta-D-galactosamine in Francisella novicida and its function in lipid A modification. Biochemistry. 2009;48:1162–72. doi:10.1021/bi802211k.
110. Wiese A, Gutsmann T, Seydel U. Towards antibacterial strategies: studies on the mechanisms of interaction between antibacterial peptides and model membranes. J Endotoxin Res. 2003;9:67–84. doi:10.1179/096805103125001441.

Sulfonamides and Trimethoprim

24

Ola E. Sköld and Göte Swedberg

1 Introduction

Sulfonamides interfere with the formation of folic acid in bacteria. Since mammalian cells lack the sequence of enzymic reactions leading to folic acid, including that catalyzed by dihydropteroate synthase, the target of sulfonamides, they are dependent on an external source of folic acid. This is the basis for the selective action of sulfonamides on bacteria.

The first demonstration of the antibacterial (antistreptococcal) effect of the chemically synthesized sulfonamides in mice was performed by Gerhard Domagk at the University of Münster in Germany in 1932 [1, 2]. This can be regarded as the very first demonstration of the selective antibacterial action of a drug. This work was highly valued and Domagk was nominated for the Nobel Prize in 1939, but since the Nazi regime of that time in Germany did not want any German to receive the Nobel Prize, Nazi officials put pressure on the Nobel Committee at the Karolinska Institute not to award him. The Nobel Committee under its chairman, Folke Henschen, stood up to the pressure and recommended the medical faculty at the Karolinska Institute to award the Nobel Prize to Domagk. In his memoirs from 1957, Henschen has described, that when this had been announced in October 1939, soldiers came to Domagk's home in Wuppertal in the middle of the night to arrest him and put him in jail. On his round the next morning the astonished prison director found Domagk there and asked him: "How come you are here?" Domagk replied: "*Ich habe den Nobelpreis bekommen*." Domagk was not allowed to leave Germany at the time, but finally came to Stockholm in 1947 to receive his Nobel diploma.

Chemically synthesized sulfonamides with Domagk's Prontosil rubrum (4-sulfonamide-2′,4′-diaminoazobenzene) [1] as the first have been widely used as efficient and inexpensive antibacterial drugs for the treatment of both gram-positive and gram-negative pathogens. The many sulfonamide derivatives, that have been in clinical use through the years are identical from a microbiological point of view, but differ in pharmacokinetical properties.

Sulfonamides have not been used much in later years. Some important indications still exist, for example, the combination of sulfamethoxazole and trimethoprim (SXT) is considered the drug of choice for infections caused by *Stenotrophomonas maltophilia* although other alternatives like fluoroquinolones are effective as well [3]. This use may be compromised by an increasing frequency of resistance as reported lately [4]. Also, community-associated MRSA is usually susceptible to SXT, which offers an inexpensive treatment choice [5, 6].

The distribution of sulfonamides for systemic use as a single drug in Sweden is presently nil. The combination of sulfonamide and trimethoprim is still used, however, but mostly in hospitals and only to a limited extent. The total distribution of this drug combination in the last 4 years in Sweden has been rather constant and has amounted to about 620,000 defined daily doses per year corresponding to less than 0.2 defined daily doses per 1000 inhabitants and day.

There are three main reasons for the limited use of sulfonamides today. The first is due to side effects, which are quite common in treated patients. Adverse reactions from the skin and the hemopoietic system have led to the restricted use also of the trimethoprim–sulfonamide combination. Systematic clinical studies showed blood dyscrasias, including aplastic anemia, at a frequency of 5.3 per million defined daily doses of sulfonamides, and with a fatality rate of 17 % in the affected group [7]. Sulfonamides seem to be the most commonly reported drugs for all blood dyscrasias [8]. A second reason for the small use of sulfonamides is that, after their introduction in the 1930s, penicillins and many other efficient antimicrobial agents became available. A third reason finally would be the rapid resistance development after their introduction in clinical medicine. This ought to

O.E. Sköld, M.D., Ph.D. (✉) • G. Swedberg, Ph.D.
Department of Medical Biochemistry and Microbiology, Uppsala University, PO Box 582, Uppsala SE-75123, Sweden
e-mail: Ola.Skold@imbim.uu.se; Gote.Swedberg@imbim.uu.se

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_24

mean that sulfonamide resistance in, for example, streptococci and meningococci should have disappeared in the absence of selection pressure. This is not the case, however. Detailed studies on the mechanisms and genetics of this resistance could shed light on the important question of resistance reversibility and compensatory evolution.

In the present clinical situation of increasing resistance to antibacterial agents among pathogens, sulfonamides might have to be reconsidered as remedies against infectious disease with modern vigilance and knowledge of side effects.

Trimethoprim is related to sulfonamides in the sense that it interferes with folate metabolism. Sulfonamides act by their structural analogy with *p*-aminobenzoic acid, and competitively inhibit the condensation of this folic acid component with 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate to form dihydropteroic acid under the catalysis of dihydropteroate synthase [9–11]. Trimethoprim, with its 2,4-diaminopyrimidine structure, on the other hand, is an analog of the folic acid pterin moiety, and competitively inhibits the reduction of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase, in analogy with the antifolate cytostatic drugs aminopterin and methotrexate. The selective action of trimethoprim on bacterial dihydrofolate reductases, leaving mammalian enzymes untouched, allows the clinical use of trimethoprim as an antibacterial drug [12] As a matter of fact, trimethoprim does not interfere with human dihydrofolate reductase even at concentrations 10,000-fold higher than the MIC values found for most bacteria. There is a structural explanation for this, elucidated by X-ray crystallography studies, showing that trimethoprim fits well into the nucleotide-binding site of the dihydrofolate reductase from, for example, *Escherichia coli*, but not in the corresponding site of the mammalian enzyme [13]. Trimethoprim has a broad antibacterial spectrum. This can vary slightly in analogs of it, like iclaprim [14], and epiroprim [15]. Since sulfonamides and trimethoprim attack successive steps in the same enzymic pathway, there is a synergistic effect, which has been successfully exploited in the broad spectrum combination drug, co-trimoxazole.

Some bacteria like *Campylobacter jejuni* and *Helicobacter pylori* seem to be naturally resistant to trimethoprim. It has turned out that these bacteria lack the gene *fol*A, coding for dihydrofolate reductase on their chromosomes, and do thus not offer any target for the antifolate [16]. Tetrahydrofolate-borne one-carbon units are required for RNA-, DNA-, and protein synthesis. The main drain on reduced folates in actively dividing bacterial cells is for the methylation of deoxyuridylic acid to form deoxythymidine-5′-monophosphate (thymidylate) under the catalysis of thymidylate synthase (*thy*A). In this process, the methylene tetrahydrofolate gets oxidized to dihydrofolate, which is then reduced to tetrahydrofolate by dihydrofolate reductase expressed from *fol*A, which all *thy*A-carrying bacteria also contain. There is, however, a recently discovered alternative pathway for thymidylate synthesis, catalyzed by the product of *thy*X, and which does not involve the oxidation of tetrahydrofolate, but in which reduced flavin nucleotides ($FADH_2$) have an obligatory role [17]. The *thy*X-carrying *Campylobacter jejuni* and *Helicobacter pylori* would then seem to be able to do without *fol*A, and thus without the dihydrofolate reductase target of trimethoprim [16].

2 Chromosomal Resistance to Sulfonamides

Spontaneous mutations to sulfonamide resistance changing the *dhps* (*fol*P) gene are easily observed in *Escherichia coli* [18–22]. In one of these cases, the *fol*P gene had changed by a single base pair and expressed a dihydropteroate synthase, which was temperature-sensitive and showed a 150-fold increase in the *Ki* value for the binding of sulfathiazole. The *Km* value for the *p*-aminobenzoic acid substrate, on the other hand, increased ten times resulting in a less-efficient enzyme, which could be regarded as a trade-off for acquiring resistance [23]. Sequencing of these spontaneously mutated *fol*P resistance genes showed the change of a phe to leu or to ileu at position 28 in the amino acid sequence of the enzyme [18, 21]. The resistance mutations are located to an area of *fol*P, which is highly conserved among different microorganisms [18]. Sulfonamides can also function as substrates for dihydropteroate synthase to form an abnormal pterin-sulfonamide product that cannot participate in folate metabolism. It has been suggested that this could be part of the antibacterial effect by draining dihydropterin pyrophosphate from folate synthesis. The formation of this sulfonamide adduct is much lower in resistance mutants [23].

Sulfonamide resistance is commonly found in clinical isolates of *Campylobacter jejuni*. It is mediated by chromosomal point mutations, but in a more complicated pattern than in the laboratory mutants described above. The *fol*P gene of *Campylobacter jejuni* turned out to be the largest of its kind characterized so far. Its product consists of 390 amino acid residues, and is quite similar (42 % identity) to the corresponding enzyme (380 residues) from *Helicobacter pylori*. The *fol*P from a resistant isolate differed by four mutations from that of a corresponding susceptible isolate [24]. The ensuing amino acid changes mediated a distinct effect on the sulfonamide sensitivity of the expressed dihydropteroate synthase. The *Ki* value for sulfonamide increased from 0.5 μM with the susceptibility enzyme to 500 μM for the resistance one.

In *Streptococcus pneumoniae* sulfonamide resistance is mediated by a different kind of chromosomal changes. Several years ago a spontaneous laboratory mutant of this pathogen was found to contain a six-nucleotide repeat in *fol*P

mediating the repeat of ile-glu at position 66, 67, and extending the helical stretch by two amino acid residues [25]. This could significantly alter the tertiary structure of the protein [25]. This argument was later put forward from crystallographic studies on dihydropteroate synthase [26]. Clinical isolates of sulfonamide-resistant *Streptococcus pneumoniae* showed amino acid duplications at several different locations in the protein, indicating that changes to resistance had occurred independently on many occasions [27, 28]. None of these resistant clinical isolates carried the ile_{66}-glu_{67} repeat of the laboratory mutant mentioned above, but all had 3- or 6-bp duplications in the same area of the *fol*P gene. In contrast none of the several sulfonamide-susceptible isolates had duplications in this region [27]. Transformation experiments demonstrated that the duplications were sufficient for conferring the observed sulfonamide resistance [27]. The originally characterized ile_{66}-glu_{67} repeat (*sul*-d) was eventually found in a clinical isolate of *Streptococcus pneumoniae* from the North-West of the USA [29]. When this repeat was removed by site-directed mutagenesis, susceptibility ensued. Kinetic studies on the dihydropteroate synthase showed the *Ki* for sulfonamide to drop from 18 to 0.4 μM, i.e., 35-fold, while the *Km* for *p*-aminobenzoic acid decreased 2.5-fold. The *Km* for pterin pyrophosphate did not change [29]. The enzyme characteristics for the mutated strain were identical to those of susceptible strains, demonstrating that the duplication is sufficient for resistance. The fitness cost of resistance seems to be low, as reflected in the small increase in the *Km* value. The small but discernible increase indicates the absence of compensatory mutations. Still it could be enough for counterselecting resistant strains in the absence of the drug, and might lead to an argument regarding the much-debated problem of drug resistance reversibility.

Sulfonamide resistance in clinical isolates of *Staphylococcus aureus* and *Staphylococcus haemolyticus* has been studied and been shown to involve chromosomal mutations in *fol*P in an erratic pattern [30, 31]. With *Staphylococcus aureus* the dihydropteroate synthase was purified to homogeneity and subjected to X-ray crystallographic studies [30]. In different isolates sequencing could discern four different mutational patterns and identify as many as 14 amino acid changes in the development of resistance. A simple interpretation of their role in resistance has not been possible.

The very first experiments demonstrating the selective antibacterial effect of sulfonamides were performed by Gerhart Domagk with *Streptococcus pyogenes* more than 70 years ago (see Introduction). The sulfonamides became frequently used, also for prophylaxis against streptococcal infections among soldiers in military training camps during World War II. Occasional failures of this prophylaxis were observed to be due to the appearance of resistant streptococcal strains [32]. Sulfonamides were replaced by penicillin as antistreptococcal agents in the 1940s, and the mechanism of the mentioned early resistance has not been described until in later years [33, 34]. Sulfonamide-resistant strains of *Streptococcus pyogenes* seem to have been prevalent into present times in spite of the very low or nonexistent systemic use of this drug in decades. This is an interesting illustration of the nonreversibility of resistance in the absence of the selecting effect of the drug. The drug-resistant phenotypes do not seem to have any disadvantage at competition with their drug-susceptible relatives. Sulfonamide-resistant strains of *Streptococcus pyogenes* were shown to vary substantially in resistance, displaying MIC values of 512–1024 μg/mL [34]. The mechanism of resistance turned out to be very different from the rather simple mutational *fol*P changes described above. When *fol*P genes in susceptible and highly resistant isolates were compared, a 13.8 % difference in nucleotide sequence was observed. This difference is too large to be due to accumulated mutations. The resistance gene must have been introduced by transduction or transformation. The sequence analysis of the complete genome shows that *Streptococcus pyogenes* contains at least one inducible prophage [35], indicating the possibility of phage-mediated transduction. Further studies on sulfonamide resistance [33] included sequence determination of the genes neighboring the sulfonamide target, *fol*P, in the folate operon. A comparison between five sulfonamide resistant and one susceptible isolate, the latter showing only a few differences from the sequence available through the genome sequencing project (strain SpM1) [35], demonstrated an overall difference in nucleotide sequence of 15 %. More specifically, areas of different nucleotide sequences were scattered over the folate operon in a mosaic fashion, indicating horizontal transfer of genetic material. The *fol*P gene of resistant isolates showed different areas of foreign DNA in different isolates. This imported DNA was identical between three of the five, and between two of the five of the studied resistant isolates. From examining published three-dimensional dihydropteroate synthase structures [36], and conserved amino acids in different known sequences, and also the location of substrate binding, a particular amino acid exchange could be discerned as involved in resistance, at position 213 of the *fol*P product. This is located just after a conserved sequence of Ser-Arg-Lys. In most bacteria this is an Arg as in the sequenced genome of the sulfonamide-susceptible *Streptococcus* strain of SpM1 [35]. In three of the five sulfonamide-resistant *Streptococcus pyogenes* strains, position 213 is a Gly. A change to Gly by site-directed mutagenesis at this position in a sulfonamide-susceptible strain resulted in a 50-fold rise in the *Ki* for sulfonamide, and also in an increased (1.6-fold) *Km* for *p*-aminobenzoic acid. In a parallel experiment, the Gly was changed to Arg in a resistant strain effecting a 30-fold decrease in *Ki*. These experiments

indicate that a single amino acid change could explain a large part of the resistance property. In two other sulfonamide-resistant strains there was an Arg at position 213 like in the susceptible strain. However, they carried another type of sequence change, an insertion of two additional amino acids, Val-Ala after position 67. The removal of the two amino acids by site-directed mutagenesis resulted in an enzyme with a markedly lowered activity, and while the parental strain grew at a sulfonamide concentration of 510 μg/mL, the mutationally changed one showed a MIC value of 64 μg/mL. The two extra amino acids did thus affect resistance but also seem to be involved in forming an efficient enzyme structure in the resistant strain [33].

With the addition of numerous bacterial genome sequences to available databases, the origin of the resistance genes found in *S. pyogenes* could be traced to the related *S. dysgalactiae*. Several isolates of the latter species have now been sequenced and the similarity between the sulfonamide resistance genes described above and sequences of dihydropteroate synthase from *S. dysgalactiae* is striking. Of particular interest is that the two amino acid insertion Val-Ala is found in some but not all variants of *S. dysgalactiae*. NCBI Reference Sequence: WP_012766861.1 is 99 % identical to two of the mentioned resistant isolates studied including the Val-Ala insertion, while NCBI Reference Sequence: WP_003058161.1 is also 99 % identical to these isolates but lacks the Val-Ala insertion. The sequence similarity between *S. dysgalactiae* and the sulfonamide-resistant *S. pyogenes* extends beyond the inserted amino acids and indeed outside *folP* into the other genes of the folate operon. One possible scenario is thus that the initial resistance generating mutations may have arisen in *S. dysgalactiae* and were later transferred to *S. pyogenes*.

Chromosomal resistance to sulfonamides in *Neisseria meningitidis* is related to that of *Streptococcus pyogenes* in that its mechanism is based on the horizontal transfer of genetic material. Sulfonamides were used extensively for prophylaxis and treatment of meningococcal disease since the 1930s. Sulfonamide resistance is commonly observed in clinical isolates of pathogenic *Neisseria meningitidis* today. There even seems to be an association between pathogenicity and sulfonamide resistance and possibly also between mortality rate and resistance [37]. Astonishingly large differences in the structure of *fol*P were found between resistant and susceptible strains of *Neisseria meningitidis* [37, 38]. Two classes of different resistance determinants were revealed by nucleotide sequence determinations in several clinical isolates. In one of them the *fol*P gene was about 10 % different from the corresponding gene in drug-susceptible isolates. From this it could be concluded that resistance had appeared by recombination of horizontally transferred DNA rather than by the accumulation of mutations. In this class of resistant bacteria, strains were found, showing a mosaic *fol*P, in that only the central part corresponded to the resistance gene, while the outer parts were identical to those of susceptible isolates [37]. The origin of the resistance gene or gene fragments most likely is in other *Neisseria species.* This interpretation is supported by the finding of an 80-bp sequence identical to the corresponding part of the *fol*P gene in *Neisseria gonorrhoeae*, in the *fol*P of a susceptible isolate of *Neisseria meningitidis* [39].

The mentioned class of resistance genes in *Neisseria meningitidis* is further characterized by an insertion of six nucleotides, coding for Ser-Gly, in a highly conserved part of the *fol*P gene [37]. Removal of these two amino acids by site-directed mutagenesis resulted in a tenfold drop in *Ki* resulting in susceptibility, but a concomitant tenfold increase in *Km* indicated that the two inserted extra codons could not be the sole alteration leading to resistance. Most likely, compensatory mutations have accumulated in these enzyme genes [40]. Sulfonamide resistance was further affected by amino acid changes at position 68 [41]. In these resistant strains a Ser or a Leu substituted for the well-conserved Pro at this position in the consensus sequence. When this Ser68 was changed to a Pro in a mutant lacking the Ser-Gly insertion, the already lost sulfonamide resistance was not affected. Instead, the *Km* for *p*-aminobenzoic acid was lowered almost tenfold. The amino acid change at position 68 thus seems to be involved in the meningococcal adaptation to sulfonamide resistance. When, the other way around, these results were used in an attempt to create a resistant strain from a susceptible one, the introduction of Ser-Gly had a dramatic effect on the *Km* for *p*-aminobenzoic acid in that it increased about 100-fold, while the *Ki* for sulfonamide increased so little that it did not allow growth in the presence of sulfonamide. This enzyme also performed very poorly when used to complement an *E. coli* strain, that had its *fol*P partially deleted [40]. This allowed the comparison of resistant dihydropteroate synthases with experimentally mutated variant enzymes in extracts without the interference of chromosomal background activity. The generation time doubled to 60 min, when compared to complementation with the unmutated gene [41]. Changing also Pro to Ser at position 68 mediated an increase in *Ki*, but also a *Km* increase so large that drug resistance could not be determined [41]. The pattern of changes in resistance enzyme thus seems to be more complicated than that described. These observations support the idea that the resistance gene has evolved in another bacterial species and later been introduced into *Neisseria meningitidis* by transformation and recombination [42]. This was further supported by the finding of sulfonamide-resistant *Neisseria*-commensals cultivated from throat swabs of outpatients [43].

The other mentioned class of sulfonamide resistance determinants in *Neisseria meningitidis* lacked the six bp insert and showed a lower degree of difference to susceptible isolates [39]. Several of these sulfonamide resistance *fol*P

genes were identical between themselves but distinct from the corresponding susceptibility genes. This again indicates a horizontal transfer of genes followed by recombination [39]. A comparison of amino acid sequences of dihydropteroate synthases between those from these resistant strains and those from susceptible ones showed differences at 19 positions. Three of these differences, Phe31Leu, Pro84Ser, and Gly194Cys, were in amino acids conserved in all known bacterial dihydropteroate synthases [39]. The first of these is the same alteration as was seen in the described spontaneous mutation to sulfonamide resistance in *E. coli* (Phe28Leu) [18]. The three alterations in the meningococcal enzyme were subjected to site-directed mutagenesis. When Leu31 in the resistant enzyme was mutated to Phe, the sulfonamide MIC of the host decreased from about 0.5 mM to less than 0.02 mM. The Phe31 position is localized in a *fol*P area, where six of eleven of the corresponding amino acids are conserved in all known bacterial dihydropteroate synthases. The Cys194 is also located in a very well conserved area of the enzyme. When this was experimentally changed to Gly, there was a drop in the sulfonamide MIC from more than 0.5–0.12 mM. When Ser84 finally, in the resistant strain, was changed to Pro, no effect on the sulfonamide MIC of the host could be observed [39]. In further studies on the characteristics of the dihydropteroate synthase resistance the cloned meningococcal genes were expressed in the *fol*P knock-out mutant mentioned above [40]. The obtained *Ki* data correlated well with the MIC-data described above [39]. A pronounced effect was seen with mutations at position 31. A change of the resistance Leu to the susceptibility Phe caused a more than 300-fold decrease in *Ki* and a concomitant six- to eightfold drop in the *Km* for *p*-aminobenzoic acid, measured as pseudo-first order kinetics, since the other substrate, dihydropterinpyrophosphate was added in excess. Alterations of Cys to Gly at position 194 also mediated substantial effects on kinetic characteristics. The experimental change of resistance Ser84 to susceptibility Pro84, did not decrease MIC but in several experiments effected a twofold increase in the *Km* for *p*-aminobenzoic acid. The Ser84 could then be interpreted as an amino acid change compensating for the possibly detrimental effect on the enzyme of the other two resistance-mediating amino acid changes. Later, a fourth amino acid change Arg228Ser has been observed to mediate sulfonamide resistance in *Neisseria meningitidis* [44]. All of the above described mutations were found in a larger screen of meningococcal isolates from diverse parts of the world [45].

Since the distribution of sulfonamides for systemic use in Sweden, as mentioned above, is nil, and since the combination drug co-trimoxazole (sulfonamide plus trimethoprim) is only used in hospitals, it could be concluded that the sampled patients in the mentioned study [43] had not been exposed to sulfa drugs. The studied isolates were identified to belong to the *Neisseria subflava*/*Neisseria sicca*/*Neisseria mucosa* group and showed high sulfonamide resistance with MIC values higher than 256 μg/mL. Their *fol*P genes showed resistance characteristics like those described above for *Neisseria meningitidis*, as, for example, a Leu at position 31 and a Cys at position 194. A new resistance variation was also suggested with a Met at position 66 combined with a Gly-insertion between positions 75 and 76. Experiments were performed to see if resistance could be transferred by natural transformation from these commensals with a pathogenic *Neisseria meningitidis* as recipient. No transfer could, however, be observed, in spite of positive controls showing ready transfer of resistance *fol*P with a *Neisseria meningitidis* strain as a donor [46]. A possible explanation could be that the studied isolates lacked the uptake sequence known to be necessary for efficient transformation in *Neisseria* [47]. The continued presence of sulfonamide resistance in commensal Neisseria was shown recently in a screen for resistance determinants in the microbiome of healthy humans [48].

Dapsone (4,4′-diamino-diphenyl sulfone), microbiologically a sulfonamide, has been a standard treatment for leprosy for a long period of time. As could be expected, resistance developed and has actually been known since the 1950s. It has later been defined as chromosomal mutations in the *fol*P of *Mycobacterium leprae*, resulting in Thr53Ile, Thr53Ala, or Pro55Leu [49].

Sulfonamides were initially used for the treatment of *Mycobacterium tuberculosis*, but were abandoned when the more potent drugs Streptomycin and Isoniazid were introduced. With the emergence of multiply resistant isolates of *M. tuberculosis* there has been a renewed interest in sulfonamide drugs that may be used in combination therapies. A survey by Forgacs et al. [50] found the vast majority of isolates to be highly susceptible to sulfamethoxazole. Likewise, a study on HIV patients receiving co-trimoxazole prophylaxis showed a lower risk for contracting tuberculosis during prophylaxis [51]. Co-trimoxazole was recently included as one alternative for treatment of MDR tuberculosis [52]. Remarkably, very few reports of actual sulfonamide resistance in *M. tuberculosis* exist, one of the few cited papers is from 1951 [53]. The crystal structure of M. tuberculosis DHPS was published in 2000 and can be used for rational design of more specific inhibitors [36, 54].

Among the earlier drugs used against tuberculosis is also p-aminosalicylic acid (PAS), which is similar in structure to the sulfonamides and to the DHPS substrate p-aminobenzoic acid. Its mechanism of action has been hard to elucidate, but in 2013 [55] published strong evidence that PAS is a prodrug that is activated by DHPS and the downstream enzyme dihydrofolate synthase (DHFS) and after activation acts as an inhibitor of DHFR. By enzymatic measurements, the authors could show that PAS acts as an alternative substrate for DHPS with similar efficiency as *p*-aminobenzoic acid.

They also showed that PAS itself does not inhibit DHFR but that DHFS is specifically required for activation of PAS as inhibitor. One spontaneous PAS-resistant mutant isolated had a single amino acid change Glu40Ala in DHFS. Complementation with wild-type DHFS restored susceptibility, further showing the importance of DHFS in the action of PAS. A later report showed that other mutations in DHFS as well as *thyA* mutations were present in clinical isolates showing PAS resistance. The *thyA* mutation renders thymidylate synthase inactive, and the bacteria then rely on *thyX* for dTMP production as described in the introduction. The use of *thyX* relieves the need for high levels of active DHFR making the cells less dependent on the inhibited enzyme.

Also in *Pneumocystis jiroveci* resistance to sulfonamides seems to be due to a simple chromosomal pattern of mutations hitting the *fol*P of this organism, which causes the life-threatening disease of *Pneumocystis jiroveci* pneumonia in immunosuppressed patients. Co-trimoxazole, the combination of trimethoprim and sulfonamide (sulfamethoxazole) has been the drug of choice for the prophylaxis and treatment of this disease. Life-long prophylaxis is often recommended for HIV-positive patients. The antipneumocystis effect is mainly due to the sulfonamide component, since studies on the dihydrofolate reductase of this fungus show trimethoprim to be a very poor inhibitor of this enzyme in *Pneumocystis jiroveci* [56]. Dapsone (4,4′-diamino-diphenyl sulfone) is a sulfone drug, microbiologically acting as a sulfonamide, and is also frequently used for the treatment of this infection. *Pneumocystis jiroveci* has thus been heavily exposed to sulfonamide with an increasing prevalence of resistance mutations in its *fol*P gene as a consequence. The human *Pneumocystis jiroveci* cannot be cultured and the dihydropteroate synthase protein is not available for study, but the corresponding *fol*P sequence is known [57, 58]. The most common mutations occur at nucleotide positions 165 and 171, leading to Thr55Ala and Pro57Ser. They appear either as single or double mutations in the same isolate [59, 60]. In later work the important question whether the recent emergence of resistance mutations is the result of transmission between patients or arise and are selected within the individual patient under the pressure of sulfonamide or dapsone treatment. The latter interpretation was favored, i.e., that the mutants are selected within a given patient [61], and that the mentioned mutations may be associated with reactivation of the infection [62].

3 Plasmid-Borne Resistance to Sulfonamides

Sulfonamide is a synthetic antibacterial agent. Resistance by plasmid-mediated drug-degrading or drug-modifying enzymes was not to be expected. Instead nonallelic, drug-resistant variants of the chromosomal dihydropteroate synthase target enzyme have been found to mediate high resistance to sulfonamides [20, 63]. Three genes *sul*1, *sul*2, and *sul*3 expressing enzymes of this type are known and characterized [64–67]. They are distinct from each other (similarity at the amino acid level is about 40%). Their origins are unknown. Remarkably, only *sul*1 and *sul*2 were found for a long time. In a study from 1991 on 203 human *Enterobacteriaceae* strains from different parts of the world only *sul*1 or *sul*2 or both were found [68]. The reason for this could be that there is a constraint on the dihydropteroate synthase structure in discriminating between the normal substrate *p*-aminobenzoic acid and the structurally very similar sulfonamide inhibitor. The enzymes expressed from *sul*1 and *sul*2 bind the normal substrate well, showing low *Km* values (0.6 μM), and still resist high concentrations of sulfonamide. The *sul*2 enzyme shows a particularly high acuity in distinguishing between *p*-aminobenzoic acid and very high concentrations of sulfonamide. The finding of *sul*3 in swine isolates of *E. coli* and subsequently in human isolates is very interesting in this perspective [64, 69, 70]. All three plasmid-borne *sul* genes seem to be mediated by efficient genetic transport mechanisms. The *sul*1 gene is almost always found linked to other resistance genes in the Tn*21* type integron, while *sul*2 is found on small plasmids of the *inc*Q family (e.g., RSF1010), and also on small plasmids of another type, represented by pBP1 [71]. The more recently found *sul*3 seems to be part of a composite transposon flanked by the insertion sequences IS*15delta/26* [64]. The two genes *sul*1 and *sul*2 used to be found at roughly the same frequency among sulfonamide resistant, gram-negative, clinical isolates [68]. In later years, however, a relative increase in prevalence of *sul*2 has been observed [72]. In spite of a very low use of sulfonamides in the United Kingdom, a comparison of large collections of clinically isolated *E. coli* from 1991 and 1999 showed an increase in sulfonamide resistance during this period, and this was mostly accounted for by an increase in the prevalence of *sul*2, now frequently found on large, conjugative multiresistance plasmids. An explanation for this phenomenon could be the found association between *sul*2 and multiresistance plasmids, allowing selection through the use of other antimicrobial agents [72]. The presence of these three sul genes continue to be reported from surveys of environmental bacteria with *sul*2 dominating but closely followed by *sul*1, *sul*3 is still more rare. A few notes about sulfonamide-resistant isolates where none of these *sul* genes are detected have appeared in the literature, but so far no other plasmid-borne sulfonamide resistance gene has been reported [73, 74].

Another location of *sul*2 is in *Haemophilus influenzae* mediating high sulfonamide resistance to this pathogen [75]. In the same work high sulfonamide resistance was alternatively mediated by the chromosomal insertion of five amino acids into *folP* [75]. Both *sul*1 and *sul*2 have been found in SXT-resistant isolates of *Stenotrophomonas maltophila*

[76] although *sul*1 linked to a class 1 integron appears to dominate [4].

4 Chromosomal Resistance to Trimethoprim

Resistance to trimethoprim by mutations involving *fol*A, the chromosomal gene expressing the trimethoprim target enzyme, dihydrofolate reductase is known from several pathogenic bacteria. One example of this is a clinical isolate of *Escherichia coli*, which overproduced its chromosomal dihydrofolate reductase several hundred-fold, by a combination of four types of mutations enhancing its expression [77]. One was a promoter-up mutation in the –35 region, a second was a one bp increase in the distance between –10 region and the start codon, there were also several mutations optimizing the ribosome-binding site, and finally there were mutations in the structural gene effecting changes to more frequently used codons. Now, the mere increase in the intracellular enzyme level could not be expected to decrease the susceptibility of the host more than 1000-fold to the competitively acting folate analog of trimethoprim. However, the expressed dihydrofolate reductase also showed a threefold increase in the *Ki* for the drug, which was thought to be due to the mutational substitution of a Gly for a Try at position 30 of the enzyme protein. The combined action of decreased enzyme susceptibility and enzyme overproduction could then explain the high resistance (MIC > 1000 μg/mL) observed for the mentioned isolate [77]. All the mentioned changes represent a remarkable evolutionary adaptation to the antibacterial action of trimethoprim.

A similar type of chromosomal resistance to trimethoprim has been observed in *Haemophilus influenzae*, where differences in the promoter region and also in the structural gene were seen between trimethoprim-susceptible and trimethoprim-resistant isolates [78]. Different parts of the structural gene in different isolates were changed, also in the C-terminal area, which is not known to participate in substrate or trimethoprim binding. These changes were suggested to involve changes in the secondary structure mediating a decrease in trimethoprim binding and ensuing drug resistance.

Chromosomal resistance to trimethoprim in *Streptococcus pneumoniae* is fairly common [79]. Resistant strains were shown to express dihydrofolate reductases that resisted 50-fold higher concentrations of trimethoprim. The 50 % inhibitory dose was 3.9–7.3 μM compared to 0.15 μM for the susceptible enzyme. Site-directed mutagenesis revealed that one amino acid change, Ile to Leu at position 100, resulted in the mentioned 50-fold increase in resistance to trimethoprim. Further studies on 11 trimethoprim-resistant isolates demonstrated a substantial variability in the nucleotide sequences of their dihydrofolate reductase genes. The resistant isolates could be divided into two groups with six amino acid changes in common. One group showed two extra changes, and the other, six additional changes. This high number of changes indicates horizontal transfer of resistance genes. This interpretation is experimentally supported by the ability of chromosomal DNA from resistant isolates, and cloned PCR products from resistance strains to transform a susceptible strain of *Streptococcus pneumoniae* to trimethoprim resistance [79].

The strange finding of usually plasmid-borne, foreign trimethoprim resistance genes on the chromosome of *Campylobacter jejuni* could in a way be classified as chromosomal resistance. Clinically, *Campylobacter jejuni* has always been regarded as endogenously resistant to trimethoprim. In an attempt to study the mechanism of this, it was found that a majority of clinical isolates carried foreign genes expressing drug-resistant variations of dihydrofolate reductase, the target of trimethoprim [80]. The found genes *dfr*1 and *dfr*9 are well known (see under Plasmid-Borne resistance to trimethoprim) as integron- and transposon-borne genes mediating trimethoprim resistance via plasmids. Remnants of the transposon known to carry *dfr*9 were observed in its context on the *Campylobacter* chromosome and the *dfr*1 was found as an integron cassette [80]. The occurrence of these genes would of course mediate a very high trimethoprim resistance to the bacterium, but as mentioned above it is now known that *Campylobacter jejuni* is really intrinsically resistant to trimethoprim by its different enzymatic mechanism for thymidylate synthesis obviating the need for dihydrofolate reductase, also reflected in that the *fol*A gene is missing from its chromosome [16, 17]. The trimethoprim target is thus missing from *Campylobacter jejuni*. The selective value of acquiring the resistance gene *dfr*1 or *dfr*9 (sometimes both, [80]), is then difficult to understand. Speculatively, *Campylobacter jejuni* could take advantage of the *dfr* genes, available through antibacterial selection, for acquiring a better growth potential.

A different type of chromosomal mutations leads to low trimethoprim resistance. Mutations in *thy*A expressing the enzyme thymidylate synthase make *E. coli* cells able to grow in the presence of 8–10 μg/mL of trimethoprim, provided that there is an external supply of thymine [81, 82]. The inactivated thymidylate synthase makes cells dependent on external thymine, but also relieves dihydrofolate reductase of its main task of regenerating tetrahydrofolate in the formation of N^5-, N^{10}-methylene tetrahydrofolate, which is oxidized in the deoxyuridylate methylation process. The cell can then afford to have a fraction of its dihydrofolate reductase inactivated by trimethoprim. The mentioned concentration of trimethoprim can of course, for the same reason, be used for the selection of spontaneous *thy*A mutants.

5 Plasmid-Borne Resistance to Trimethoprim

As in the case of sulfonamide resistance, plasmid-borne resistance to trimethoprim is mediated by nonallelic, drug-resistant variations of the target enzyme, that is, dihydrofolate reductase for trimethoprim. The first of these were found decades ago [83, 84], but newly found ones seem continuously to be added to the list, where now around 30 different, resistance genes (*dfr*:s) expressing these enzymes are to be found. They are numbered consecutively after the first ones found [83, 84]. These resistance genes must have moved horizontally into pathogenic bacteria and selected for by the heavy use of trimethoprim. The precise origin is not known in any case. This mechanism, with an extra resistance-mediating target enzyme is highly prevalent in enterobacteria, where *dfr*1, the one found first, seems to be most common. It occurs in a cassette in both class 1 and class 2 integrons [85]. The class 2 integron, with *dfr*1, is borne on transposon Tn*7*, which has spread very successfully, mainly because of its high-frequency insertion into a preferred site on the chromosome of *E. coli* and many other enterobacteria [85]. Transposon Tn7 in clinically isolated bacteria is usually located on the chromosome and less frequently on plasmids [86]. Among the horizontally moving trimethoprim resistance genes, there is a subclass of four genes, *dfr*2a, *dfr*2b, *dfr*2c, and *dfr*2d, which are closely related between themselves, but so different from other trimethoprim resistance genes, that they could not be included in the phylogenetic tree, where the interrelationship of the others could be demonstrated (Fig. 24.1). Their corresponding polypeptides consist of 78 amino acids and are identical to 67 % between

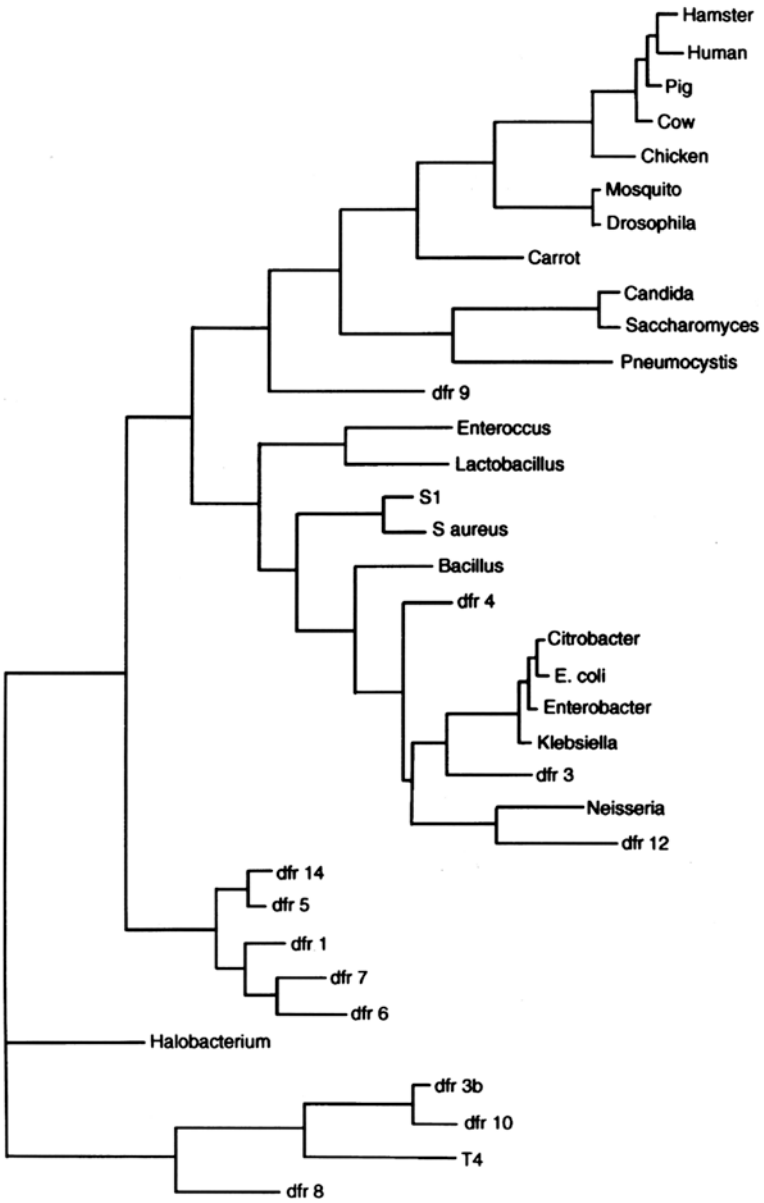


Fig. 24.1 Phylogenetic tree based on amino acid sequence alignment and parsimony analysis, showing the relationship between different dihydrofolate reductases. Resistance enzymes are marked by dfr and a number. Adapted from [65], where GenEMBL accession numbers to the different sequences could be found

themselves, and are active in the form of tetramers showing dihydrofolate reductase activity, that is almost insensitive to trimethoprim IC_{50}s > 1 mM, making hosts so drug resistant that MIC cannot be determined for solubility reasons [69, 70, 87–89]. All of these group 2 genes have been found as integron cassettes [69, 70, 85]. The phylogenetic tree mentioned above and shown in Fig. 24.1 relates different dihydrofolate reductases and is based on amino acid alignment and parsimony analysis [90]. In this tree, *dfr*1, *dfr*5, *dfr*6, *dfr*7, and *dfr*14 form a well-supported group of similar enzymes. Otherwise the trimethoprim resistance enzymes are diverse and scattered all over the tree. This is consistent with the notion that these resistance genes have their origins in a large variety of different organisms. One, however, *dfr*3, is rather closely related to the chromosomal dihydrofolate reductases of enterobacteria, which could hint at its origin. In staphylococci, extrachromosomally mediated high level resistance to trimethoprim is effected by the drug insensitive dihydrofolate reductase S1 borne on the ubiquitous transposon Tn*4003* [91, 92]. This trimethoprim-resistant enzyme is almost identical with the chromosomal dihydrofolate reductase of *Staphylococcus epidermidis*. It differs by only three amino acid substitutions, and it has therefore been suggested that a mutated form of the *S. epidermidis* enzyme has moved horizontally into other staphylococcal species [93]. A second trimethoprim-resistant and plasmid-encoded dihydrofolate reductase, S2, was later isolated from *Staphylococcus haemolyticus*. Its similarity with other staphylococcal enzymes indicates that its origin is similar to that of S1 [94]. The S2 enzyme was later found, also in *Listeria monocytogenes* [95]. Low to intermediate levels of trimethoprim resistance in *Staphylococcus aureus* were observed as a consequence of a Phe98Tyr mutation in the chromosomal dihydrofolate reductase gene [96]. This change is identical to one of the differences between S1 and the chromosomal dihydrofolate reductase of *S. epidermidis* [93].

Further studies of clinically isolated aerobic gram-negative enterobacteria have extended the list of trimethoprim resistance genes. In a survey of trimethoprim-resistant isolates from commensal fecal flora a gene numbered *dfr*13 was found. Its gene product showed 84 % amino acid identity with *dfr*12, and also a similar trimethoprim inhibition profile. It appeared as an integron cassette in a class 1 integron [97]. Another, *dfr*15, was also found as a cassette in a class 1 integron, and in a commensal, fecal *Escherichia coli*. Its predicted protein showed 90 % amino acid identity with *dfr*1, i.e., the first extrachromosomal trimethoprim resistance enzyme found and it thus belongs to the prevalent group that can be discerned as a well-supported cluster of similar enzymes in the phylogenetic tree of Fig. 24.1 [98]. Another member of this group, *dfr*17, was observed, again as a cassette in a class 1 integron, and in urinary tract-infecting *Escherichia coli* isolated in Taiwan and Australia, respectively. The *dfr*17 cassette showed 91 % identity with the earlier characterized *dfr*7 cassette [99, 100].

An obviously transferable dihydrofolate reductase gene *dfr*F has been observed to be located on the chromosome of highly trimethoprim resistant, clinical isolates of *Enterococcus faecalis* [101]. The characterized *dfr*F codes for a predicted polypeptide showing 38–64 % similarity with other dihydrofolate reductases from gram-positive and gram-negative organisms.

One of the resistance enzymes of the phylogenetic tree, *dfr*9, only distantly related to the earlier mentioned main group (Fig. 24.1), was originally found expressed from *dfr*9 on large transferable plasmids in isolates of *Escherichia coli* from swine [102]. The *dfr*9 was observed at a frequency of 11 % among these trimethoprim resistant, veterinary isolates of *E. coli*, but only very rarely among corresponding human isolates [103]. The spread of *dfr*9 among swine bacteria is most likely due to the frequent veterinarian prescription of trimethoprim in swine rearing. A subsequent spread into human commensals might then have taken place [104, 105]. One case of such a spread into a human pathogen was actually observed. The *dfr*9 gene was observed in an isolate of *E. coli* from the urine of a patient with urinary tract infection. This patient, an elderly woman living in a small town, had no contacts with husbandry or farming. Modern stock breeding creates very large populations of bacteria, which under attainable hygienic conditions could communicate genetically and efficiently exchange genetic material between themselves [106].

Origin of *dfr*9 is unknown, but further study of its surroundings in many plasmids from several strains showed that it is borne on a truncated transposon, Tn*5393*, previously found on a plasmid in the plant pathogen *Erwinia amylovora*, causing fire blight on apple trees [107]. This transposon carries two streptomycin resistance genes *str*A and *str*B, and it most probably evolved under the selection pressure of streptomycin ubiquitously used for the control of the mentioned plant disease in many countries [108, 109]. The *dfr*9 gene was found inserted in the *str*A gene at the right hand end of Tn*5393*. The occurrence of *dfr*9, expressing trimethoprim resistance in *E. coli* from swine in Sweden, and its location on a genetic structure, closely related to transposon Tn*5393*, originally observed to mediate streptomycin resistance in a plant pathogen in the USA, could be regarded as a powerful demonstration of bacterial adaptation to the heavy use of antibacterial agents in agriculture and stock breeding. Modern pig rearing in large stables with many animals could be regarded as gigantic genetic laboratories creating very large populations of genetically communicating bacteria, allowing also very rare genetic events to surface, like mobilizing a trimethoprim resistance gene under the selection pressure of the heavy use of this drug in animal husbandry.

As mentioned above, *dfr*9 has been found in a Tn*5393* context also on the chromosome of *Campylobacter jejuni* [80].

It could be mentioned that *Campylobacter jejuni* is a commensal in the gut of swine.

Would it be possible to reverse the frequency of clinical resistance to trimethoprim by limiting its use? The volume of antibiotic use and the biological fitness cost conferred by the resistance mechanism are important determinants of the development and maintenance of antibiotic resistance. Laboratory isolates of spontaneous sulfonamide-resistant mutants of *E. coli* described earlier in this chapter showed a clear fitness cost of resistance in that the mutationally changed resistance enzyme, the sulfonamide target, dihydropteroate synthase, showed an increased *Km* value, that is, was less efficient. This trade-off between resistance and fitness seems to be a logical outcome when a bacterium adapts its evolutionary optimized genotype to one acutely needed in the presence of an antibiotic. On the other hand, as described in this chapter, the properties of clinical isolates of *N. meningitides* seemed to show that resistant strains were not selected against in the absence of sulfonamide. The very important question of possible reversion of resistance should antibiotic use be discontinued or reduced was tested experimentally in a large clinical experiment in a county (Kronoberg) in Sweden. This is a rural part of the country with a population of 178,000. The healthcare system is funded at the county level and includes two hospitals and 25 primary healthcare centers. All 464 physicians in the area were asked to substitute trimethoprim with other antibacterials in the treatment of urinary tract infections. Existing alternatives to trimethoprim for the treatment of urinary tract infections were carefully described. This experiment or drug intervention study was performed over 24 months. A prompt and sustained decrease of 85 % in the total trimethoprim prescription was reached rapidly, as judged from the sales figures of the central distributor. There was, however, no significant trend break in the trimethoprim resistance rate in consecutive isolates of *E. coli*. This apparent lack of effect of the intervention on trimethoprim resistance could be explained by the lack of fitness cost, combined with coselection by plasmid-associated resistance genes. These results indicate that the cyclic use of antibiotics will not be an adequate method for curbing antibiotic resistance development [110].

6 Development of New Antifolate Agents

The elucidation of crystal structures for both the sulfonamide and trimethoprim target enzymes, DHPS and DHFR, has opened the area of rational drug design to develop more potent inhibitors that may overcome the problem of drug resistance. One careful study of the substrate binding and catalysis of DHPS published in Science in 2012 [111] led to the conclusion that the dihydropteridine pyrophosphate substrate must bind first, and that this binding creates the binding pocket for the other substrate, PABA. The authors also showed the effect of defined amino acid changes in DHPS in excluding the sulfonamide inhibitors from binding, thus leading to resistance. The same research group has also tried to develop new inhibitors, mainly based on competitive binding to the pteridine-binding site. This development is reviewed in a later publication [112]. Although many of these inhibitors show good activity against the purified enzyme, their inhibition potential against live bacteria is limited, probably due to poor penetration through the bacterial envelope. The authors also review the development of new PABA analogs as well as transition state mimics as inhibitors.

Similarly, development of new DHFR inhibitors is proceeding. With special focus on the naturally trimethoprim-resistant DHFR from *B. anthracis*, a series of new inhibitors were presented based on the structure of this particular enzyme [113]. A similar development of inhibitors specifically against multidrug-resistant *S. aureus* has recently been published [114]. Although a number of new antifolates have been described [115] no new antifolate has been released on the market to replace the traditional DHPS and DHFR inhibitors.

7 Conclusions

The study of resistance to sulfonamides and trimethoprim is of interest, in spite of the fact that their clinical importance has diminished dramatically in recent years. The present limited use of sulfonamides is due to the allergic side effects that were evident already several decades ago. For trimethoprim, there has been a steep increase in resistance. For both drugs the mechanisms of resistance and its spread among pathogenic bacteria reveals a remarkable adaptation to the presence of these antibacterial agents. In the case of sulfonamides, laboratory experiments showed that spontaneous mutations to drug resistance always exacted a trade-off price in the form of a less-efficient target enzyme dihydropteroate synthase that would cause counterselection of its host in the absence of the drug. In sulfonamide-resistant clinical isolates this price seems to be discounted, however, in that compensatory changes in the target enzyme make it as efficient as its wild-type counterpart. Further studies of this phenomenon could be an inroad to the understanding of evolutionary adaptation, which is most important for judging reversibility of resistance and for assessing the future of antibacterial agents in general. Trimethoprim resistance in clinical samples of pathogenic bacteria is most commonly mediated by cassette-borne genes expressing drug-resistant variations of the target enzyme, dihydrofolate reductase. A better understanding of the diverse origins of these genes, and their horizontal transfer as integron-borne cassettes could shed light on the important question of how antibiotic resistance integrons have originated and developed.

Sulfonamides might be forced back into clinical use by the general increase in antibiotic resistance, and then with a better understanding and vigilance regarding the allergic side effects. The selective effect of trimethoprim could possibly be developed further in derivatives like epiroprim and iclaprim mentioned above. One example of recent activity is the new analogs of pyrimethamine and cycloguanil that show inhibitory effect on the dihydrofolate reductase from drug-resistant mutants of *Plasmodium falciparum* [116].

References

1. Domagk G. Beitrag zur Chemotherapie der bakteriellen Infektionen. Dtsch med Wschr. 1935;7:250–3.
2. Domagk G, Hegler C. Chemotherapie bakterieller Infektionen, second edition Band I. In: Lendle L, Schoen R, editors. Beiträge zur Arzneimitteltherapie. Leipzig: Verlag von S. Hirzel; 1942.
3. Wang YL, Scipione MR, Dubrovskaya Y, Papadopoulos J. Monotherapy with fluoroquinolone or trimethoprim-sulfamethoxazole for treatment of Stenotrophomonas maltophilia infections. Antimicrob Agents Chemother. 2014;58:176–82.
4. Chung HS, Kim K, Hong SS, Hong SG, Lee K, Chong Y. The sul1 gene in stenotrophomonas maltophilia with high-level resistance to trimethoprim/sulfamethoxazole. Ann Lab Med. 2015;35:246–9.
5. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated meticillin-resistant Staphylococcus aureus. Lancet. 2010;375:1557–68.
6. Li V, Chui L, Simmonds K, Nguyen T, Golding GR, Yacoub W, Ferrato C, Louie M. Emergence of new CMRSA7/USA400 methicillin-resistant Staphylococcus aureus spa Types in Alberta, Canada, from 2005 to 2012. J Clin Microbiol. 2014;52:2439–46.
7. Keisu M, Wiholm BE, Palmblad J. Trimethoprim-sulfamethoxazole-associated blood dyscrasias. Ten years experience of the Swedish spontaneous reporting system. J Int Med. 1990;228:353–60.
8. Wiholm B-E, Emanuelsson S. Drug-related blood dyscrasias in a Swedish reporting system, 1985–1994. Eur J Haematol. 1996;57 Suppl 60:42–6.
9. Brown GM. The biosynthesis of folic acid II. Inhibition by sulfonamides. J Biol Chem. 1962;237:536–40.
10. Sköld O. Sulfonamide resistance: mechanisms and trends. Drug Res Updates. 2000;3:155–60.
11. Woods DD. The relation of p-aminobenzoic acid to the mechanism of the action of sulfanilamide. Br J Exp Pathol. 1940; 21:74–90.
12. Burchall JJ, Hitchings GH. Inhibitor binding analysis of dihydrofolate reductases from various species. Mol Pharmacol. 1965;1:126–36.
13. Matthews DA, Bolin JT, Burridge JM, Filman DJ, Volz KW, Kraut J. Dihydrofolate reductase. The stereochemistry of inhibitor selectivity. J Biol Chem. 1985;260:392–9.
14. Schneider P, Hawser S, Islam K. Iclaprim, a novel diaminopyrimidine with potent activity on trimethoprim sensitive and resistant bacteria. Bioorg Med Chem Lett. 2003;13:4217–21.
15. Locher HH, SchluneggerH HPG, Angehrn P. Antibacterial activities of epiroprim, a new dihydrofolate reductase inhibitor, alone and in combination. Antimicrob Agents Chemother. 1996;40:1376–81.
16. Myllykallio H, Leduc D, Filee J, Liebl U. Life without dihydrofolate reductase folA. Trends Microbiol. 2003;11:220–3.
17. Myllykallio H, Lipowski G, Leduc D, Filee J, Forterre P, Liebl U. An alternative flavin-dependent mechanism for thymidylate synthesis. Science. 2002;297:105–7.
18. Dallas WS, Gowen JC, Ray PH, Cox MJ, Dev JK. Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of Escherichia coli MC 4100. J Bacteriol. 1992;174:5961–70.
19. Pato ML, Brown GM. Mechanisms of resistance of Escherichia coli to sulfonamides. Arch Biochem Biophys. 1963;103:443–8.
20. Sköld O. R-factor-mediated resistance to sulfonamides by a plasmid-borne, drug-resistant dihydropteroate synthase. Antimicrob Agents Chemother. 1976;9:49–54.
21. Swedberg G, Fermér C, Sköld O. Point mutations in the dihydropteroate synthase gene causing sulfonamide resistance. Adv Exp Med Biol. 1993;338:555–8.
22. Vedantam G, Nichols BP. Characterization of a mutationally altered dihydropteroate synthase contributing to sulfathiazole resistance in Escherichia coli. Microb Drug Resist. 1998;4:91–7.
23. Swedberg G, Castensson S, Sköld O. Characterization of mutationally altered dihydropteroate synthase and its ability to form a sulfonamide-containing dihydrofolate analogue. J Bacteriol. 1979;137:29–136.
24. Gibreel A, Sköld O. Sulfonamide resistance in clinical isolates of Campylobacter jejuni: mutational changes in the chromosomal dihydropteroate synthase. Antimicrob Agents Chemother. 1999; 43:2156–60.
25. Lopez P, Espinosa M, Greenberg B, Lacks SA. Sulfonamide resistance in Streptococcus pneumoniae: DNA sequence of the gene encoding dihydropteroate synthase and characterization of the enzyme. J Bacteriol. 1987;169:4320–6.
26. Achari A, Somers DO, Champness JN, Bryant PK, Rosemond J, Stammers DK. Crystal structure of the anti-bacterial sulfonamide drug target dihydropteroate synthase. Nat Struct Biol. 1997;4:490–7.
27. Maskell JP, Sefton AM, Hall LMC. Mechanisms of sulfonamide resistance in clinical isolates of Streptococcus pneumoniae. Antimicrob Agents Chemother. 1997;41:2121–6.
28. Padayachee T, Klugman KP. Novel expansions of the gene encoding dihydropteroate synthase in trimethoprim-sulfamethoxazole-resistant Streptococcus pneumoniae. Antimicrob Agents Chemother. 1999;43:2225–30.
29. Haasum Y, Ström K, Wehelie R, Luna V, Roberts MC, Maskell JP, Hall LMC, Swedberg G. Amino acid repetitions in the dihydropteroate synthase of Streptococcus pneumoniae lead to sulfonamide resistance with limited effects on substrate Km. Antimicrob Agents Chemother. 2001;45:805–9.
30. Hampele JC, D'arcy A, Dale GE, et al. Structure and function of the dihydropteroate synthase from Staphylococcus aureus. J Mol Biol. 1997;268:21–30.
31. Kellam P, Dallas WS, Ballantine SP, Delves CJ. Functional cloning of the dihydropteroate synthase gene of Staphylococcus haemolyticus. FEMS Microbiol Lett. 1995;134:165–9.
32. Damrosch DS. Chemoprophylaxis and sulfonamide resistant streptococci. J Am Med Assoc. 1946;130:124–8.
33. Jönsson M, Ström K, Swedberg G. Mutations and horizontal transmission have contributed to Sulfonamide resistance in Streptococcus pyogenes. Microb Drug Resist. 2003;9:147–53.
34. Swedberg G, Ringertz S, Sköld O. Sulfonamide resistance in Streptococcus pneumoniae is associated with differences in the amino acid of its chromosomal dihydropteroate synthase. Antimicrob Agents Chemother. 1998;42:1062–9.
35. Ferretti JJ, McShan WM, Ajdic D, et al. Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc Natl Acad Sci U S A. 2001;98:4658–63.
36. Baca AM, Sirawaraporn R, Turley S, Sirawaraporn W, Hol WGJ. Crystal structure of Mycobacterium tuberculosis 6-hydroxy methyl-7,8-dihydropteroate synthase in complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. J Mol Biol. 2000;302:1193–212.
37. Rådstrom P, Fermér C, Kristiansen B-E, Jenkins A, Skold O, Swedberg G. Transformational exchanges in the dihydropteroate

synthase gene of Neisseria meningitidis: a novel mechanism for acquisition of sulfonamide resistance. J Bacteriol. 1992;174: 6386–93.
38. Kristiansen B-E, Radstrom P, Jenkins A, Ask E, Facinelli B, Skold O. Cloning and characterization of a DNA fragment that confers sulfonamide resistance in a serogroup B, serotype 15 strain of Neisseria meningitidis. Antimicrob Agents Chemother. 1990;34:2277–9.
39. Fermér C, Kristiansen B-E, Sköld O, Swedberg G. Sulfonamide resistance in Neisseria meningitidis as defined by site-directed mutagenesis could have its origin in other species. J Bacteriol. 1995;177:4669–75.
40. Fermér C, Swedberg G. Adaptation to sulfonamide resistance in Neisseria meningitidis may have required compensatory changes to retain enzyme function: kinetic analysis of dihydropteroate synthases from Neisseria meningitidis expressed in a knock-out mutant of Escherichia coli. J Bacteriol. 1997;179:831–7.
41. Qvarnström Y, Swedberg G. Additive effects of a two-amino-acid insertion and a single-amino-acid substitution in dihydropteroate synthase for the development of sulphonamide-resistant Neisseria meningitidis. Microbiology. 2000;146:1151–6.
42. Linz B, Schenker M, Zhu P, Achtman M. Frequent interspecific genetic exchange between commensal Neisseriae and Neisseria meningitidis. Mol Microbiol. 2000;36:1049–58.
43. Qvarnström Y, Swedberg G. Sulphonamide resistant commensal Neisseria with alterations in the dihydropteroate synthase can be isolated from carriers not exposed to sulphonamides. BMC Microbiol. 2002;2:34–8.
44. Bennett DE, Cafferkey MT. PCR and restriction endonuclease assay for detection of a novel mutation associated with sulfonamide resistance in Neisseria meningitidis. Antimicrob Agents Chemother. 2003;47:3336–8.
45. Fiebelkorn KR, Crawford SA, Jorgensen JH. Mutations in folP associated with elevated MICs for Neisseria meningitidis clinical isolates from five continents. Antimicrob Agents Chemother. 2005;49:536–40.
46. Qvarnström Y, Swedberg G. Variations in gene organization and DNA uptake signal sequence in the folP region between commensal and pathogenic Neisseria species. BMC Microbiol. 2006;6:11.
47. Elkins C, Thomas CE, Seifert HS, Sparling PF. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J Bacteriol. 1991;173:3911–3.
48. Card RM, Warburton PJ, MacLaren N, Mullany P, Allan E, Anjum MF. Application of microarray and functional-based screening methods for the detection of antimicrobial resistance genes in the microbiomes of healthy humans. PLoS One. 2014;9, e86428.
49. Kai M, Matsuoka M, Nakata N. Diaminodiphenylsulfone resistance in Mycobacterium leprae due to mutations in the dihydropteroate synthase gene. FEMS Microbiol Lett. 1999;177:231–5.
50. Forgacs P, Wengenack NL, Hall L, Zimmerman SK, Silverman ML, Roberts GD. Tuberculosis and trimethoprim-sulfmethoxazole. Antimicrob Agents Chemother. 2009;53:4789–93.
51. Hasse B, Walker AS, Fehr J, et al. Co-trimoxazole prophylaxis is associated with reduced risk of incident tuberculosis in participants in the Swiss HIV cohort study. Antimicrob Agents Chemother. 2014;58:2363–8.
52. Alsaad N, Wilffert B, van Altena R, de Lange WCM, van der Werf TS, Kosterink JGW, Alffenaar J-WC. Potential antimicrobial agents for the treatment of multidrug-resistant tuberculosis. Eur Respir J. 2014;43:884–97.
53. Yegian D, Long RT. The specific resistance of tubercle bacilli to para-aminosalicylic acid and sulfonamides. J Bacteriol. 1951;61:747–9.
54. Wolff KA, Nguyen L. Strategies for potentiation of ethionamide and folate antagonists against Mycobacterium tuberculosis. Expert Rev Anti Infect Ther. 2012;10:971–81.
55. Zheng J, Rubin EJ, Bifani P, Mathys V, Lim V, Au M, Jang J, Nam J, Dick T, Walker JR, Pethe K, Camacho LR. para-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in Mycobacterium tuberculosis. J Biol Chem. 2013;288:23447–56.
56. Edman JC, Edman U, Cao M, Lundgren B, Kovacs JA, Santi DV. Isolation and expression of the Pneumocystis carinii dihydrofolate reductase gene. Proc Natl Acad Sci. 1989;86:8625–9.
57. Lane BR, Ast JC, Hossler PA, et al. Dihydropteroate synthase polymorphisms in Pneumocystis carinii. J Infect Dis. 1997;175: 482–5.
58. Volpe F, Ballantine SP, Delves CJ. The multifunctional folic acid synthesis fas gene of Pneumocystis carinii encodes dihydroneopterin aldolase, hydroxymethyldihydropterin pyrophosphokinase and dihydropteroate synthase. Eur J Biochem. 1993;216:449–58.
59. Helweg-Larsen J, Benfield TL, Eugen-Olsen J, Lundgren JD, Lundgren B. Effects of mutations in Pneumocystis carinii dihydropteroate synthase gene on outcome of AIDS-associated P carinii pneumonia. Lancet. 1999;354:1347–51.
60. Mei Q, Gurunathan S, Masur H, Kovacs JA. Failure of cotrimoxazole in Pneumocystis carinii infection and mutations in dihydropteroate synthase gene. Lancet. 1998;351:1631–2.
61. Ma L, Kovacs JA. Genetic analysis of multiple loci suggests that mutations in the Pneumocystis carinii f. sp. hominis dihydropteroate synthase gene arose independently in multiple strains. Antimicrob Agents Chemother. 2001;45:3213–5.
62. Nahimana A, Rabodonirina M, Helweg-Larsen J, Meneau I, Francioli P, Bille J, Hauser PM. Sulfa resistance and dihydropteroate synthase mutants in recurrent Pneumocystis carinii pneumonia. Emerg Infect Dis. 2003;9:864–7.
63. Wise EM, Abou-Donia MM. Sulfonamide resistance mechanism in Escherichia coli. R-plasmids can determine sulfonamide-resistant dihydropteroate synthases. Proc Natl Acad Sci U S A. 1975;72:2621–5.
64. Perreten V, Boerlin P. A new sulfonamide resistance gene (sul3) in Escherichia coli is widespread in the pig population of Switzerland. Antimicrob Agents Chemother. 2003;47:1169–72.
65. Rådström P, Swedberg G. RSF1010 and a conjugative plasmid contain sul2, one of two known genes for plasmid-borne sulfonamide resistance dihydropteroate synthase. Antimicrob Agents Chemother. 1988;32:1684–92.
66. Sundström L, Rådström P, Swedberg G, Sköld O. Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of dfr5 and sul1 and a recombination active locus of Tn21. Mol Gen Genet. 1988;213:191–201.
67. Swedberg G, Sköld O. Plasmid-borne sulfonamide resistance determinants studied by restriction enzyme analysis. J Bacteriol. 1983;153:1228–37.
68. Rådström P, Swedberg G, Sköld O. Genetic analyses of sulfonamide resistance and its dissemination in gram-negative bacteria illustrate new aspects of R plasmid evolution. Antimicrob Agents Chemother. 1991;35:1840–8.
69. Grape M, Sundström L, Kronvall G. New dfr2 gene as a single-gene cassette in a class 1 integron from a trimethoprim-resistant Escherichia coli isolate. Microb Drug Resist. 2003;9:317–22.
70. Grape M, Sundström L, Kronvall G. Sulfonamide resistance gene sul3 found in Escherichia coli isolates from human sources. J Antimicrob Chemother. 2003;52:1022–4.
71. Van Treeck UF, Schmidt F, Wiedemann B. Molecular nature of a streptomycin and sulfonamide resistance plasmid (pBP1) prevalent in clinical Escherichia coli strains and integration of an ampicillin resistance transposon (TnA). Antimicrob Agents Chemother. 1981;19:371–80.
72. Enne VI, Livermore DM, Stephens P, Hall LMC. Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restrictions. Lancet. 2001;357:1325–8.

73. Machado E, Coque TM, Cantón R, Sousa JC, Peixe L. Commensal Enterobacteriaceae as reservoirs of extended-spectrum beta-lactamases, integrons, and sul genes in Portugal Front. Microbiology. 2013;4:80.
74. Zhang T, Wang CG, Zhong XH. Survey on sulfonamide antibiotic-resistant genotype and phenotype of avian Escherichia coli in North China. Poult Sci. 2012;91:884–7.
75. Enne VI, King A, Livermore DM, Hall LMC. Sulfonamide resistance in Haemophilus influenzae mediated by acquisition of sul2 or a short insertion in chromosomal folP. Antimicrob Agents Chemother. 2002;46:1934–9.
76. Chang YT, Lin CY, Chen YH, Hsueh PR. Update on infections caused by Stenotrophomonas maltophilia with particular attention to resistance mechanisms and therapeutic options. Front Microbiol. 2015;6:893.
77. Flensburg J, Sköld O. Massive overproduction of dihydrofolate reductase as a response to the use of trimethoprim. Eur J Biochem. 1987;162:473–6.
78. de Groot R, Sluijter M, de Bruyn A, Camps J, Goessens WHF, Smith AL, Hermans PWM. Genetic characterization of trimethoprim resistance in Haemophilus influenzae. Antimicrob Agents Chemother. 1996;40:2131–6.
79. Adrian PV, Klugman KP. Mutations in the dihydrofolate reductase gene of trimethoprim-resistant isolates of Streptococcus pneumoniae. Antimicrob Agents Chemother. 1997;41:2406–13.
80. Gibreel A, Sköld O. High-level resistance to trimethoprim in clinical isolates of Campylobacter jejuni by acquisition of foreign genes (dfr1 and dfr9) expressing drug-insensitive dihydrofolate reductases. Antimicrob Agents Chemother. 1998;42:3059–64.
81. Hamilton-Miller JMT. Resistance to antibacterial agents acting on folate metabolism. In: Bryan LE, editor. Antimicrobial drug resistance. New York: Academic Press; 1984. p. 173–90.
82. King CH, Shlaes DM, Dul MJ. Infection caused by thymidine-requiring, trimethoprim-resistant bacteria. J Clin Microbiol. 1983;18:79–83.
83. Amyes SGB, Smith JT. R-factor trimethoprim resistance mechanism: an insusceptible target site. Biochem Biophys Res Commun. 1974;58:412–8.
84. Sköld O, Widh A. A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R-factor mediating high resistance to trimethoprim. J Biol Chem. 1974;249:4324–5.
85. Huovinen P, Sundström L, Swedberg G, Sköld O. Trimethoprim and sulfonamide resistance. Antimicrob Agents Chemother. 1995;39:279–89.
86. Heikkilä E, Sundström L, Skurnik M, Huovinen P. Analysis of genetic localization of the type I trimethoprim resistance gene from Escherichia coli isolated in Finland. Antimicrob Agents Chemother. 1991;35:1562–9.
87. Brisson N, Hohn T. Nucleotide sequence of the dihydrofolate reductase gene borne by the plasmid R67 and conferring methotrexate resistance. Gene. 1984;28:271–5.
88. Flensburg J, Steen R. Nucleotide sequence analysis of the trimethoprim resistant dihydrofolate reductase encoded by R plasmid R751. Nucleic Acids Res. 1986;14:5933.
89. Swift G, McCarthy BJ, Heffron F. DNA sequence of a plasmid-encoded dihydrofolate reductase. Mol Gen Genet. 1981;181:441–7.
90. Sundström L, Jansson C, Bremer K, Heikkilä E, Olsson-Liljequist B, Sköld O. A new dhfrVIII trimethoprim-resistance gene flanked by IS26, whose product is remote from other dihydrofolate reductases in parsimony analysis. Gene. 1995;154:7–14.
91. Burdeska A, Ott M, Bannwarth W, Then R. Identical genes for trimethoprim-resistant dihydrofolate reductase from Staphylococcus aureus in Australia and central Europe. FEBS Lett. 1990;266:159–62.
92. Rouch DA, Messerotti LJ, Loo LSL, Jackson CA, Skurray RA. Trimethoprim resistance transposon Tn4003 from Staphylococccus aureus encodes genes for a dihydrofolate reductase and thymidylate synthetase flanked by three copies of IS257. Mol Microbiol. 1989;3:161–75.
93. Dale GE, Broger C, Hartman PG, Langen H, Page MGP, Then RL, Stüber D. Characterization of the gene for the chromosomal dihydrofolate reductase (DHFR) of Staphylococcus epidermidis ATCC 14990: the origin of the trimethoprim-resistant S1 DHFR from Staphylococcus aureus? J Bacteriol. 1995;177:2965–70.
94. Dale GE, Langen H, Page MGP, Then RL, Stüber D. Cloning and characterization of a novel, plasmid-encoded trimethoprim-resistant dihydrofolate reductase from Staphylococcus haemolyticus MUR313. Antimicrob Agents Chemother. 1995;39:1920–4.
95. Charpentier E, Courvalin P. Emergence of a new class of trimethoprim resistance gene dfrD in Listeria monocytogenes BM4293. Antimicrob Agents Chemother. 1997;41:1134–6.
96. Dale GE, Broger C, Dárcy A, Hartman PG, DeHoogt R, Jolidon S, Kompis I, Labhardt AM, Langen H, Locher H, Page MGP, Stüber D, Then RL, Wipf B, Oefner C. A single amino acid substitution in Staphylococcus aureus dihydrofolate reductase determines trimethoprim resistance. J Mol Biol. 1997;266:23–30.
97. Adrian PV, Thomson CJ, Klugman KP, Amyes SGB. New gene cassettes for trimethoprim resistance, dfr13, and streptomycin-spectinomycin resistance, aadA4, inserted in a class 1 integron. Antimicrob Agents Chemother. 2000;44:355–61.
98. Adrian PV, duPlessis M, Klugman KP, Amyes SGB. New trimethoprim-resistant dihydrofolate reductase cassette, dfrXV, inserted in a class 1 integron. Antimicrob Agents Chemother. 1998;42:2221–4.
99. Chang C-Y, Chang L-L, Chang Y-H, Lee T-M, Li Y-H, Chang S-F. Two new gene cassettes, dfr17 (for trimethoprim resistance) and aadA4 (for spectinomycin/streptomycin resistance), inserted in an Escherichia coli class 1 integron. J Antimicrob Chemother. 2000;46:87–9.
100. White PA, McIver CJ, Deng Y-M, Rawlinson WD. Characterization of two new gene cassettes, aadA5 and dfrA17. FEMS Microbiol Lett. 2000;182:265–9.
101. Coque TM, Singh KV, Weinstock GM, Murray BE. Characterization of dihydrofolate reductase genes from trimethoprim-susceptible and trimethoprim-resistant strains of Enterococcus faecalis. Antimicrob Agents Chemother. 1999;43:141–7.
102. Jansson C, Sköld O. Appearance of a new trimethoprim resistance gene, dhfrIX, in Escherichia coli from swine. Antimicrob Agents Chemother. 1991;35:1891–9.
103. Jansson C, Franklin A, Sköld O. Spread of a new trimethoprim resistance gene, dhfrIX, among porcine isolates and human pathogens. Antimicrob Agents Chemother. 1992;36:2704–8.
104. Hummel R, Tschäpe H, Witte W. Spread of plasmid-mediated nourseothricin resistance due to antibiotic use in animal husbandry. J Basic Microbiol. 1986;26:461–6.
105. Marshall B, Petrowski D, Levy SB. Inter and intraspecies spread of Escherichia coli in a farm environment in the absence of antibiotic usage. Proc Natl Acad Sci U S A. 1990;87:6609–13.
106. Jansson C, Franklin A, Sköld O. Trimethoprim resistance arising in animal bacteria and transferring into human pathogens. J Infect Dis. 1993;167:785–7.
107. Chiou CS, Jones AL. Nucleotide sequence analysis of a transposon (Tn 5393) carrying streptomycin resistance genes in Erwinia amylovora and other Gram-negative bacteria. J Bacteriol. 1993;175:732–40.
108. McManus PS. Antibiotic use in plant disease control. APUA (Alliance for the prudent use of antibiotics). Newsletter. 1999;17:1–3.
109. Sundin GW, Bender CL. Dissemination of the strA-strB streptomycin resistance genes among commensal and pathogenic bacteria from humans, animals and plants. Mol Ecol. 1996;5:133–43.

110. Sundqvist M, Geli P, Andersson DI, Sjölund-Karlsson M, Runehagen A, Cars H, Abelson-Storby K, Cars O, Kahlmeter G. Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. J Antimicrob Chemother. 2010;65:350–60.
111. Yun MK, Wu Y, Li Z, Zhao Y, Waddell MB, Ferreira AM, Lee RE, Bashford D, White SW. Catalysis and sulfa drug resistance in dihydropteroate synthase. Science. 2012;335:1110–4.
112. Hammoudeh DI, Zhao Y, White SW, Lee RE. Replacing sulfa drugs with novel DHPS inhibitors. Future Med Chem. 2013;5:1331–40.
113. Nammalwar B, Muddala NP, Bourne CR, Henry M, Bourne PC, Bunce RA, Barrow EW, Berlin KD, Barrow WW. Synthesis and biological evaluation of 2,4-diaminopyrimidine-based antifolate drugs against Bacillus anthracis. Molecules. 2014;19:3231–46.
114. Kobayashi M, Kinjo T, Koseki Y, Bourne CR, Barrow WW, Aoki S. Identification of novel potential antibiotics against Staphylococcus using structure-based drug screening targeting dihydrofolate reductase. J Chem Inf Model. 2014;54:1242–53.
115. Wright DL, Anderson AC. Antifolate agents: a patent review (2006–2010). Expert Opin Ther Pat. 2014;6:687–97.
116. Yuthavong Y, Tarnchompoo B, Vilaivan T, Chitnumsub P, Kamchonwongpaisan S, Charman SA, McLennan DN, White KL, Vivas L, Bongard E, Thongphanchang C, Taweechai S, Vanichtanankul J, Rattanajak R, Arwon U, Fantauzzi P, Yuvaniyama J, Charman WN, Matthews D. Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. Proc Natl Acad Sci U S A. 2012;109:16823–8.

Mechanisms of Action and Resistance of the Antimycobacterial Agents

25

Noton K. Dutta and Petros C. Karakousis

1 Introduction

Historically, mycobacterial infections have been associated with significant morbidity and mortality worldwide. In particular, *Mycobacterium tuberculosis* is a highly successful human pathogen, causing ~8.7 million cases of active tuberculosis (TB) and ~1.4 million deaths annually [1]. The organism is unique in its ability to establish persistent infection, requiring prolonged treatment with antimicrobials in order to achieve clinical cure. In general, the goals of antituberculosis therapy include rapid reduction of the massive numbers of actively multiplying bacilli in the diseased host, prevention of acquired drug resistance, and sterilization of infected host tissues to prevent clinical relapse. In order to achieve these goals, currently accepted guidelines recommend administration of multiple active drugs for a minimum duration of 6 months [2]. In areas where drug resistance is prevalent and resources permit, *M. tuberculosis* clinical isolates should be routinely tested for susceptibility to first-line antituberculosis agents in order to optimally guide therapy. The emergence of multidrug-resistant TB (MDR-TB) [3], defined as resistance to the first-line drugs isoniazid and rifampicin, extensively drug-resistant TB (XDR-TB), defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the injectable second-line drugs (capreomycin, kanamycin, and amikacin), and totally drug-resistant tuberculosis (TDR-TB), loosely used for TB strains resistant to a wider range of drugs than those classified as XDR-TB, poses formidable challenges to global TB control efforts [4–6]. The global incidence of MDR-TB is estimated to be ~500,000 cases annually, of which 5–7 % represent XDR-TB.

Prior to the advent of highly active antiretroviral therapy (HAART), disseminated infection with *M. avium* complex was the most common bacterial opportunistic infection in adults infected with HIV-1 in the developed world, occurring annually in 10–20 % of individuals with AIDS [7, 8]. The availability of HAART, as well as the use of effective prophylaxis with azithromycin or clarithromycin, has reduced the annual incidence of disseminated *M. avium* complex infection among individuals with advanced HIV disease to less than 1 % per year [9]. Nevertheless, *M. avium* complex continues to cause disseminated disease in persons with HIV and advanced immunosuppression not receiving or unable to tolerate HAART. In addition, *M. avium* complex is an important cause of pulmonary infection, particularly in HIV-negative persons with underlying lung disease or other immunosuppression [10]. Infections with other mycobacteria, including *M. kansasii*, *M. genavense*, *M. hemophilum*, *M. fortuitum*, *M. xenopi*, *M. chelonae*, have been reported with increasing frequency, particularly in the setting of HIV infection [11–13].

This chapter will review the mechanisms of action and resistance of the antimycobacterial agents (Table 25.1), with an emphasis on the four first-line antituberculosis drugs isoniazid, rifampin, pyrazinamide, and ethambutol. The mechanisms of action of other drugs used to treat mycobacterial infections, including the fluoroquinolones, aminoglycosides, and the macrolides, will be reviewed elsewhere in this book and this chapter will focus on specific mutations associated with resistance to these agents in *M. tuberculosis* and *M. avium* complex. This chapter includes mechanistic studies carried out in *M. smegmatis*, which, because of its relatedness to *M. tuberculosis*, its fast-growing nature and lack of pathogenicity, and its relative genetic tractability, is widely used as a model system to study mycobacterial physiology. The phenomenon of *M. tuberculosis* phenotypic drug tolerance will not be addressed in this section, and discussion will be limited to genotypic mechanisms of drug resistance.

N.K. Dutta, Ph.D. • P.C. Karakousis, M.D. (✉)
Division of Infectious Diseases, Department of Medicine, Center for Tuberculosis Research, Johns Hopkins University School of Medicine, 1551 E. Jefferson Street, Room 110, Baltimore, MD 21287, USA
e-mail: petros@jhmi.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_25

Table 25.1 Mechanisms of action and resistance of the antimycobacterial agents

Drug/drug class	Cellular process inhibited	Drug target	Resistance mutations in clinical isolates	Frequency	Comments
Isoniazid (INH)	Mycolic acid synthesis	InhA	*katG* (S315T)	50–80 %	INH is a pro-drug requiring activation by the *M. tuberculosis* catalase-peroxidase KatG [26]
			inhA	15–34 %	
Rifampin	mRNA synthesis	RNA polymerase β subunit	*rpoB* (codons 507–533)	>90 %	>90 % of rifampin-resistant isolates are also resistant to INH
Pyrazinamide (PZA)	Depletion of membrane energy	Unknown	*pncA*	70–90 %	PZA is a pro-drug requiring activation by *M. tuberculosis* pyrazinamidase, which is encoded by *pncA* [153]
Ethambutol	Arabinogalactan synthesis	EmbB	*embB*	50–70 %	*embB* mutations may not be sufficient to confer resistance to EMB [202]
Streptomycin	Translation	30 S ribosomal subunit	*rpsL* (codons 43 and 88)	~50 %	Cross-resistance may not be observed with kanamycin or amikacin
			rrs	~20 %	
Amikacin/ Kanamycin	Translation	30 S ribosomal subunit	*rrs* (codon 1400)		Cross-resistance is observed with capreomycin, but not with streptomycin
Fluoroquinolones	DNA synthesis and transcription	DNA gyrase	*gyrA*	42–85 %	Cross-resistance is generally observed among the fluoroquinolones
			gyrB		
Macrolides	Translation	50 S ribosomal subunit	23S rRNA gene (Domain V loop)		Mechanisms of action and resistance listed are for *M. avium* complex; *M. tuber-culosis* is inherently resistant to the macrolides
Ethionamide	Mycolic acid synthesis	InhA	*ethA*		Ethionamide is a pro-drug requiring activation by the monooxygenase EthA [290, 318]
			inhA		
Capreomycin	Translation	16S rRNA	*rrs*		Cross-resistance is observed with kanamycin/amikacin
Cycloserine	Peptidoglycan synthesis	AlrA	*alrA*		Mechanisms of resistance have been shown in *M. smegmatis*, but not in *M. tuberculosis*
		Ddl			
Paraaminosalicylic acid (PAS)	Folic acid biosynthesis	Unknown	*thyA*		The mechanisms of action and resistance for PAS remain poorly characterized
	Iron uptake?				

2 Isoniazid

Isoniazid (isonicotinic acid hydrazide [INH]) has been the most commonly used drug in the armamentarium against *M. tuberculosis* since recognition of its clinical activity by Robitzek and Selikoff in 1952 [14]. Consisting of a pyridine ring and a hydrazide group (Fig. 25.1), INH is a nicotinamide analog structurally related to the antituberculosis drugs ethionamide and pyrazinamide [15]. Because of its significant bactericidal activity, it has become a critical component of first-line antituberculous chemotherapy. However, in the last two decades, resistance to INH has been reported with increasing frequency, ranging from 3 % to as high as one-quarter of all *M. tuberculosis* isolates from previously untreated individuals [16–20], with the highest rates of resistance reported from southeast Asia and the Russian Federation [19, 21].

2.1 Mechanism of Action

Despite the widespread use of INH for more than half a century, its mechanism of action has only recently begun to be elucidated. The drug appears to penetrate host cells readily [22, 23], and diffuses across the *M. tuberculosis* membrane [24, 25]. INH is a pro-drug, requiring oxidative activation by the *M. tuberculosis katG*-encoded catalase-peroxidase enzyme [26]. The resulting isonicotinoyl radical reacts non-enzymatically with oxidized NAD^+ to generate several different 4-isonicotinoyl-NAD adducts [27]. Although the active metabolites of INH have been reported to inhibit multiple essential cellular pathways, including synthesis of nucleic acids [28] and phospholipids [29], and NAD metabolism [30, 31], the primary pathway inhibited by the drug appears to be the synthesis of mycolic acids [32–34], manifesting as a loss of acid-fast staining of the organisms following INH treatment [35].

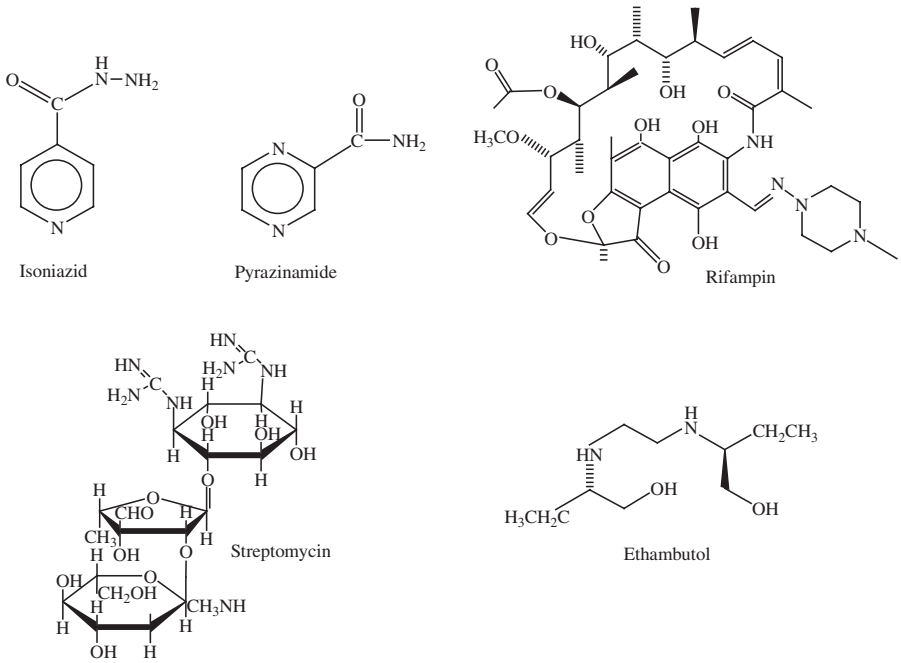


Fig. 25.1 Chemical structures of frontline TB drugs

Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids, which are unique outer cell wall components of mycobacteria and other Actinomycetales [36]. Mycolic acids are covalently attached to arabinogalactan and, together with other lipids of the outer leaflet, constitute a very hydrophobic barrier [37] responsible for resistance to certain drugs [38, 39]. Disruption of this hydrophobic barrier is believed to result in a loss of cellular integrity [40]. INH interrupts mycolic acid synthesis by binding tightly to the NADH-dependent enoyl acyl carrier protein (ACP) reductase InhA [41], a component of the fatty acid synthase II system of mycobacteria, which is essential for fatty acid elongation [42]. Genetic, biochemical, and structural data provide compelling evidence that InhA is the primary target for INH in the mycolic acid synthesis pathway. When transferred on a multicopy plasmid, the wild-type *inhA* gene of *M. tuberculosis* or *M. smegmatis* confers INH resistance to *M. smegmatis* and *M. bovis* BCG [41], as well as to *M. tuberculosis* [43]. A missense mutation within the mycobacterial *inhA* gene leading to the amino acid substitution S94A confers INH resistance to *M. smegmatis* [41] and *M. bovis* [44]. In addition, the same single point mutation in *inhA* (S94A) was sufficient to cause fivefold increased resistance to INH and inhibition of mycolic acid biosynthesis in *M. tuberculosis* [45]. Interestingly, overexpression of or mutation within *inhA* also confers resistance to the structurally related second-line antituberculosis drug ethionamide in *M. tuberculosis*, *M. smegmatis*, and *M. bovis*, suggesting that *inhA* encodes the target of both INH and ethionamide in these mycobacteria [41]. In addition, enoyl reductases, and specifically mycobacterial InhA, have been shown to be targets for the widely used topical disinfectant triclosan, and particular *M. smegmatis* mutants in *inhA* are cross-resistant to INH and triclosan [46]. However, although it affects InhA function, INH does not directly interact with InhA. Biochemical and structural studies have shown that InhA catalyzes the NADH-specific reduction of 2-trans-enoyl-ACP, and that the INH-resistant phenotype of S94A mutant InhA is related to reduced NADH binding [42, 47]. X-ray crystallographic and mass spectrometry data revealed that the activated form of INH covalently attaches to the nicotinamide ring of NAD bound within the active site of InhA, causing NADH to dissociate from InhA [47, 48].

Although inhibition of DNA synthesis by INH had been observed long ago [28], only relatively recently was a mechanism of action for this phenomenon proposed. Argyrou and colleagues cloned and overexpressed the *M. tuberculosis* gene encoding dihydrofolate reductase (DHFR), *dfrA*, in *M. smegmatis* and demonstrated a twofold increase in MIC [49]. *M. tuberculosis* DHFR was shown to selectively bind and co-crystallize with an active INH metabolite which is distinct from that which binds InhA [48]. However, this work requires further biochemical and genetic confirmation. Mutations in *dfrA* have yet to be reported among INH-resistant clinical isolates of *M. tuberculosis*.

Despite the identification of specific cellular targets in the last 15 years, the precise mechanism by which INH kills *M. tuberculosis* remains elusive. Interestingly, depletion of mycolic acids does not necessarily result in loss of viability in other mycobacteria in vitro [50, 51]. However, inhibition of mycolic acid synthesis may more severely compromise the intracellular survival of *M. tuberculosis* in vivo. It remains

to be shown that inhibition of mycolic acid synthesis is both necessary and sufficient for the highly potent in vivo bactericidal activity of INH against *M. tuberculosis*.

2.2 Mechanisms of Drug Resistance

Spontaneous INH resistance may be observed at a rate of 10^{-5} to 10^{-6} per bacterium per generation in *M. tuberculosis* cultures grown in vitro [52]. Because INH is the most commonly used antituberculosis drug, resistance to INH occurs more frequently among clinical isolates than to any other agent [53]. INH resistance varies geographically [54], and may be as high as 20–30 % in some parts of the world [19, 21]. Mutations are most commonly detected in the *katG* gene, occurring in 50–80 % of INH-resistant clinical isolates, or in the *inhA* gene, accounting for 15–34 % of INH resistance [53]. Depending on the mutation, the degree of INH resistance may vary from low (0.2 μg/mL) to high (100 μg/mL) [55]. Interestingly, a report from South Africa noted a higher frequency of mutations in the *inh*A region among patients with XDR-TB than among those with MDR-TB. In this study, mutations in the *inh*A promoter region accounted for ~50 to 60 % of cases of MDR-TB and 85–90 % of XDR-TB [56].

2.3 katG

INH resistance among clinical isolates of *M. tuberculosis* has long been associated with loss of catalase and peroxidase enzyme activities [57]. In general, there is a strong inverse correlation between degree of INH resistance and catalase activity [58]. Zhang and colleagues first demonstrated that deficiency of *katG*, which encodes the *M. tuberculosis* catalase-peroxidase enzyme, accounts for the observed resistance to INH in drug-resistant clinical isolates of *M. tuberculosis* [26, 59]. Mutations in KatG reduce the ability of the enzyme to activate the pro-drug INH, thus leading to resistance. The *M. tuberculosis katG* gene is situated in a highly variable and unstable region of the genome, perhaps because of the presence of repetitive DNA sequences [60], thereby potentially predisposing to a high frequency of *katG* mutations. Point mutations in *katG* are more common than deletions in INH-resistant clinical isolates, and a single point mutation resulting in substitution of threonine for serine at residue 315 (S315T) accounts for the majority of INH-resistant clinical isolates [61–63]. The S315T mutation is associated with a 50 % reduction in catalase and peroxidase activity and high-level INH resistance (MIC = 5–10 μg/mL) [58, 64]. The availability of the crystal structure for *M. tuberculosis* KatG [65] has provided greater insight into the process of INH activation and may permit a more accurate interpretation of structural and functional effects of mutations implicated in causing INH resistance in clinical isolates. Down-regulation of *katG* expression has also been shown recently to be associated with resistance to INH [66]. Three novel mutations in the *furA-katG* intergenic region were identified in 4 % of 108 INH-resistant strains studied; none of these was present in 51 INH-susceptible strains. Reconstructing these mutations in the *furA-katG* intergenic region of isogenic strains decreased the expression of *katG* and conferred resistance to INH.

2.4 inhA

INH resistance may arise either from mutations in *inhA*, resulting in reduced affinity of the enzyme for NADH without affecting its enoyl reductase activity [67], or in the promoter region of the *mabAinhA* operon [63], resulting in overexpression of the wild-type enzyme. Mutations in the *mabA* promoter region appear to be more frequent, but overexpression of MabA alone does not confer INH resistance in mycobacteria [68]. Unlike mutations in *katG*, which can confer low-level or high-level INH resistance, depending on the extent to which catalase-peroxidase enzyme activity is affected, mutations in *inhA* or in the promoter region of its operon usually confer low-level resistance (MIC = 0.2–1 μg/mL) [69]. Similarly, mutations in the intergenic region *oxyR-ahpC* can reduce the level of expression of *inhA* and have been associated with resistance to INH. A study by Dalla Costa et al. [70] found mutations in the intergenic region *oxyR-ahpC* in 8.9 % of 224 INH-resistant strains studied, confirming its less frequent involvement as a cause of resistance to INH. However, the precise role of these genes in INH resistance has not been completely elucidated.

2.5 Other Genes

The role of mutations in *kasA*, which encodes a β-ketoacyl ACP synthase of the type II fatty acid synthase system, with respect to INH resistance is controversial. Initial reports identified an association between clinical INH resistance and four independent mutations in *kasA* [71], but subsequent studies reported the presence of three of these mutations in INH-sensitive *M. tuberculosis* strains [72, 73]. In addition, although one group reported a fivefold increase in the MIC of INH following *kasA* overexpression in *M. tuberculosis* [74], another group found that overexpression of *kasA* conferred resistance to thiolactomycin, a known KasA inhibitor, but no increased resistance to INH in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* [43]. Using radioactive INH, Mdluli and colleagues reported KasA to be covalently associated with INH and ACP in *M. tuberculosis* [71], but Kremer and colleagues used anti-KasA antibodies to show that INH

treatment in mycobacteria does not result in significant KasA sequestering, and also demonstrated that activated INH does not inhibit KasA activity in an in vitro assay [75]. Although the preponderance of evidence suggests that InhA, and not KasA, is the primary target of INH in the mycolic acid synthesis pathway, the role of *kasA* mutations in INH resistance of clinical *M. tuberculosis* isolates requires further investigation.

Mutations in *ndh*, encoding a NADH dehydrogenase, were first shown in *M. smegmatis* to confer resistance to INH and ethionamide, as well as to exhibit other phenotypes, including thermosensitive lethality and auxotrophy [76]. Subsequently, *ndh* mutations were detected in almost 10 % of INH-resistant *M. tuberculosis* clinical isolates, which did not contain mutations in *katG*, *inhA*, or *kasA* [72]. Defective NADH dehydrogenase, which normally oxidizes NADH and transfers electrons to quinones of the respiratory chain, could lead to an increased ratio of NADH/NAD, which may interfere with KatG-mediated peroxidation of the drug, or displace the INH/NAD adduct from the InhA active site [76].

Mutations in the promoter region of *ahpC*, leading to overexpression of an alkylhydroperoxide reductase, have been observed in INH-resistant *M. tuberculosis* [77]. Although rarely found in some INH-resistant strains with apparently intact KatG [78], the *ahpC* mutation is usually found in KatG-negative INH-resistant *M. tuberculosis*, presumably as a compensatory mechanism for the loss of catalase-peroxidase activity in such strains [79–81]. AhpC does not appear to play a direct role in INH resistance, since *ahpC* overexpression in a wild-type reference strain of *M. tuberculosis* does not appreciably increase the MIC of INH, but mutations in the *ahpC* promoter region may serve as a useful marker for detection of INH resistance [78].

The *M. tuberculosis iniA* gene (Rv0342), part of a three-gene operon (Rv0341, Rv0342, Rv0343) induced in the presence of INH, appears to contribute to the development of tolerance to both INH and ethambutol, perhaps functioning through an MDR-pump-like mechanism, although IniA does not appear to directly transport INH from the cell [82]. INH also induces several other genes, including an operon cluster of five genes that code type II fatty acid synthase enzymes and *fbpC*, which encodes trehalose dimycolyl transferase. Other genes also induced are *efpA*, *fadE23*, *fadE24*, and *ahpC*, which mediate processes linked to the toxic activity of the drug and efflux mechanisms [83].

Despite the identification of several genetic mutations associated with resistance to INH, as many as a quarter of all clinical INH-resistant isolates do not have mutations in any of the above genes, suggesting alternative mechanisms of INH resistance. Tessema et al. reported 8 % of phenotypically defined isoniazid-resistant strains had no mutations in codon 315 of the *katG* gene and in the regulatory region of the *inhA* gene, demonstrating that other mechanisms or mutations in other codons of the katG gene may be responsible for the development of INH resistance in *M. tuberculosis* strains [84].

3 Rifampin and Other Rifamycins

The rifamycins were first isolated in 1957 from *Amycolatopsis* (formerly *Streptomyces*) *mediterranei* as part of an antibiotic screening program in Italy [85]. Their discovery and widespread use has revolutionized antituberculosis therapy, allowing for the reduction of the duration of treatment from 18 months to 9 months [86]. Although the early bactericidal activity of the rifamycins is inferior to that of INH [87–89], the former are the most potent sterilizing agents available in TB chemotherapy, continuing to kill persistent tubercle bacilli throughout the duration of therapy [90, 91]. Rifampin is a broad-spectrum antibiotic and the most widely used rifamycin to treat tuberculosis. Rifabutin, another rifamycin with reduced induction of the hepatic cytochrome P-450 enzyme system, was originally shown to be effective for the prophylaxis [92] and treatment [93] of *M. avium-intracellulare* complex infection in persons with advanced HIV disease. Rifapentine is a rifamycin with favorable pharmacokinetic properties [94, 95], including substantially greater maximum serum concentration and extended half-life, which permits highly intermittent therapy for HIV-negative patients who do not have cavitation on chest radiograph and who are sputum culture-negative after 2 months of therapy [96].

3.1 Mechanism of Action

The rifamycins are characterized by a unique chemical structure consisting of an aromatic nucleus linked on both sides by an aliphatic bridge (see figure) [97]. Although structural changes at positions C-21, C-23, C-8, or C-1 may significantly reduce microbiological activity, modifications at C-3 do not alter antituberculous activity. Rifampin is a 3-formyl derivative of rifamycin S; rifabutin is a spiropiperidyl derivative of rifamycin S; and rifapentine is a cyclopentyl-substituted rifampin [97].

The rifamycins are highly protein-bound in plasma, but easily diffuse across the *M. tuberculosis* cell membrane due to their lipophilic nature [69]. The bactericidal activity of the rifamycins has been attributed to their ability to inhibit mRNA synthesis by binding with high affinity to bacterial DNA-dependent RNA polymerase [98]. The core structure of RNA polymerase, comprising the subunits $\alpha_2\beta\beta'\omega$, is evolutionarily conserved among prokaryotes [99], explaining the antimicrobial activity of the rifamycins against a broad range of bacteria. X-ray crystallographic data examining the interaction of rifampin and RNA polymerase from *Thermus*

aquaticus revealed that rifampin exerts its effect by binding in a pocket between two structural domains of the RNA polymerase β subunit and directly blocking the path of the elongating RNA transcript at the 5′ end beyond the second or third nucleotide [100].

Although the molecular target of rifampin has been well characterized, the precise mechanism by which this interaction leads to mycobacterial killing remains unclear. Interestingly, transcriptional inhibition of the toxin-antitoxin *mazEF* module by rifampin was shown to trigger programmed cell death in *Escherichia coli* by reducing cellular levels of the labile antitoxic protein MazE, allowing the unrestrained lethal action of the long-lived toxic protein MazF [101]. Although *M. tuberculosis* contains homologous toxin-antitoxin gene modules [102, 103], it appears that these modules may play a role in *M. tuberculosis* growth arrest and persistence under adverse conditions, rather than in programmed cell death, as originally suggested [103].

3.2 Mechanism of Resistance

Although resistance to INH alone is common in *M. tuberculosis*, resistance to rifampin alone is rare, and more than 90 % of rifampin-resistant isolates are also resistant to INH. Therefore, rifampin resistance has been used as a surrogate marker for multidrug-resistant tuberculosis [104]. Resistance to rifampin develops in a single step at a frequency of 10^{-7} to 10^{-8} organisms in *M. tuberculosis* [105].

As in *E. coli* [106–108], resistance to rifampin in *M. tuberculosis* arises from mutations in *rpoB*, which encodes the β-subunit of RNA polymerase [109]. Over 90 % of rifampin-resistant clinical isolates contain point mutations clustered in an 81-base pair region between codons 507 and 533 of the *rpoB* gene [110, 111]. Although at least 35 distinct *rpoB* mutant allelic variants have been described [110], amino acid substitutions at one of two positions (Ser_{531} and His_{526}) account for the great majority of mutations conferring clinical resistance to rifampin [109, 111–113]. Consistent with the clinical data, selection of spontaneous rifampin resistance in vitro in the *M. tuberculosis* laboratory reference strain H37Rv yields *rpoB* mutations only at Ser_{531} and His_{526}, with the Ser_{531}Leu mutation predominating [114]. Strains with the point mutations CAC → TAC (His → Tyr) at codon 526 and TCG → TTG (Ser → Leu) at codon 531 account for 30 % and 25 %, respectively, of rifampin-resistant clinical isolates in the USA [112], while the same mutations represent 12 % and 47 %, respectively, of predominantly foreign rifampin-resistant isolates [109], suggesting that there may be geographic variation in the frequency of occurrence of particular *rpoB* mutations [110, 115]. Unlike mutations in codons 531 and 526, which confer high-level resistance to rifampin (MIC > 32 μg/mL) and cross-resistance to all rifamycins [69], mutations in codons 511, 516, and 522 are associated with low- or high-level resistance to rifampin and rifapentine (MIC 2–32 μg/mL), but preservation of susceptibility to rifabutin and the new rifamycin rifalazil [116–118]. In particular, MDR strains with the *rpoB* point mutation Asp516Val were almost always identified as rifabutin-susceptible [119]. Rare mutations in *M. tuberculosis* also have been reported in the 5′ region of the *rpoB* gene, and one such mutation at V176F confers intermediate- to high-level resistance to rifampin [120–123].

Several fast-growing strains of mycobacteria, including *M. smegmatis*, *M. chelonae*, *M. flavescens*, and *M. vaccae*, are able to inactivate rifampin by ribosylation, leading to inherent resistance to this antibiotic [124, 125]. However, this mechanism of rifampin resistance has not been described in *M. tuberculosis*. Nevertheless, a small percentage of rifampin-resistant isolates (<5 %) do not contain any mutations in the *rpoB* gene, suggesting additional molecular mechanisms of rifampin resistance in *M. tuberculosis*, such as altered rifampin permeability or mutations in other RNA polymerase subunits [110].

An important finding related to resistance to rifampicin is that almost all rifampicin-resistant strains also show resistance to other drugs, particularly to isoniazid. For this reason, rifampicin resistance detection has been proposed as a surrogate molecular marker for MDR [126].

4 Pyrazinamide

The use of pyrazinamide (PZA) in combination with rifampin in modern antituberculosis regimens has permitted shortening the duration of therapy from the previous 9–12 months to the current 6 months [127]. PZA is one of the key components of primary drug therapy against TB, especially when MDR has been diagnosed [128, 129]. Although its bactericidal activity is inferior to that of isoniazid and rifampin [130], the reduction of relapse rates associated with the addition of PZA in 6-month regimens is attributed to the drug's unique ability to target semi-dormant populations of bacilli residing within an acidic environment [131]. Consistent with this hypothesis, the drug was shown to be more active against old non-growing tubercle bacilli than against young, actively replicating organisms [132]. Interestingly, despite its established activity in vivo [133–136], PZA is inactive against *M. tuberculosis* grown under normal conditions in vitro [137], and requires acidification of the medium pH to demonstrate antituberculosis activity [138].

4.1 Mechanism of Action

PZA is an amide derivative of pyrazine-2-carboxylic acid and a nicotinamide analog (see figure) [139]. Despite recognition of its antituberculosis activity more than half a century

ago [133], the mechanism of action of PZA remains poorly understood. Because of the strict requirement for an acidic microenvironment, it was originally hypothesized that the site of action of PZA was in the macrophage phagolysosome [140], where intracellular *M. tuberculosis* resides. However, the interior pH of these organelles may be neutral or only slightly acidic [141, 142], well above the range where PZA is active [143]. In addition, although older studies suggested otherwise [140, 144], more recent studies have demonstrated that PZA has neither bacteriostatic nor bactericidal activity against intracellular *M. tuberculosis* in human monocyte-derived macrophages [145]. An alternative hypothesis is that PZA acts against bacilli residing in acidified compartments of the lung that are present during the early inflammatory stages of infection [131], which is consistent with the clinical observation that the potent sterilizing activity of PZA is limited to the first 2 months of therapy [146–148]. Anaerobic and microaerophilic conditions in vitro have been shown to enhance the activity of PZA against *M. tuberculosis*, suggesting an alternative explanation for the higher sterilizing activity of PZA against in vivo organisms residing within oxygen-deprived granulomas as compared to bacilli grown under in vitro conditions with ambient oxygen tension [149].

PZA enters *M. tuberculosis* through passive diffusion and via an ATP-dependent transport system [150]. The drug accumulates intracellularly because of an inefficient efflux system unique to *M. tuberculosis* [151]. Similar to INH, PZA is a pro-drug, which requires activation to its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PncA) [152, 153]. PncA is expressed constitutively in the cytoplasm of *M. tuberculosis* [154]. After conversion of PZA into POA, the drug exerts its cidal effect on tubercle bacilli by destabilizing the membrane potential and affecting membrane transport function [155]. The uptake and accumulation of POA in *M. tuberculosis* is enhanced when the extracellular pH is acidic [151]. The inhibitory effects of POA accumulation initially were attributed to direct inhibition of the mycobacterial fatty acid synthase I (FAS-I) enzyme [156], which is responsible for de novo synthesis of C_{16} fatty acids from acetyl-CoA primers and their elongation to $C_{24–26}$ fatty acyl-CoA derivatives [157, 158]. However, subsequent studies showed that, although the PZA analog 5-chloropyrazinamide irreversibly inhibits fatty acid synthesis through inhibition of FAS-I, POA does not directly inhibit purified mycobacterial FAS-I, suggesting that the enzyme is not the immediate target of PZA [159]. It has been proposed that the antituberculosis activity of PZA is not attributable to inhibition of a specific cellular target, but rather may reflect disruption of the proton motive force required for essential membrane transport functions by POA at acidic pH [160]. These findings could explain the enhanced susceptibility to PZA of old, non-replicating bacilli, which have a relatively low membrane potential [151] and reduced ability to maintain membrane energetics [161], as compared to young, actively replicating organisms [160]. Alternatively, the accumulation of POA or other weak organic acids has been hypothesized to lower the intracellular pH sufficiently to inactivate FAS-I or other vital enzymes required for cellular metabolism [159].

Recently, the ribosomal protein S1 (RpsA), a vital protein involved in protein translation and the ribosome-sparing process of trans-translation, was identified as a target of POA [162]. RpsA overexpression in *M. tuberculosis* conferred increased PZA resistance, and POA was shown to bind RpsA, thereby inhibiting trans-translation. Since the latter process is essential for freeing scarce ribosomes in nonreplicating organisms, inhibition of RpsA by POA may explain the sterilizing activity of PZA against persistent bacilli.

4.2 Mechanisms of Resistance

It has been known for some time that PZA resistance in *M. tuberculosis* is associated with loss of PZase activity [152]. More recently, pyrazinamide resistance has been attributed to mutations in *pncA*, the gene encoding PZase [153]. Consistent with these findings, integration of wild-type *pncA* into a pyrazinamide-resistant *pncA* mutant of *M. tuberculosis* is sufficient to restore susceptibility to PZA [163]. *M. bovis*, another member of the *M. tuberculosis* complex, is inherently resistant to PZA, most frequently because of a point mutation at codon 169 of the *pncA* gene, which renders the enzyme nonfunctional [164]. In contrast, studies of PZA-resistant clinical isolates of *M. tuberculosis* revealed that 72–97 % of these strains may contain various missense mutations, insertions, deletions, or termination mutations throughout the *pncA* gene or its promoter [165–167]. Recent data indicate that three mutations (D8G, S104R, and C138Y) in PncA confer excessive hydrogen bonding between PZA-binding residues and their neighboring residues, creating a rigid binding cavity, which in turn abolishes conversion of PZA into POA [168].

Resistance to PZA is also mediated by mutations in *rpsA* [162], which encodes a protein required for trans-translation in nonreplicating bacilli. A rare type of PZA-resistant isolate was found containing a deleted alanine at the C terminus of RpsA, preventing binding of the protein to tmRNA [162, 169, 170].

A small percentage of isolates with high-level PZA resistance contain no mutation in *pncA* or its promoter, suggesting other potential mechanisms of resistance to the drug [165], including perhaps deficient uptake [150], enhanced efflux, or altered *pncA* regulation. Alternatively, these findings may reflect the intrinsic problems associated with PZA susceptibility testing, since PZA resistance may be reported erroneously when the culture medium contains excessive bovine serum albumin, or a high inoculum of *M. tuberculosis*,

as both of these conditions may raise the pH of the medium and falsely elevate the MIC of the drug [132]. In fact, reliable methods for susceptibility testing of PZA have only recently been developed, using media with slightly higher pH (6.0–6.2) and higher concentrations of PZA (ranging from 300 μg/mL to as high as 1200 μg/mL, depending on the culture broth) [171].

5 Ethambutol

Ethambutol (EMB; dextro-2,2′-(ethylenediimino)-di-1-butanol), a synthetic compound structurally similar to D-arabinose (see figure) [172], was initially reported to have antituberculosis activity in 1961 [173]. In addition to its role as a first-line agent against *M. tuberculosis*, EMB is an important component of combination therapy against *M. avium* complex [9], and the drug exhibits activity against other mycobacteria, including *M. kansasii*, *M. xenopi*, and *M. marinum* [174]. EMB kills only actively multiplying bacilli [175], although its early bactericidal activity is not as potent as that of INH [176, 177]. EMB has poor sterilizing activity, as its addition to a regimen of INH, rifampin, and streptomycin does not improve culture conversion rates after 2 months of therapy [178], and its substitution for PZA increases clinical relapse rates [179]. Because of its modest contribution to the standard regimen of INH, rifampin, and PZA, the principal role of EMB is in the empiric treatment of individuals who are deemed at increased risk for harboring INH-resistant or multidrug-resistant *M. tuberculosis*, until drug susceptibility results become available.

5.1 Mechanism of Action

The mechanism of action of EMB remains incompletely understood. EMB has been reported to inhibit numerous mycobacterial cellular pathways, including RNA metabolism [175, 180], transfer of mycolic acids into the cell wall [181], phospholipid synthesis [182, 183], and spermidine biosynthesis [184]. However, the primary pathway affected by EMB appears to be that of arabinogalactan biosynthesis [185], through inhibition of cell wall arabinan polymerization [186].

Initial studies showed that treatment of *M. smegmatis* with EMB results in rapid bacterial disaggregation and morphological changes, consistent with alterations in cell wall composition [187]. A potential explanation for this phenomenon was provided by the observations that EMB inhibits transfer of mycolic acids to the cell wall in *M. smegmatis* [181], leading to rapid accumulation of trehalose monomycolate, trehalose dimycolate, and free mycolic acids in the culture medium [188]. Subsequently, EMB was shown to inhibit arabinogalactan synthesis, since MIC levels of the drug immediately inhibited the transfer of label from D-[^{14}C] glucose into the D-arabinose residue of arabinogalactan in EMB-susceptible *M. smegmatis*, but not in a drug-resistant strain [185]. In addition to inhibiting the synthesis of the arabinan component of the mycobacterial cell wall core polymer arabinogalactan, EMB inhibits biosynthesis of the arabinan of lipoarabinomannan, a lipoglycan noncovalently associated with the cell envelope [189, 190]. The observations that the latter effect is delayed relative to the former [186], and that EMB treatment results in rapid accumulation of β-D-arabinofuranosyl-1-monophosphoryldecaprenol (decaprenol phosphoarabinose) [191], an intermediate in arabinan biosynthesis, suggested that the primary site of EMB action is not on de novo synthesis of D-arabinose or on its activation, but rather in the final polymerization steps [186].

Using target overexpression by a plasmid vector as a selection tool, Belanger et al. demonstrated that the translationally coupled *embA* and *embB* genes of *M. avium* are both necessary and sufficient to render a naturally susceptible *M. smegmatis* strain resistant to EMB [192]. Subsequently, the *embCAB* gene cluster encoding the homologous arabinosyl transferase enzymes EmbA, EmbB, and EmbC was cloned, sequenced, and characterized in *M. tuberculosis* [193]. Although it has been proposed that these genes constitute an operon, there is evidence to suggest that the *embB* gene can be expressed from a unique promoter [194], the location of which remains unknown. The Emb proteins are thought to be integral membrane proteins with 12 transmembrane domains and a large carboxyl-terminal globular region of approximately 375 amino acids [193, 195]. Genetic and biochemical studies have shown that the EmbA and EmbB proteins are involved in the formation of the proper terminal hexaarabinofuranoside motif during arabinogalactan synthesis [196], while EmbC is involved in lipoarabinomannan synthesis [197]. Since the majority of EMB-resistant clinical isolates contain mutations in *embB* (see below) [193, 195, 198], the EmbB protein has been proposed as the main target of EMB, although X-ray crystallographic data supporting this interaction are lacking.

5.2 Mechanisms of Resistance

In *M. smegmatis*, high-level resistance to EMB appears to require multiple steps, including overexpression of the Emb proteins, as well as mutations in the conserved region of EmbB or further increases in protein expression levels [193]. Resistance to EMB in *M. tuberculosis* is usually associated with point mutations in the *embCAB* operon, commonly involving amino acid substitutions at codon Met306 of the *embB* gene [193, 195, 198]. EmbB mutations have been identified in 30–70 % of EMB-resistant isolates of *M. tuberculosis* [193, 198–200]. Mutations in the *embB* gene were

reported to be associated with high-level EMB resistance [201], with the mutations Met306Leu or Met306Val yielding a higher MIC (40 μg/mL) than the Met306Ile substitution (20 μg/mL) [198]. However, a study of 183 epidemiologically unlinked *M. tuberculosis* isolates collected in St. Petersburg, Russia, detected the presence of *embB* mutations at codon 306 in 48 % of EMB-resistant isolates, but also in 31 % of EMB-susceptible isolates, suggesting that *embB* mutations may not be sufficient to confer resistance to EMB, or the presence of a compensatory mutation that reverses the EMB resistance phenotype of *embB* mutants [202]. Interestingly, the discrepancy in phenotypic and genotypic EMB resistance tests was restricted to strains already resistant to other antituberculosis drugs; specifically, *embB* mutations at codon 306 were noted in 40 of the 69 (60 %) of EMB-susceptible strains resistant to isoniazid, rifampin, and streptomycin but none of the 43 pan-susceptible strains [202]. Huang et al. identified several novel mutations in *embB*, including at codon 319 and codon 497 [203]. Interestingly, Lacoma et al. found that two EMB-susceptible strains harbored a mutation at codon 306 [204].

Nucleotide polymorphisms in the *embC-embA* intergenic region have been reported in association with resistance-associated amino acid replacements in EmbA or EmbB, suggesting that these intergenic mutations represent secondary or compensatory changes [195]. Other potential mutations involved in EMB resistance include a Gln379Arg replacement in *M. tuberculosis embR*, a homologue of the synonymous gene encoding a putative transcriptional activator of *embAB* in *M. avium* [192], as well as mutations in *rmlD* and *rmlA2*, which encode proteins involved in rhamnose modification [195]. In addition, mutations associated with EMB resistance have been described in *Rv0340* [195], a gene transcribed in the same orientation and upstream of the *iniBAC* operon, which is significantly upregulated following exposure to EMB in vitro [205]. As many as one-quarter of all EMB-resistant *M. tuberculosis* isolates do not harbor mutations in any of the genes described above, suggesting alternative mechanisms of EMB resistance [206].

6 Aminoglycosides

The discovery of streptomycin (see figure) in the early 1940s represented the first breakthrough in the chemotherapy of tuberculosis [207]. Patients treated with streptomycin and bed rest improved initially compared to those assigned to bed rest alone, but streptomycin monotherapy led inevitably to relapses with streptomycin-resistant *M. tuberculosis* [208]. Although relapse rates are comparable when streptomycin is substituted for ethambutol as the fourth drug in addition to INH, rifampin, and PZA, the poor oral absorption of streptomycin, which necessitates parenteral administration, as well as the toxicity profile of the aminoglycosides have favored the use of ethambutol in first-line antituberculosis therapy [209]. Other aminoglycosides with significant antimycobacterial activity include kanamycin and amikacin [210]. The detailed mechanisms of action of the aminoglycosides will be addressed elsewhere, and this section will cover mechanisms of aminoglycoside resistance identified specifically in *M. tuberculosis*.

As in other bacteria, the mode of action of the aminoglycosides against mycobacterial species is through their binding to the 30S ribosomal subunit, which affects polypeptide synthesis and ultimately results in inhibition of translation [211]. In clinically relevant bacteria, resistance to the aminoglycosides most often results from modification of the aminoglycoside molecule. Although genes encoding aminoglycoside-modifying enzymes have been identified in the chromosome of slow-growing mycobacteria [212, 213], and disruption of aminoglycoside 2′-*N*-acetyltransferase genes has been correlated with increased aminoglycoside susceptibility in *M. smegmatis* [214], this mechanism of resistance has not been described for *M. tuberculosis* [105]. Instead, resistance to streptomycin and the other aminoglycosides in *M. tuberculosis* usually develops by mutation of the ribosome target binding sites. Interestingly, although cross-resistance is observed between amikacin and kanamycin [215], these drugs are not cross-resistant with streptomycin [216], suggesting distinct mechanisms of resistance. Amikacin is a derivative of kanamycin, and the two drugs are structurally related, each containing a 2-deoxystreptamine moiety, while streptomycin is structurally distinct, containing a streptidine moiety. High-level resistance to amikacin and kanamycin with preserved susceptibility to streptomycin has been reported in *M. abscessus* and *M. chelonae* [217], and in *M. tuberculosis* [218] in association with a point mutation at position 1400 (corresponding to position 1408 in *E. coli*) of the *rrs* gene, which encodes 16S rRNA [216, 217]. On the other hand, streptomycin resistance in mycobacteria is most commonly associated with mutations in the *rpsL* gene, which encodes the ribosomal protein S12 [219–224]. Specifically, a missense mutation resulting in a substitution of an arginine for a lysine at codon 43, as well as point mutations in codon 88 account for the majority of *rpsL* mutations in *M. tuberculosis* [224]. As in *E. coli*, streptomycin resistance in *M. tuberculosis* also commonly arises from *rrs* mutations, which are usually clustered in the regions surrounding nucleotides 530 or 912 [219, 220, 225]. Unlike most other bacteria, which have multiple copies of the *rrs* gene, *M. tuberculosis* and other slow-growing mycobacteria have a single copy of the gene, making it an easily selected mutation site. Thus, alterations in the drug target arising from reduced association of the 16S rRNA with the S12 ribosomal protein lead to an inability of aminoglycosides to disrupt translation of mycobacterial mRNA, thereby resulting

in antibiotic resistance. Mutations in *rpsL* and *rrs*, which occur in about 50 % and 20 %, respectively, of streptomycin-resistant *M. tuberculosis* clinical isolates, are usually associated with intermediate-(MIC 64–512 μg/mL) or high-level resistance (MIC > 1000 μg/mL) [105]. The mechanisms responsible for streptomycin resistance in other *M. tuberculosis* isolates, particularly those with low-level resistance (MIC 4–32 μg/mL), are unknown, but may involve changes in cell envelope permeability and diminished drug uptake [219, 221].

Reeves et al. recently identified aminoglycoside cross-resistance in *M. tuberculosis* due to mutations in the 5′ untranslated region of *whiB7*. These mutations led to an increase in the number of *whiB7* transcripts and increased expression of both *eis* (Rv2416c) and *tap* (Rv1258c) [226]. An association has been reported between *M. tuberculosis* clinical isolates harboring a variety of mutations in the *gidB* gene (Rv3919c) and low-level streptomycin resistance [227–229]. These data show that a mutation at either the *whiB7* or *gidB* locus leads to the acquisition of high-level streptomycin resistance at an elevated frequency, which may partly explain why streptomycin resistance can develop so quickly in the host [226, 229]. However, recent results suggest that for *gidB* this may remain problematic both because the number of mutations required to accurately assess *gidB* status is large and also because the impact of specific mutations in *gidB* on the resistance level of the isolate remains unclear [230]. A recent systematic review described additional mutations in the *rrs*, *tlyA*, *eis* promoter, and *gidB* genes appear to be associated with resistance to the injectable agents amikacin, kanamycin, and/or capreomycin [231]. Mutations in the gene *tlyA* encoding a 2′-*O*-methyltransferase of 16S rRNA and 23S rRNA have been implicated in resistance to capreomycin and viomycin [232]. Based on our understanding of aminoglycoside cross-resistance, the best order in which to introduce an injectable agent with the hope of preventing cross-resistance to other injectables would be streptomycin first, then capreomycin, then kanamycin, and finally amikacin [233].

7 Fluoroquinolones

The fluoroquinolones demonstrate excellent activity against several mycobacterial species, including *M. tuberculosis*, *M. kansasii*, and *M. fortuitum*, but not against others, such as *M. avium*, *M. marinum*, *M. chelonae*, and *M. abscessus* [234]. In particular, drugs of the fluoroquinolone class are highly active against *M. tuberculosis* both *in vitro* [235, 236] and in animal models [237–239]. In descending order of activity, fluoroquinolones active against *M. tuberculosis* include moxifloxacin, sparfloxacin, levofloxacin, ofloxacin, and ciprofloxacin [240]. The 8-methoxy-fluoroquinolone moxifloxacin has bactericidal activity similar to that of INH against *M. tuberculosis* both in vitro and in the murine model of TB [239, 241, 242], as well as early bactericidal activity comparable to INH in patients with pulmonary TB [243–245]. Unlike gatifloxacin, which appears to lack sterilizing activity against stationary-phase cultures of *M. tuberculosis* [246], moxifloxacin, when substituted for INH, is able to shorten the duration of therapy needed to effect stable cure in murine TB [247, 248], suggesting that the drug has significant sterilizing activity. Until recently, the fluoroquinolones have been recommended primarily as second-line agents in the treatment of multidrug-resistant tuberculosis [2]. However, the use of a fluoroquinolone as the only active agent in a failing regimen for treatment of multidrug-resistant *M. tuberculosis* constitutes the most frequent cause of fluoroquinolone resistance [240]. Resistance to fluoroquinolones also may arise extremely rapidly following use of these drugs for other infections [249, 250]. Despite the widespread use of fluoroquinolones to treat a variety of bacterial infections, fluoroquinolone resistance is detected in fewer than 2 % of *M. tuberculosis* isolates in the United States and Canada [251]. Because of its potent bactericidal and sterilizing activities, moxifloxacin is currently under investigation as a first-line agent in the treatment of tuberculosis. The mechanism of action and detailed mechanisms of resistance to this class of drugs will be discussed in another chapter, and this section will highlight specific mutations identified in fluoroquinolone-resistant *M. tuberculosis*.

Fluoroquinolones exert their powerful antibacterial activity by trapping gyrase and topoisomerase IV on DNA as ternary complexes and blocking the movement of replication forks and transcription complexes [252]. Unlike most other bacterial species, *M. tuberculosis* lacks topoisomerase IV but does contain the genes *gyrA* and *gyrB*, which encode the A and B subunits, respectively, of DNA gyrase [212]. Consequently, fluoroquinolone resistance in *M. tuberculosis* is most commonly associated with mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*, conserved regions involved in the interaction between the drug and DNA gyrase [240]. Spontaneous fluoroquinolone resistance develops in laboratory strains of *M. tuberculosis* at frequencies of 2×10^{-6} to 1×10^{-8} [253]. The most frequent mutations associated with high-level fluoroquinolone resistance involve substitutions at codons 88, 90, 91, and 94 of the *gyrA* gene [254–256].

The degree of resistance to fluoroquinolones depends on the specific amino acid substitution in the QRDR, and on the number of resistance mutations present. Therefore, while single mutations in *gyrA* may confer low-level resistance (MIC > 2 μg/mL) [257], high-level resistance to fluoroquinolones usually requires a stepwise process of at least two

mutations in *gyrA* or the combination of mutations in *gyrA* and *gyrB* [255, 257]. Mutations in the QRDR of *gyrA* do not occur following exposure of *M. tuberculosis* to low concentrations of fluoroquinolones in vitro, and the selection pressure for mutants in *gyrA* increases when *M. tuberculosis* is exposed to high concentrations of fluoroquinolones in vitro [258]. However, mutations in the QRDR region of *gyrA* are identified in only 42–85 % of fluoroquinolone-resistant clinical isolates, suggesting alternative mechanisms of resistance.

Mutations in the QRDR of the *gyrB* gene in the absence of *gyrA* mutations have been identified in some laboratory isolates [257, 258], but are rare in fluoroquinolone-resistant *M. tuberculosis* clinical isolates [259]. Pantel et al. reported four GyrB substitutions in fluoroquinolone-resistant *M. tuberculosis* clinical strains (D500A, N538T, T539P, and E540V) suggesting that the GyrB QRDR may extend from positions 500–540 [260]. Functional genetic analysis and structural modeling of GyrB suggest that N538D, E540V, and R485C/T539N conferred resistance to four different fluoroquinolones in at least one genetic background. The GyrB D500H and D500N mutations conferred resistance only to levofloxacin and ofloxacin while N538K and E540D consistently conferred resistance to moxifloxacin only. These findings indicate that certain mutations in *gyrB* may be sufficient to confer fluoroquinolone resistance, but the level and pattern of resistance varies among different mutations [261].

The *lfrA* gene, which encodes a multidrug efflux pump, has been shown to confer low-level resistance to fluoroquinolones when expressed on multicopy plasmids in *M. smegmatis* [262, 263]. Furthermore, expression of MfpA, a member of the pentapeptide repeat family of bacterial proteins [264], which includes McbG in *E. coli* and Qnr in *K. pneumoniae*, confers low-level resistance (four- to eightfold increase in the MIC) in *M. smegmatis* to ciprofloxacin and sparfloxacin [265]. Fluoroquinolone resistance related to MfpA has been attributed to DNA mimicry, as MfpA can directly bind to and inhibit DNA gyrase, thus preventing the formation of the DNA gyrase-DNA complex required for fluoroquinolone binding [266]. High-level resistance of *M. smegmatis* to ciprofloxacin (MIC = 64 2 μg/mL) also has been associated with overexpression and chromosomal amplification of the *pstB* gene, which encodes a putative ATPase subunit of the phosphate-specific transport (Pst) system, and disruption of this gene in *M. smegmatis* results in a twofold increase in sensitivity to fluoroquinolones relative to the isogenic wild-type strain [267]. Although homologues of *lfrA*, *mfpA*, and *pstB* appear to be present in *M. tuberculosis* [212], mutations or amplifications of these genes have not been identified in fluoroquinolone-resistant clinical isolates.

8 Macrolides

Clinical outcomes of patients with AIDS and disseminated *M. avium* complex have improved substantially since the introduction of the extended-spectrum macrolides, which are now considered the cornerstone of any potent regimen [268–270]. However, combination therapy with at least one other antimycobacterial agent, usually ethambutol, is necessary to prevent the emergence of macrolide resistance [271–273]. Although clarithromycin and azithromycin are both effective against disseminated *M. avium* complex infection, several studies directly comparing these two drugs when used in combination with ethambutol suggest trends toward more rapid clearance of bacteremia with clarithromycin [271, 272]. The mechanism of action of the macrolide antibiotics will be covered elsewhere in this book, and this section will focus on known macrolide resistance mutations occurring in *M. avium* complex.

The macrolides exert their antibacterial effect by binding to the bacterial 50S ribosomal subunit and inhibiting RNA-dependent protein synthesis [274]. However, these drugs have limited activity against wild-type *M. tuberculosis* [275]. This intrinsic resistance is believed to be associated with expression of the *erm* gene [276, 277], which is induced upon exposure of *M. tuberculosis* to clarithromycin [278]. Interestingly, disruption of the *pks12* gene, which encodes a polyketide synthase required for synthesis of the major cell wall lipid dimycocerosyl phthiocerol, results in increased susceptibility of *M. tuberculosis* to clarithromycin relative to its parent strain, but no change in susceptibility to ciprofloxacin or penicillin [279].

In *M. avium*, spontaneous resistance to clarithromycin has been estimated to occur at a rate of 10^{-8} to 10^{-9} organisms [280, 281]. Clarithromycin resistance in *M. avium* isolated from patients with pulmonary disease has been associated with point mutations in the generally conserved loop of domain V of 23S rRNA [282], corresponding to position 2058 in *E. coli* 23S rRNA, which confer resistance to erythromycin and the macrolides-lincomide-streptogramin B antibiotics [283]. Similarly, clarithromycin-resistant *M. avium* isolates obtained from patients with AIDS and disseminated *M. avium* infection contained point mutations in the domain V sequences of 23S rRNA at position 2274 [284]. Mutations in the *M. avium* 23S rRNA gene are associated with high-level resistance (MIC ≥ 128 μg/mL) [285]. As in *M. avium*, clarithromycin resistance in *M. chelonae* and *M. abscessus* has been associated with point mutations in the 23S rRNA peptidyltransferase region at positions 2058 or 2059 in strains with a single chromosomal copy of the rRNA operon [286, 287]. However, a few clarithromycin-resistant *M. avium* isolates, particularly with low-level resistance, have been described in which no mutation can be identified in the peptidyltransferase region of the 23S rRNA [281, 288], suggesting alternative mechanisms of drug resistance.

8.1 Cross-Resistance of Antimycobacterial Agents

In general, there is low cross-resistance among most antituberculosis drugs. When present, the degree of cross-resistance depends on the particular mutations and mechanism of drug resistance. Although the most commonly observed INH resistance mutations, i.e., those involving *katG*, do not generate cross-resistance to other agents, mutations in *inhA* itself or in its promoter region confer resistance to the second-line antituberculosis drug ethionamide [41, 43, 289]. Mutations in *ethA*, which confer ethionamide resistance, also yield cross-resistance to thiacetazone and thiocarlide [290].

Mutations in the *rpoB* gene of *M. tuberculosis*, particularly in codons Ser531 and His526, have been associated with high-level resistance (MIC > 32 μg/mL) to rifampin and cross-resistance to all the rifamycins. On the other hand, the *rpoB* mutations L511P, D516Y, D516V, or S522L, which are associated with low- to high-level resistance to rifampin and rifapentine, do not significantly alter susceptibility to rifabutin (MIC 0.5 μg/mL) or rifalazil (MIC 0.01–0.04 μg/mL) [118, 291]. In one study of 25 rifampin-resistant *M. tuberculosis* isolates (MIC > 2 μg/mL), three of these isolates (12 %) retained susceptibility to rifabutin [292]. Another study of 112 *M. tuberculosis* clinical isolates detected 73 % cross-resistance between rifabutin and rifampin [293], suggesting that rifabutin may have a role in the therapy of multidrug-resistant tuberculosis in cases where the isolate retains susceptibility to rifabutin.

Cross-resistance among the aminoglycosides is variable. Thus, cross-resistance is usually seen between the 2-deoxystreptamine aminoglycosides amikacin and kanamycin [218], but not between these two drugs and the streptidine aminoglycoside streptomycin [216]. In addition, cross-resistance may be observed between kanamycin and capreomycin or viomycin [294, 295]. Although cross-resistance has not been reported between fluoroquinolones and other classes of antituberculosis agents, mutations associated with individual fluoroquinolone resistance appear to confer cross-resistance to the entire class of drugs [240]. Similarly, resistance to clarithromycin or azithromycin in *M. avium* complex is usually associated with class-wide resistance to the macrolides [275, 284].

8.2 Mechanism of Spread of Resistance

Although drug resistance may be spread by plasmids or transposons among many bacterial species, including the fast-growing *M. fortuitum* [262], these mobile genetic elements are not known to cause drug resistance in *M. tuberculosis* [105]. As described above, drug resistance in *M. tuberculosis* is caused by mutations in specific chromosomal genes. In general, genetic resistance of *M. tuberculosis* to specific antimycobacterial drugs does not alter the fitness or virulence of the organism [105], suggesting that drug-resistant isolates may spread to previously uninfected individuals and cause disease equivalent to that caused by drug-susceptible isolates. One notable exception to this rule is in the case of certain INH-resistant *M. tuberculosis* isolates with reduced catalase activity, which demonstrate decreased virulence in the guinea pig model of tuberculosis [57]. Reduced catalase activity in these isolates correlates well with increased INH resistance, as well as decreased virulence [105]. Molecular genetic studies have shown that integration of a functional *katG* gene into the genome of INH-resistant, catalase-defective *M. bovis* restores INH susceptibility, as well as virulence in the guinea pig model [44]. Consistent with these findings, KatG-deficient *M. tuberculosis* is attenuated relative to a wild-type strain during infection of immunocompetent mice and mouse-derived macrophages, as a result of exposure to the peroxides generated by the phagocyte NADPH oxidase [296]. Although *M. tuberculosis* clinical isolates containing the S315T mutation appear to retain full virulence and transmissibility in humans [297], it is unknown if other *katG* mutants, with more greatly reduced catalase activity, are less transmissible or virulent in humans. On the other hand, restoration of virulence may be associated with promoter-up mutations in the *ahpC* gene, which may compensate for loss of catalase activity resulting from mutations in *katG* [79]. Full transmissibility and virulence are expected among *M. tuberculosis* strains in which INH resistance is mediated by mutations in genes other than *katG*, such as *inhA* or *ndh* [105].

The efficient spread of drug-resistant isolates certainly may occur from person to person, as evidenced by the ecologically successful strain W. This strain, which is resistant to as many as 11 antimycobacterial drugs, caused a multidrug-resistant outbreak of tuberculosis in New York City and spread across the United States [298]. However, the emergence of drug resistance in a particular individual is most often not due to primary infection with a drug-resistant isolate, but rather a result of human error. Thus, a prior history of tuberculosis and antituberculosis therapy has been implicated strongly in the causation of multidrug-resistant *M. tuberculosis* [299]. Factors associated with acquisition of drug resistance include incomplete and inadequate treatment, such as the use of a single drug to treat tuberculosis, the addition of a single drug to a failing regimen and the failure to identify preexisting resistance, as well as inadequate treatment adherence on the part of the patient [300]. Mathematical models predict that the future of the MDR and XDR-TB epidemic will depend to a large extent on the transmission efficiency or relative fitness of drug-resistant *M. tuberculosis* compared to drug-susceptible strains [301].

8.3 Alternative Agents

M. tuberculosis strains that are resistant to either isoniazid or rifampin may be treated effectively with other first-line drugs. However, strains that are resistant to both drugs, termed "multidrug-resistant" strains, require the use of "second-line drugs," which are generally less effective and more toxic [299]. These drugs include ethionamide, capreomycin, cycloserine, and paraaminosalicylic acid. Promising new antituberculosis drugs [302, 303], such as the nitroimidazoles (PA-824, OPC-67683) [304, 305], a diarylquinoline (TMC207/bedaquiline) [306], an ethylene diamine (SQ-109) [307], oxazolidinones (Linezolid, PNU-100480/sutezolid) [308, 309], benzothiazinones [310], clofazimine, and thioridazine [311–313], are currently being tested in preclinical or clinical trials [314]. Known mechanisms of action and resistance for each of these drugs will be discussed briefly in this section.

Ethionamide, a synthetic compound structurally related to INH, was shown to have antituberculosis activity in the late 1950s [315]. Although less potent than INH, ethionamide also inhibits mycolic acid synthesis [41, 316]. Ethionamide is a pro-drug requiring activation by the monooxygenase EthA [290, 317, 318], which itself is negatively regulated by the transcriptional repressor EthR. [317] Similarly to INH, ethionamide inhibits mycolic acid synthesis by binding the ACP reductase InhA [41]. Using a cell-based activation method, Wang et al. recently showed that the thioamide drugs ethionamide and prothionamide form covalent adducts with NAD, which are tight-binding inhibitors of *M. tuberculosis* and *M. leprae* InhA [319]. Approximately three-quarters of *M. tuberculosis* isolates with high-level ethionamide resistance (MIC > 50 μg/mL) have mutations in *ethA* or *inhA* [289]. Recently, other potential mechanisms of resistance have been identified, as *M. tuberculosis mshA* deletion mutants were found to be defective in mycothiol biosynthesis and resistant to ethionamide, likely due to defective activation of the drug [320].

Although often grouped together with the aminoglycosides because of similar activity and toxicities, capreomycin is a macrocyclic polypeptide antibiotic isolated from *Streptomyces capreolus* [210]. Like streptomycin and kanamycin, capreomycin inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA [69]. Recent studies using site-directed mutagenesis have identified the binding site of capreomycin on 16S rRNA helix 44 [321]. In *M. smegmatis*, mutations in *vicA* and *vicB*, which encode components of the 50S and 30S ribosomal subunits, confer resistance to capreomycin and viomycin [294, 295]. In *M. tuberculosis*, mutations in the *rrs* gene encoding 16S rRNA have been associated with resistance to capreomycin as well as kanamycin [218, 322]. The *rrs* mutation A1401G causes high-level amikacin/kanamycin and low-level capreomycin resistance. C1402T is associated with capreomycin (and viomycin) resistance and low-level kanamycin resistance. G1484T has been linked to high-level amikacin/kanamycin and capreomycin/viomycin resistance [323–325]. Various single-nucleotide polymorphisms (SNPs) in the *tlyA* gene have been also associated with capreomycin resistance [326].

Cycloserine interrupts peptidoglycan synthesis by inhibiting the enzymes D-alanine racemase (AlrA) and D-alanine:alanine ligase (Ddl) [327, 328]. Overexpression of *M. tuberculosis* AlrA and Ddl on a multicopy vector results in resistance to D-cycloserine in *M. smegmatis* and *M. bovis* BCG [327, 328], and *M. smegmatis alrA* mutants lacking D-alanine racemase activity display hypersusceptibility to D-cycloserine [329]. In *E. coli*, cycloserine resistance has been attributed to mutations in *cycA*, which encodes a permease responsible for uptake of the drug [330], but such a mechanism of resistance has not been described for mycobacteria. In addition, mutations in a gene homologous to that encoding *E. coli* penicillin binding protein 4 (PBP4) were shown to confer resistance to D-cycloserine, as well as to vancomycin in *M. smegmatis* [331]. However, the mechanism of cycloserine resistance in *M. tuberculosis* remains unknown.

Paraaminosalicylic acid (PAS) was introduced in 1945 [332, 333]. Although its activity was inferior to that of streptomycin when used alone, the combination of PAS with streptomycin significantly reduced the emergence of streptomycin-resistant organisms [334]. The mechanisms of action and resistance to PAS have not been well characterized, but it has been suggested that the drug may inhibit folic acid biosynthesis and uptake of iron [69]. Recently, PAS-resistant transposon mutants of *M. bovis* BCG were found to harbor insertions in the *thyA* gene, which encodes the enzyme thimidylate synthesis in the folate biosynthesis pathway [335]. In addition, mutations in the *thyA* gene resulting in diminished enzymatic activity were identified in PAS-resistant *M. tuberculosis* clinical isolates, suggesting that PAS may act as a folate antagonist and that *thyA* mutations may mediate clinical PAS resistance [335]. However, only slightly more than a third of the evaluated PAS-resistant strains had mutations in *thyA*, suggesting the existence of additional mechanisms of PAS resistance. Thr202Ala has been reported as the most common mutation associated with PAS resistance, although this mutation has also been identified in several PAS-susceptible isolates [336].

PA-824, a small molecule nitroimidazopyran related to metronidazole, was recently shown to have bactericidal activity against replicating and static *M. tuberculosis* cultures in vitro, as well as in murine and guinea pig models of tuberculosis [337]. In the mouse model, PA-824 has bactericidal activity comparable to that of INH [338, 339]. However, unlike INH, but like metronidazole, the drug also has potent activity against nonreplicating bacilli exposed to microaerophilic conditions [337, 338]. In addition, PA-824 is highly

active against multidrug-resistant clinical isolates of *M. tuberculosis* (MIC < 1 μg/mL), suggesting no cross-resistance with current antituberculosis drugs [338]. Like metronidazole, PA-824 is a pro-drug which requires bioreductive activation of an aromatic nitro group in order to exert an antitubercular effect [337]. Although the precise mechanism by which PA-824 exerts its lethal effect is unknown, the drug appears to inhibit the oxidation of hydroxymycolates to ketomycolates, a terminal step in mycolic acid synthesis [337]. Similar to INH, resistance to PA-824 is most commonly mediated by mutations which lead to loss of pro-drug activation. Mutations in *fgd1* and *fbiC* result in the loss of a specific glucose-6-phosphate dehydrogenase and its deazaflavin cofactor F_{420}, respectively, which together provide electrons for the reductive activation of PA-824 [340]. In addition, resistance to PA-824 has been associated with mutations in *Rv3547*, a gene encoding a conserved hypothetical protein which appears to be involved in PA-824 activation [340, 341]. Among laboratory strains, the frequency of resistance to PA-824 is slightly less than that to INH, approximately 9.0×10^{-7} [337].

As in the case of PA-824, mutations in the Rv3547 gene have been identified in strains resistant to OPC-67683, indicating defective drug activation [342]. Resistance to TMC207 is mediated by mutations in the *atpE* gene encoding the transmembrane and oligomeric C subunit of ATP synthase, typically at positions 63 or 66 [343]. However, more recent studies have shown that a majority of in vitro-generated mutants resistant to TMC207 lacked mutations in *atpE*, indicating alternative mechanisms of drug resistance [344].

Whether upregulation of *ahpC* expression, observed in strains resistant to INH, EMB, and SQ109, plays a role in resistance to SQ109 or merely reflects a compensatory metabolic mechanism remains to be determined [345].

While resistance to linezolid in *M. tuberculosis* clinical isolates is rarely reported, in vitro-selected mutants with high-level resistance to linezolid (MIC = 16–32 mg/L) have been found to contain mutations at G2061T and G2576T in the 23S rRNA gene [346]. On the other hand, mutants with lower level linezolid resistance (MIC = 4–8 mg/L) lack mutations in the 23S rRNA gene, implicating other possible mechanisms of resistance, such as the possible involvement of efflux pumps or other non-ribosomal alterations, as has been shown in *M. smegmatis* mutants [347, 348]. Sutezolid (PNU-100480) [349] is undergoing Phase I studies and resistance mechanisms are expected to be similar to those of linezolid.

Although spontaneous benzothiazinone-resistant laboratory mutants were found to have a Ser or Gly substitution at codon Cys387 of *dprE1*, resistance to benzothiazinones has not been reported in clinical *M. tuberculosis* isolates [350].

Several existing drugs used for other medical conditions have been "repurposed" for the treatment of tuberculosis. A recent study showed that the antitubercular activity of clofazimine, including against MDR-TB, is due to the generation of reactive oxygen species formed as a consequence of Ndh-mediated reduction of clofazimine [351]. Xu et al. reported that clofazimine mutants resistant to 0.48 and 1.92 μg/mL were not observed using the indirect method in mice during 90 days of treatment [352]. The mechanism of antitubercular activity of thioridazine is likely multifactorial [353, 354], as the drug appears to act on enzymes involved in fatty acid metabolism and membrane proteins, particularly efflux pumps, in addition to inhibiting type II NADH:menaquinone oxidoreductase as a phenothiazine [355]. Mechanisms of *M. tuberculosis* resistance to the phenothiazines remain to be elucidated.

Acknowledgements This work was supported by NIH grants UH2AI122309, R21AI114507A and R21AI122922 to PCK.

References

1. World Health Organization. WHO: Global Tuberculosis Report 2012. WHO/HTM/TB/2012.6.
2. Blumberg HM, Burman WJ, Chaisson RE, Daley CL, Etkind SC, Friedman LN, Fujiwara P, Grzemska M, Hopewell PC, Iseman MD, Jasmer RM, Koppaka V, Menzies RI, O'Brien RJ, Reves RR, Reichman LB, Simone PM, Starke JR, Vernon AA. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. Am J Respir Crit Care Med. 2003;167:603–62.
3. Lanzas F, Karakousis PC, Sacchettini JC, Ioerger TR. Multidrug-resistant tuberculosis in panama is driven by clonal expansion of a multidrug-resistant Mycobacterium tuberculosis strain related to the KZN extensively drug-resistant M. tuberculosis strain from South Africa. J Clin Microbiol. 2013;51:3277–85.
4. Royce S, Falzon D, van Weezenbeek C, Dara M, Hyder K, Hopewell P, Richardson MD, Zignol M. Multidrug resistance in new tuberculosis patients: burden and implications [Short communication]. Int J Tuberc Lung Dis. 2013;17:511–3.
5. Udwadia ZF. MDR, XDR, TDR tuberculosis: ominous progression. Thorax. 2012;67:286–8.
6. Zar HJ, Udwadia ZF. Advances in tuberculosis 2011–2012. Thorax. 2013;68:283–7.
7. Chaisson RE, Moore RD, Richman DD, Keruly J, Creagh T, The Zidovudine Epidemiology Study Group. Incidence and natural history of Mycobacterium avium-complex infections in patients with advanced human immunodeficiency virus disease treated with zidovudine. Am Rev Respir Dis. 1992;146:285–9.
8. Nightingale SD, Byrd LT, Southern PM, Jockusch JD, Cal SX, Wynne BA. Incidence of Mycobacterium avium-intracellulare complex bacteremia in human immunodeficiency virus-positive patients. J Infect Dis. 1992;165:1082–5.
9. Karakousis PC, Moore RD, Chaisson RE. Mycobacterium avium complex in patients with HIV infection in the era of highly active antiretroviral therapy. Lancet Infect Dis. 2004;4:557–65.
10. Field SK, Fisher D, Cowie RL. Mycobacterium avium complex pulmonary disease in patients without HIV infection. Chest. 2004;126:566–81.
11. Canueto-Quintero J, Caballero-Granado FJ, Herrero-Romero M, Dominguez-Castellano A, Martin-Rico P, Verdu EV, Santamaria DS, Cerquera RC, Torres-Tortosa M. Epidemiological, clinical,

and prognostic differences between the diseases caused by Mycobacterium kansasii and Mycobacterium tuberculosis in patients infected with human immunodeficiency virus: a multicenter study. Clin Infect Dis. 2003;37:584–90.
12. Bottger EC, Teske A, Kirschner P, Bost S, Chang HR, Beer V, Hirschel B. Disseminated "Mycobacterium genavense" infection in patients with AIDS. Lancet. 1992;340:76–80.
13. Bessesen MT, Shlay J, Stone-Venohr B, Cohn DL, Reves RR. Disseminated Mycobacterium genavense infection: clinical and microbiological features and response to therapy. Aids. 1993;7:1357–61.
14. Robitzek EH, Selikoff IJ. Hydrazine derivatives of isonicotinic acid (rimifon marsilid) in the treatment of active progressive caseous-pneumonic tuberculosis; a preliminary report. Am Rev Tuberc. 1952;65:402–28.
15. Slayden RA, Barry III CE. The genetics and biochemistry of isoniazid resistance in mycobacterium tuberculosis. Microbes Infect. 2000;2:659–69.
16. Al-Tawfiq JA, Al-Muraikhy AA, Abed MS. Susceptibility pattern and epidemiology of Mycobacterium tuberculosis in a Saudi Arabian hospital: a 15-year study from 1989 to 2003. Chest. 2005;128:3229–32.
17. Anuradha B, Aparna S, Hari Sai Priya V, Vijaya Lakshmi V, Akbar Y, Suman Latha G, Murthy KJ. Prevalence of drug resistance under the DOTS strategy in Hyderabad, South India, 2001–2003. Int J Tuberc Lung Dis. 2006;10:58–62.
18. Perez del Molino Bernal ML, Tunez V, Cruz-Ferro E, Fernandez-Villar A, Vazquez-Gallardo R, Diaz-Cabanela D, Anibarro L. Study of Mycobacterium tuberculosis drug resistance in the region of Galicia, Spain. Int J Tuberc Lung Dis. 2005;9:1230–5.
19. Quy HT, Buu TN, Cobelens FG, Lan NT, Lambregts CS, Borgdorff MW. Drug resistance among smear-positive tuberculosis patients in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis. 2006;10:160–6.
20. Swaminathan S, Paramasivan CN, Ponnuraja C, Iliayas S, Rajasekaran S, Narayanan PR. Anti-tuberculosis drug resistance in patients with HIV and tuberculosis in South India. Int J Tuberc Lung Dis. 2005;9:896–900.
21. Balabanova Y, Drobniewski F, Fedorin I, Zakharova S, Nikolayevskyy V, Atun R, Coker R. The directly observed therapy short-course (DOTS) strategy in Samara Oblast, Russian Federation. Respir Res. 2006;7:44.
22. Mackaness GB, Smith N. The action of isoniazid (isonicotinic acid hydrazide) on intracellular tubercle bacilli. Am Rev Tuberc. 1952;66:125–33.
23. Suter E. Multiplication of tubercle bacilli within phagocytes cultivated in vitro, and effect of streptomycin and isonicotinic acid hydrazide. Am Rev Tuberc. 1952;65:775–6.
24. Tsukamura M, Tsukamura S, Nakano E. The uptake of isoniazid by mycobacteria and its relation to isoniazid susceptibility. Am Rev Respir Dis. 1963;87:269–75.
25. Bardou F, Raynaud C, Ramos C, Laneelle MA, Laneelle G. Mechanism of isoniazid uptake in Mycobacterium tuberculosis. Microbiology. 1998;144(Pt 9):2539–44.
26. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature. 1992;358:591–3.
27. Nguyen M, Claparols C, Bernadou J, Meunier B. A fast and efficient metal-mediated oxidation of isoniazid and identification of isoniazid-NAD(H) adducts. Chembiochem. 2001;2:877–83.
28. Gangadharam PR, Harold FM, Schaefer WB. Selective inhibition of nucleic acid synthesis in Mycobacterium tuberculosis by isoniazid. Nature. 1963;198:712–4.
29. Brennan PJ, Rooney SA, Winder FG. The lipids of Mycobacterium tuberculosis BCG: fractionation, composition, turnover and the effects of isoniazid. Ir J Med Sci. 1970;3:371–90.
30. Zatman LJ, Kaplan NO, Colowick SP, Ciotti MM. Effect of isonicotinic acid hydrazide on diphosphopyridine nucleotidases. J Biol Chem. 1954;209:453–66.
31. Bekierkunst A. Nicotinamide-adenine dinucleotide in tubercle bacilli exposed to isoniazid. Science. 1966;152:525–6.
32. Winder FG, Collins PB. Inhibition by isoniazid of synthesis of mycolic acids in Mycobacterium tuberculosis. J Gen Microbiol. 1970;63:41–8.
33. Takayama K, Wang L, David HL. Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1972;2:29–35.
34. Takayama K, Schnoes HK, Armstrong EL, Boyle RW. Site of inhibitory action of isoniazid in the synthesis of mycolic acids in Mycobacterium tuberculosis. J Lipid Res. 1975;16:308–17.
35. Middlebrook G. Sterilization of tubercle bacilli by isonicotinic acid hydrazide and the incidence of variants resistant to the drug in vitro. Am Rev Tuberc. 1952;65:765–7.
36. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem. 1995;64:29–63.
37. Draper P. The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci. 1998;3:D1253–61.
38. Nikaido H. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol. 2001;12:215–23.
39. Karakousis PC, Williams EP, Bishai WR. Altered expression of isoniazid-regulated genes in drug-treated dormant Mycobacterium tuberculosis. J Antimicrob Chemother. 2008;61:323–31.
40. Barry III CE, Lee RE, Mdluli K, Sampson AE, Schroeder BG, Slayden RA, Yuan Y. Mycolic acids: structure, biosynthesis and physiological functions. Prog Lipid Res. 1998;37:143–79.
41. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle G, Jacobs Jr WR. inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science. 1994;263:227–30.
42. Quemard A, Sacchettini JC, Dessen A, Vilcheze C, Bittman R, Jacobs Jr WR, Blanchard JS. Enzymatic characterization of the target for isoniazid in Mycobacterium tuberculosis. Biochemistry. 1995;34:8235–41.
43. Larsen MH, Vilcheze C, Kremer L, Besra GS, Parsons L, Salfinger M, Heifets L, Hazbon MH, Alland D, Sacchettini JC, Jacobs Jr WR. Overexpression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in Mycobacterium smegmatis, M. bovis BCG and M. tuberculosis. Mol Microbiol. 2002;46:453–66.
44. Wilson TM, de Lisle GW, Collins DM. Effect of inhA and katG on isoniazid resistance and virulence of Mycobacterium bovis. Mol Microbiol. 1995;15:1009–15.
45. Vilcheze C, Wang F, Arai M, Hazbon MH, Colangeli R, Kremer L, Weisbrod TR, Alland D, Sacchettini JC, Jacobs Jr WR. Transfer of a point mutation in Mycobacterium tuberculosis inhA resolves the target of isoniazid. Nat Med. 2006;12:1027–9.
46. McMurry LM, McDermott PF, Levy SB. Genetic evidence that InhA of Mycobacterium smegmatis is a target for triclosan. Antimicrob Agents Chemother. 1999;43:711–3.
47. Dessen A, Quemard A, Blanchard JS, Jacobs Jr WR, Sacchettini JC. Crystal structure and function of the isoniazid target of Mycobacterium tuberculosis. Science. 1995;267:1638–41.
48. Rozwarski DA, Grant GA, Barton DH, Jacobs Jr WR, Sacchettini JC. Modification of the NADH of the isoniazid target (InhA) from Mycobacterium tuberculosis. Science. 1998;279:98–102.
49. Argyrou A, Vetting MW, Aladegbami B, Blanchard JS. Mycobacterium tuberculosis dihydrofolate reductase is a target for isoniazid. Nat Struct Mol Biol. 2006;13:408–13.
50. Liu J, Nikaido H. A mutant of Mycobacterium smegmatis defective in the biosynthesis of mycolic acids accumulates meromycolates. Proc Natl Acad Sci U S A. 1999;96:4011–6.

51. Mdluli K, Swanson J, Fischer E, Lee RE, Barry III CE. Mechanisms involved in the intrinsic isoniazid resistance of Mycobacterium avium. Mol Microbiol. 1998;27:1223–33.
52. Winder F. The antibacterial action of streptomycin, isoniazid, and PAS. In: Barry V, editor. Chemotherapy of tuberculosis. London: Butterworth; 1964. p. 111–49.
53. Zhang Y. Isoniazid. In: Rom W, Garay S, editors. Tuberculosis. Philadelphia: Lippincott Williams & Wilkins; 2004. p. 739–58.
54. Cohn DL, Bustreo F, Raviglione MC. Drug-resistant tuberculosis: review of the worldwide situation and the WHO/IUATLD Global Surveillance Project. International Union Against Tuberculosis and Lung Disease. Clin Infect Dis. 1997;24 Suppl 1:S121–30.
55. Hobby GL, Lenert TF. Resistance to isonicotinic acid hydrazide. Am Rev Tuberc. 1952;65:771–4.
56. Muller B, Streicher EM, Hoek KG, Tait M, Trollip A, Bosman ME, Coetzee GJ, Chabula-Nxiweni EM, Hoosain E, Gey van Pittius NC, Victor TC, van Helden PD, Warren RM. inhA promoter mutations: a gateway to extensively drug-resistant tuberculosis in South Africa? Int J Tuberc Lung Dis. 2011;15:344–51.
57. Middlebrook G. Isoniazid-resistance and catalase activity of tubercle bacilli; a preliminary report. Am Rev Tuberc. 1954; 69:471–2.
58. Rouse DA, DeVito JA, Li Z, Byer H, Morris SL. Site-directed mutagenesis of the katG gene of Mycobacterium tuberculosis: effects on catalase-peroxidase activities and isoniazid resistance. Mol Microbiol. 1996;22:583–92.
59. Zhang Y, Garbe T, Young D. Transformation with katG restores isoniazid-sensitivity in Mycobacterium tuberculosis isolates resistant to a range of drug concentrations. Mol Microbiol. 1993;8:521–4.
60. Zhang Y, Young D. Strain variation in the katG region of Mycobacterium tuberculosis. Mol Microbiol. 1994;14:301–8.
61. Abate G, Hoffner SE, Thomsen VO, Miorner H. Characterization of isoniazid-resistant strains of Mycobacterium tuberculosis on the basis of phenotypic properties and mutations in katG. Eur J Clin Microbiol Infect Dis. 2001;20:329–33.
62. Marttila HJ, Soini H, Eerola E, Vyshnevskaya E, Vyshnevskiy BI, Otten TF, Vasilyef AV, Viljanen MK. A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant Mycobacterium tuberculosis isolates originating from the St. Petersburg area in Russia. Antimicrob Agents Chemother. 1998;42:2443–5.
63. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, van Embden JD. Characterization of the catalase-peroxidase gene (katG) and inhA locus in isoniazid-resistant and -susceptible strains of Mycobacterium tuberculosis by automated DNA sequencing: restricted array of mutations associated with drug resistance. J Infect Dis. 1996;173:196–202.
64. Saint-Joanis B, Souchon H, Wilming M, Johnsson K, Alzari PM, Cole ST. Use of site-directed mutagenesis to probe the structure, function and isoniazid activation of the catalase/peroxidase, KatG, from Mycobacterium tuberculosis. Biochem J. 1999;338(Pt 3): 753–60.
65. Bertrand T, Eady NA, Jones JN, Jesmin Nagy JM, Jamart-Gregoire B, Raven EL, Brown KA. Crystal structure of Mycobacterium tuberculosis catalase-peroxidase. J Biol Chem. 2004;279:38991–9.
66. Ando H, Kitao T, Miyoshi-Akiyama T, Kato S, Mori T, Kirikae T. Downregulation of katG expression is associated with isoniazid resistance in Mycobacterium tuberculosis. Mol Microbiol. 2011;79:1615–28.
67. Basso LA, Zheng R, Musser JM, Jacobs Jr WR, Blanchard JS. Mechanisms of isoniazid resistance in Mycobacterium tuberculosis: enzymatic characterization of enoyl reductase mutants identified in isoniazid-resistant clinical isolates. J Infect Dis. 1998;178:769–75.
68. Banerjee A, Sugantino M, Sacchettini JC, Jacobs Jr WR. The mabA gene from the inhA operon of Mycobacterium tuberculosis encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance. Microbiology. 1998;144(Pt 10):2697–704.
69. Wade MM, Zhang Y. Mechanisms of drug resistance in Mycobacterium tuberculosis. Front Biosci. 2004;9:975–94.
70. Dalla Costa ER, Ribeiro MO, Silva MS, Arnold LS, Rostirolla DC, Cafrune PI, Espinoza RC, Palaci M, Telles MA, Ritacco V, Suffys PN, Lopes ML, Campelo CL, Miranda SS, Kremer K, da Silva PE, Fonseca Lde S, Ho JL, Kritski AL, Rossetti ML. Correlations of mutations in katG, oxyR-ahpC and inhA genes and in vitro susceptibility in Mycobacterium tuberculosis clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. BMC Microbiol. 2009;9:39.
71. Mdluli K, Slayden RA, Zhu Y, Ramaswamy S, Pan X, Mead D, Crane DD, Musser JM, Barry III CE. Inhibition of a Mycobacterium tuberculosis beta-ketoacyl ACP synthase by isoniazid. Science. 1998;280:1607–10.
72. Lee AS, Teo AS, Wong SY. Novel mutations in ndh in isoniazid-resistant Mycobacterium tuberculosis isolates. Antimicrob Agents Chemother. 2001;45:2157–9.
73. Piatek AS, Telenti A, Murray MR, El-Hajj H, Jacobs Jr WR, Kramer FR, Alland D. Genotypic analysis of Mycobacterium tuberculosis in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrob Agents Chemother. 2000;44:103–10.
74. Slayden RA, Lee RE, Barry III CE. Isoniazid affects multiple components of the type II fatty acid synthase system of Mycobacterium tuberculosis. Mol Microbiol. 2000;38:514–25.
75. Kremer L, Dover LG, Morbidoni HR, Vilcheze C, Maughan WN, Baulard A, Tu SC, Honore N, Deretic V, Sacchettini JC, Locht C, Jacobs Jr WR, Besra GS. Inhibition of InhA activity, but not KasA activity, induces formation of a KasA-containing complex in mycobacteria. J Biol Chem. 2003;278:20547–54.
76. Miesel L, Weisbrod TR, Marcinkeviciene JA, Bittman R, Jacobs Jr WR. NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in Mycobacterium smegmatis. J Bacteriol. 1998;180:2459–67.
77. Wilson TM, Collins DM. ahpC, a gene involved in isoniazid resistance of the Mycobacterium tuberculosis complex. Mol Microbiol. 1996;19:1025–34.
78. Telenti A, Honore N, Bernasconi C, March J, Ortega A, Heym B, Takiff HE, Cole ST. Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at reference laboratory level. J Clin Microbiol. 1997;35:719–23.
79. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry III CE, Stover CK. Compensatory ahpC gene expression in isoniazid-resistant Mycobacterium tuberculosis. Science. 1996;272:1641–3.
80. Kelley CL, Rouse DA, Morris SL. Analysis of ahpC gene mutations in isoniazid-resistant clinical isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1997;41:2057–8.
81. Guimaraes BG, Souchon H, Honore N, Saint-Joanis B, Brosch R, Shepard W, Cole ST, Alzari PM. Structure and mechanism of the alkyl hydroperoxidase AhpC, a key element of the Mycobacterium tuberculosis defense system against oxidative stress. J Biol Chem. 2005;280:25735–42.
82. Colangeli R, Helb D, Sridharan S, Sun J, Varma-Basil M, Hazbon MH, Harbacheuski R, Megjugorac NJ, Jacobs Jr WR, Holzenburg A, Sacchettini JC, Alland D. The Mycobacterium tuberculosis iniA gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. Mol Microbiol. 2005;55:1829–40.
83. Wilson M, DeRisi J, Kristensen HH, Imboden P, Rane S, Brown PO, Schoolnik GK. Exploring drug-induced alterations in gene

expression in Mycobacterium tuberculosis by microarray hybridization. Proc Natl Acad Sci U S A. 1999;96:12833–8.
84. Tessema B, Beer J, Emmrich F, Sack U, Rodloff AC. Analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among Mycobacterium tuberculosis isolates from Ethiopia. BMC Infect Dis. 2012;12:37.
85. Sensi P. History of the development of rifampin. Rev Infect Dis. 1983;5 Suppl 3:S402–6.
86. Lounis N, Roscigno G. In vitro and in vivo activities of new rifamycin derivatives against mycobacterial infections. Curr Pharm Des. 2004;10:3229–38.
87. Donald PR, Sirgel FA, Botha FJ, Seifart HI, Parkin DP, Vandenplas ML, Van de Wal BW, Maritz JS, Mitchison DA. The early bactericidal activity of isoniazid related to its dose size in pulmonary tuberculosis. Am J Respir Crit Care Med. 1997;156:895–900.
88. Sirgel FA, Donald PR, Odhiambo J, Githui W, Umapathy KC, Paramasivan CN, Tam CM, Kam KM, Lam CW, Sole KM, Mitchison DA. A multicentre study of the early bactericidal activity of anti-tuberculosis drugs. J Antimicrob Chemother. 2000;45:859–70.
89. Chan SL, Yew WW, Ma WK, Girling DJ, Aber VR, Felmingham D, Allen BW, Mitchison DA. The early bactericidal activity of rifabutin measured by sputum viable counts in Hong Kong patients with pulmonary tuberculosis. Tuber Lung Dis. 1992;73:33–8.
90. Grosset J, Lounis N, Truffot-Pernot C, O'Brien RJ, Raviglione MC, Ji B. Once-weekly rifapentine-containing regimens for treatment of tuberculosis in mice. Am J Respir Crit Care Med. 1998;157:1436–40.
91. Mitchison DA. Mechanisms of the action of drugs in the short-course chemotherapy. Bull Int Union Tuberc. 1985;60:36–40.
92. Nightingale SD, Cameron DW, Gordin FM, Sullam PM, Cohn DL, Chaisson RE, Eron LJ, Sparti PD, Bihari B, Kaufman DL, et al. Two controlled trials of rifabutin prophylaxis against Mycobacterium avium complex infection in AIDS. N Engl J Med. 1993;329:828–33.
93. Sullam PM, Gordin FM, Wynne BA, The Rifabutin Treatment Group. Efficacy of rifabutin in the treatment of disseminated infection due to Mycobacterium avium complex. Clin Infect Dis. 1994;19:84–6.
94. Dutta NK, Alsultan A, Peloquin CA, Karakousis PC. Preliminary pharmacokinetic study of repeated doses of rifampin and rifapentine in guinea pigs. Antimicrob Agents Chemother. 2013;57:1535–7.
95. Dutta NK, Illei PB, Peloquin CA, Pinn ML, Mdluli KE, Nuermberger EL, Grosset JH, Karakousis PC. Rifapentine is not more active than rifampin against chronic tuberculosis in guinea pigs. Antimicrob Agents Chemother. 2012;56:3726–31.
96. Benator D, Bhattacharya M, Bozeman L, Burman W, Cantazaro A, Chaisson R, Gordin F, Horsburgh CR, Horton J, Khan A, Lahart C, Metchock B, Pachucki C, Stanton L, Vernon A, Villarino ME, Wang YC, Weiner M, Weis S. Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. Lancet. 2002;360:528–34.
97. Vernon A. Rifamycin antibiotics, with a focus on newer agents. In: Rom W, Garay S, editors. Tuberculosis. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 759–71.
98. Hartmann G, Honikel KO, Knusel F, Nuesch J. The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochim Biophys Acta. 1967;145:843–4.
99. Archambault J, Friesen JD. Genetics of eukaryotic RNA polymerases I, II, and III. Microbiol Rev. 1993;57:703–24.
100. Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. Cell. 2001;104:901–12.
101. Sat B, Hazan R, Fisher T, Khaner H, Glaser G, Engelberg-Kulka H. Programmed cell death in Escherichia coli: some antibiotics can trigger mazEF lethality. J Bacteriol. 2001;183:2041–5.
102. Gerdes K, Christensen SK, Lobner-Olesen A. Prokaryotic toxin-antitoxin stress response loci. Nat Rev Microbiol. 2005;3:371–82.
103. Zhu L, Zhang Y, Teh JS, Zhang J, Connell N, Rubin H, Inouye M. Characterization of mRNA interferases from Mycobacterium tuberculosis. J Biol Chem. 2006;281:18638–43.
104. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tuber Lung Dis. 1998;79:3–29.
105. Zhang Y, Telenti A. Genetics of drug resistance in Mycobacterium tuberculosis. In: Hatfull G, Jacobs Jr WR, editors. Molecular genetics of mycobacteria. Washington, DC: ASM Press; 2000. p. 235–54.
106. Jin DJ, Gross CA. Mapping and sequencing of mutations in the Escherichia coli rpoB gene that lead to rifampicin resistance. J Mol Biol. 1988;202:45–58.
107. Lisitsyn NA, Sverdlov ED, Moiseyeva EP, Danilevskaya ON, Nikiforov VG. Mutation to rifampicin resistance at the beginning of the RNA polymerase beta subunit gene in Escherichia coli. Mol Gen Genet. 1984;196:173–4.
108. Ovchinnikov YA, Monastyrskaya GS, Guriev SO, Kalinina NF, Sverdlov ED, Gragerov AI, Bass IA, Kiver IF, Moiseyeva EP, Igumnov VN, Mindlin SZ, Nikiforov VG, Khesin RB. RNA polymerase rifampicin resistance mutations in Escherichia coli: sequence changes and dominance. Mol Gen Genet. 1983;190:344–8.
109. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet. 1993;341:647–50.
110. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin Microbiol Rev. 1995;8:496–514.
111. Kapur V, Li LL, Hamrick MR, Plikaytis BB, Shinnick TM, Telenti A, Jacobs Jr WR, Banerjee A, Cole S, Yuen KY, et al. Rapid Mycobacterium species assignment and unambiguous identification of mutations associated with antimicrobial resistance in Mycobacterium tuberculosis by automated DNA sequencing. Arch Pathol Lab Med. 1995;119:131–8.
112. Kapur V, Li LL, Iordanescu S, Hamrick MR, Wanger A, Kreiswirth BN, Musser JM. Characterization by automated DNA sequencing of mutations in the gene (rpoB) encoding the RNA polymerase beta subunit in rifampin-resistant Mycobacterium tuberculosis strains from New York City and Texas. J Clin Microbiol. 1994;32:1095–8.
113. Williams DL, Waguespack C, Eisenach K, Crawford JT, Portaels F, Salfinger M, Nolan CM, Abe C, Sticht-Groh V, Gillis TP. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob Agents Chemother. 1994;38:2380–6.
114. Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced in vitro in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1999;43:1866–9.
115. Chia BS, Lanzas F, Rifat D, Herrera A, Kim EY, Sailer C, Torres-Chavolla E, Narayanaswamy P, Einarsson V, Bravo J, Pascale JM, Ioerger TR, Sacchettini JC, Karakousis PC. Use of multiplex allele-specific polymerase chain reaction (MAS-PCR) to detect multidrug-resistant tuberculosis in Panama. PLoS One. 2012;7, e40456.
116. Bodmer T, Zurcher G, Imboden P, Telenti A. Mutation position and type of substitution in the beta-subunit of the RNA polymerase influence in-vitro activity of rifamycins in rifampicin-resistant Mycobacterium tuberculosis. J Antimicrob Chemother. 1995;35:345–8.
117. Moghazeh SL, Pan X, Arain T, Stover CK, Musser JM, Kreiswirth BN. Comparative antimycobacterial activities of rifampin,

rifapentine, and KRM-1648 against a collection of rifampin-resistant Mycobacterium tuberculosis isolates with known rpoB mutations. Antimicrob Agents Chemother. 1996;40:2655–7.
118. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PR, Gillis TP. Contribution of rpoB mutations to development of rifamycin cross-resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1998;42:1853–7.
119. Yoshida S, Suzuki K, Iwamoto T, Tsuyuguchi K, Tomita M, Okada M, Sakatani M. Comparison of rifabutin susceptibility and rpoB mutations in multi-drug-resistant Mycobacterium tuberculosis strains by DNA sequencing and the line probe assay. J Infect Chemother. 2010;16:360–3.
120. Heep M, Brandstatter B, Rieger U, Lehn N, Richter E, Rusch-Gerdes S, Niemann S. Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical Mycobacterium tuberculosis isolates. J Clin Microbiol. 2001;39:107–10.
121. Heep M, Odenbreit S, Beck D, Decker J, Prohaska E, Rieger U, Lehn N. Mutations at four distinct regions of the rpoB gene can reduce the susceptibility of Helicobacter pylori to rifamycins. Antimicrob Agents Chemother. 2000;44:1713–5.
122. Yang B, Koga H, Ohno H, Ogawa K, Fukuda M, Hirakata Y, Maesaki S, Tomono K, Tashiro T, Kohno S. Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and rpoB mutations of Mycobacterium tuberculosis. J Antimicrob Chemother. 1998;42:621–8.
123. Cavusoglu C, Karaca-Derici Y, Bilgic A. In-vitro activity of rifabutin against rifampicin-resistant Mycobacterium tuberculosis isolates with known rpoB mutations. Clin Microbiol Infect. 2004;10:662–5.
124. Dabbs ER, Yazawa K, Mikami Y, Miyaji M, Morisaki N, Iwasaki S, Furihata K. Ribosylation by mycobacterial strains as a new mechanism of rifampin inactivation. Antimicrob Agents Chemother. 1995;39:1007–9.
125. Quan S, Venter H, Dabbs ER. Ribosylative inactivation of rifampin by Mycobacterium smegmatis is a principal contributor to its low susceptibility to this antibiotic. Antimicrob Agents Chemother. 1997;41:2456–60.
126. Traore H, Fissette K, Bastian I, Devleeschouwer M, Portaels F. Detection of rifampicin resistance in Mycobacterium tuberculosis isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. Int J Tuberc Lung Dis. 2000;4:481–4.
127. Steele MA, Des Prez RM. The role of pyrazinamide in tuberculosis chemotherapy. Chest. 1988;94:845–50.
128. Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR, Feldman K, Hoffner S, Drobniewski F, Barrera L, van Soolingen D, Boulabhal F, Paramasivan CN, Kam KM, Mitarai S, Nunn P, Raviglione M. Epidemiology of antituberculosis drug resistance 2002–07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Lancet. 2009;373:1861–73.
129. Simons SO, van Ingen J, van der Laan T, Mulder A, Dekhuijzen PN, Boeree MJ, van Soolingen D. Validation of pncA gene sequencing in combination with the mycobacterial growth indicator tube method to test susceptibility of Mycobacterium tuberculosis to pyrazinamide. J Clin Microbiol. 2012;50:428–34.
130. Jindani A, Aber VR, Edwards EA, Mitchison DA. The early bactericidal activity of drugs in patients with pulmonary tuberculosis. Am Rev Respir Dis. 1980;121:939–49.
131. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 1985;66:219–25.
132. Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility testing of Mycobacterium tuberculosis to pyrazinamide. J Med Microbiol. 2002;51:42–9.
133. Yeager RL, Munroe WG, Dessau FI. Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis. Am Rev Tuberc. 1952;65:523–46.
134. Results at 5 years of a controlled comparison of a 6-month and a standard 18-month regimen of chemotherapy for pulmonary tuberculosis. Am Rev Respir Dis. 1977;116:3–8.
135. Controlled clinical trial of four short-course regimens of chemotherapy for two durations in the treatment of pulmonary tuberculosis. Second report. Third East African/British Medical Research Council Study. Tubercle. 1980;61:59–69.
136. Santha T, Nazareth O, Krishnamurthy MS, Balasubramanian R, Vijayan VK, Janardhanam B, Venkataraman P, Tripathy SP, Prabhakar R. Treatment of pulmonary tuberculosis with short course chemotherapy in south India—5-year follow up. Tubercle. 1989;70:229–34.
137. Tarshis MS, Weed Jr WA. Lack of significant in vitro sensitivity of Mycobacterium tuberculosis to pyrazinamide on three different solid media. Am Rev Tuberc. 1953;67:391–5.
138. McDermott W, Tompsett R. Activation of pyrazinamide and nicotinamide in acidic environments in vitro. Am Rev Tuberc. 1954;70:748–54.
139. Kushner S, Dalalian H, Sanjurjo J, Bach Jr F, Safir S, Smith Jr V, Williams J. Experimental chemotherapy of tuberculosis. II. The synthesis of pyrazinamides and related compounds. J Am Chem Soc. 1952;74:3617–21.
140. Salfinger M, Crowle AJ, Reller LB. Pyrazinamide and pyrazinoic acid activity against tubercle bacilli in cultured human macrophages and in the BACTEC system. J Infect Dis. 1990;162:201–7.
141. Crowle AJ, Dahl R, Ross E, May MH. Evidence that vesicles containing living, virulent Mycobacterium tuberculosis or Mycobacterium avium in cultured human macrophages are not acidic. Infect Immun. 1991;59:1823–31.
142. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, Allen RD, Gluck SL, Heuser J, Russell DG. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994;263:678–81.
143. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity in vitro against semidormant Mycobacterium tuberculosis bacterial populations. Am Rev Respir Dis. 1992;145:1223–5.
144. Crowle AJ, Sbarbaro JA, May MH. Inhibition by pyrazinamide of tubercle bacilli within cultured human macrophages. Am Rev Respir Dis. 1986;134:1052–5.
145. Heifets L, Higgins M, Simon B. Pyrazinamide is not active against Mycobacterium tuberculosis residing in cultured human monocyte-derived macrophages. Int J Tuberc Lung Dis. 2000;4:491–5.
146. Controlled clinical trial of 4 short-course regimens of chemotherapy (three 6-month and one 8-month) for pulmonary tuberculosis: final report. East and Central African/British Medical Research Council Fifth Collaborative Study. Tubercle. 1986;67:5–15.
147. Long-term follow-up of a clinical trial of six-month and four-month regimens of chemotherapy in the treatment of pulmonary tuberculosis. Singapore Tuberculosis Service/British Medical Research Council. Am Rev Respir Dis. 1986;133:779–83.
148. Controlled trial of 2, 4, and 6 months of pyrazinamide in 6-month, three-times-weekly regimens for smear-positive pulmonary tuberculosis, including an assessment of a combined preparation of isoniazid, rifampin, and pyrazinamide. Results at 30 months. Hong Kong Chest Service/British Medical Research Council. Am Rev Respir Dis. 1991;143:700–6.
149. Wade MM, Zhang Y. Anaerobic incubation conditions enhance pyrazinamide activity against Mycobacterium tuberculosis. J Med Microbiol. 2004;53:769–73.
150. Raynaud C, Laneelle MA, Senaratne RH, Draper P, Laneelle G, Daffe M. Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity. Microbiology. 1999;145(Pt 6):1359–67.
151. Zhang Y, Scorpio A, Nikaido H, Sun Z. Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of

Mycobacterium tuberculosis to pyrazinamide. J Bacteriol. 1999;181:2044–9.
152. Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis. 1967;95:461–9.
153. Scorpio A, Zhang Y. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med. 1996;2:662–7.
154. Chang KC, Yew WW, Zhang Y. Pyrazinamide susceptibility testing in Mycobacterium tuberculosis: a systematic review with meta-analyses. Antimicrob Agents Chemother. 2011;55:4499–505.
155. Zhang JL, Zheng QC, Li ZQ, Zhang HX. Molecular dynamics simulations suggest ligand's binding to nicotinamidase/pyrazinamidase. PLoS One. 2012;7, e39546.
156. Zimhony O, Cox JS, Welch JT, Vilcheze C, Jacobs Jr WR. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of Mycobacterium tuberculosis. Nat Med. 2000;6:1043–7.
157. Kikuchi S, Rainwater DL, Kolattukudy PE. Purification and characterization of an unusually large fatty acid synthase from Mycobacterium tuberculosis var. bovis BCG. Arch Biochem Biophys. 1992;295:318–26.
158. Zimhony O, Vilcheze C, Jacobs Jr WR. Characterization of Mycobacterium smegmatis expressing the Mycobacterium tuberculosis fatty acid synthase I (fas1) gene. J Bacteriol. 2004;186:4051–5.
159. Boshoff HI, Mizrahi V, Barry III CE. Effects of pyrazinamide on fatty acid synthesis by whole mycobacterial cells and purified fatty acid synthase I. J Bacteriol. 2002;184:2167–72.
160. Zhang Y, Wade MM, Scorpio A, Zhang H, Sun Z. Mode of action of pyrazinamide: disruption of Mycobacterium tuberculosis membrane transport and energetics by pyrazinoic acid. J Antimicrob Chemother. 2003;52:790–5.
161. Zhang Y, Zhang H, Sun Z. Susceptibility of Mycobacterium tuberculosis to weak acids. J Antimicrob Chemother. 2003;52:56–60.
162. Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry III CE, Wang H, Zhang W, Zhang Y. Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. Science. 2011;333:1630–2.
163. Boshoff HI, Mizrahi V. Expression of Mycobacterium smegmatis pyrazinamidase in Mycobacterium tuberculosis confers hypersensitivity to pyrazinamide and related amides. J Bacteriol. 2000;182:5479–85.
164. Cheng SJ, Thibert L, Sanchez T, Heifets L, Zhang Y. pncA mutations as a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis: spread of a monoresistant strain in Quebec, Canada. Antimicrob Agents Chemother. 2000;44:528–32.
165. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in pncA is a major mechanism of pyrazinamide resistance in Mycobacterium tuberculosis. Tuber Lung Dis. 1997;78:117–22.
166. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, Zhang Y. Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1997;41:540–3.
167. Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN, Musser JM. Mutations associated with pyrazinamide resistance in pncA of Mycobacterium tuberculosis complex organisms. Antimicrob Agents Chemother. 1997;41:636–40.
168. Rajendran V, Sethumadhavan R. Drug resistance mechanism of PncA in Mycobacterium tuberculosis. J Biomol Struct Dyn. 2013;32(2):209–21.
169. Cole ST. Microbiology. Pyrazinamide—old TB drug finds new target. Science. 2011;333:1583–4.
170. Zhang Y, Yew WW, Barer MR. Targeting persisters for tuberculosis control. Antimicrob Agents Chemother. 2012;56:2223–30.
171. Heifets L. Susceptibility testing of Mycobacterium tuberculosis to pyrazinamide. J Med Microbiol. 2002;51:11–2.
172. Maddry JA, Suling WJ, Reynolds RC. Glycosyltransferases as targets for inhibition of cell wall synthesis in M. tuberculosis and M. avium. Res Microbiol. 1996;147:106–12.
173. Thomas JP, Baughn CO, Wilkinson RG, Shepherd RG. A new synthetic compound with antituberculous activity in mice: ethambutol (dextro-2,2′-(ethylenediimino)-di-l-butanol). Am Rev Respir Dis. 1961;83:891–3.
174. Falkinham III JO. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev. 1996;9:177–215.
175. Forbes M, Kuck NA, Peets EA. Mode of action of ethambutol. J Bacteriol. 1962;84:1099–103.
176. Gangadharam PR, Pratt PF, Perumal VK, Iseman MD. The effects of exposure time, drug concentration, and temperature on the activity of ethambutol versus Mycobacterium tuberculosis. Am Rev Respir Dis. 1990;141:1478–82.
177. Liss RH. Bactericidal activity of ethambutol against extracellular Mycobacterium tuberculosis and bacilli phagocytized by human alveolar macrophages. S Afr Med J. 1982;Spec No.15–9.
178. Controlled trial of 6-month and 8-month regimens in the treatment of pulmonary tuberculosis: the results up to 24 months. Tubercle. 1979;60:201–10.
179. Five-year follow-up of a controlled trial of five 6-month regimens of chemotherapy for pulmonary tuberculosis. Hong Kong Chest Service/British Medical Research Council. Am Rev Respir Dis. 1987;136:1339–42.
180. Forbes M, Kuck NA, Peets EA. Effect of ethambutol on nucleic acid metabolism in Mycobacterium smegmatis and its reversal by polyamines and divalent cations. J Bacteriol. 1965;89:1299–305.
181. Takayama K, Armstrong EL, Kunugi KA, Kilburn JO. Inhibition by ethambutol of mycolic acid transfer into the cell wall of Mycobacterium smegmatis. Antimicrob Agents Chemother. 1979;16:240–2.
182. Cheema S, Khuller GK. Phospholipid composition and ethambutol sensitivity of Mycobacterium smegmatis ATCC 607. Indian J Exp Biol. 1985;23:511–3.
183. Cheema S, Khuller GK. Metabolism of phospholipids in Mycobacterium smegmatis ATCC 607 in the presence of ethambutol. Indian J Med Res. 1985;82:207–13.
184. Paulin LG, Brander EE, Poso HJ. Specific inhibition of spermidine synthesis in Mycobacteria spp. by the dextro isomer of ethambutol. Antimicrob Agents Chemother. 1985;28:157–9.
185. Takayama K, Kilburn JO. Inhibition of synthesis of arabinogalactan by ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother. 1989;33:1493–9.
186. Mikusova K, Slayden RA, Besra GS, Brennan PJ. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob Agents Chemother. 1995;39:2484–9.
187. Kilburn JO, Greenberg J. Effect of ethambutol on the viable cell count in Mycobacterium smegmatis. Antimicrob Agents Chemother. 1977;11:534–40.
188. Kilburn JO, Takayama K. Effects of ethambutol on accumulation and secretion of trehalose mycolates and free mycolic acid in Mycobacterium smegmatis. Antimicrob Agents Chemother. 1981;20:401–4.
189. Deng L, Mikusova K, Robuck KG, Scherman M, Brennan PJ, McNeil MR. Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. Antimicrob Agents Chemother. 1995;39:694–701.
190. Khoo KH, Douglas E, Azadi P, Inamine JM, Besra GS, Mikusova K, Brennan PJ, Chatterjee D. Truncated structural variants of lipoarabinomannan in ethambutol drug-resistant strains of Mycobacterium smegmatis. Inhibition of arabinan biosynthesis by ethambutol. J Biol Chem. 1996;271:28682–90.
191. Wolucka BA, McNeil MR, de Hoffmann E, Chojnacki T, Brennan PJ. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J Biol Chem. 1994;269:23328–35.

192. Belanger AE, Besra GS, Ford ME, Mikusova K, Belisle JT, Brennan PJ, Inamine JM. The embAB genes of Mycobacterium avium encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci U S A. 1996;93:11919–24.
193. Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, Musser JM, Jacobs Jr WR. The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. Nat Med. 1997;3:567–70.
194. Lety MA, Nair S, Berche P, Escuyer V. A single point mutation in the embB gene is responsible for resistance to ethambutol in Mycobacterium smegmatis. Antimicrob Agents Chemother. 1997;41:2629–33.
195. Ramaswamy SV, Amin AG, Goksel S, Stager CE, Dou SJ, El Sahly H, Moghazeh SL, Kreiswirth BN, Musser JM. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2000;44:326–36.
196. Escuyer VE, Lety MA, Torrelles JB, Khoo KH, Tang JB, Rithner CD, Frehel C, McNeil MR, Brennan PJ, Chatterjee D. The role of the embA and embB gene products in the biosynthesis of the terminal hexaarabinofuranosyl motif of Mycobacterium smegmatis arabinogalactan. J Biol Chem. 2001;276:48854–62.
197. Zhang N, Torrelles JB, McNeil MR, Escuyer VE, Khoo KH, Brennan PJ, Chatterjee D. The Emb proteins of mycobacteria direct arabinosylation of lipoarabinomannan and arabinogalactan via an N-terminal recognition region and a C-terminal synthetic region. Mol Microbiol. 2003;50:69–76.
198. Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs Jr WR, Telenti A, Musser JM. Ethambutol resistance in Mycobacterium tuberculosis: critical role of embB mutations. Antimicrob Agents Chemother. 1997;41:1677–81.
199. Kiet VS, Lan NT, An DD, Dung NH, Hoa DV, van Vinh Chau N, Chinh NT, Farrar J, Caws M. Evaluation of the MTBDRsl test for detection of second-line-drug resistance in Mycobacterium tuberculosis. J Clin Microbiol. 2010;48:2934–9.
200. Zhang Y, Yew WW. Mechanisms of drug resistance in Mycobacterium tuberculosis. Int J Tuberc Lung Dis. 2009;13:1320–30.
201. Alcaide F, Pfyffer GE, Telenti A. Role of embB in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob Agents Chemother. 1997;41:2270–3.
202. Mokrousov I, Otten T, Vyshnevskiy B, Narvskaya O. Detection of embB306 mutations in ethambutol-susceptible clinical isolates of Mycobacterium tuberculosis from Northwestern Russia: implications for genotypic resistance testing. J Clin Microbiol. 2002;40:3810–3.
203. Huang WL, Chi TL, Wu MH, Jou R. Performance assessment of the GenoType MTBDRsl test and DNA sequencing for detection of second-line and ethambutol drug resistance among patients infected with multidrug-resistant Mycobacterium tuberculosis. J Clin Microbiol. 2011;49:2502–8.
204. Lacoma A, Garcia-Sierra N, Prat C, Maldonado J, Ruiz-Manzano J, Haba L, Gavin P, Samper S, Ausina V, Dominguez J. GenoType MTBDRsl for molecular detection of second-line-drug and ethambutol resistance in Mycobacterium tuberculosis strains and clinical samples. J Clin Microbiol. 2012;50:30–6.
205. Alland D, Kramnik I, Weisbrod TR, Otsubo L, Cerny R, Miller LP, Jacobs Jr WR, Bloom BR. Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 1998;95:13227–32.
206. Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W. Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant Mycobacterium tuberculosis complex isolates. J Clin Microbiol. 2010;48:1683–9.
207. Schatz A, Bugie E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc Soc Exptl Biol Med. 1944;55:66–9.
208. Streptomycin treatment of pulmonary tuberculosis. BMJ. 1948;2:769–82.
209. Iseman MD. Tuberculosis chemotherapy, including directly observed therapy. In: A clinician's guide to tuberculosis. Philadelphia: Lippincott Williams & Wilkins; 2000. p. 271–321.
210. Chan E, Chatterjee D, Iseman M, Heifets L. Pyrazinamide, ethambutol, ethionamide, and aminoglycosides. In: Rom W, Garay S, editors. Tuberculosis. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2004. p. 773–89.
211. Winder FG. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of mycobacteria. In: Ratledge C, Stanford J, editors. The biology of the mycobacteria, vol. 1. New York: Academic; 1982. p. 354–438.
212. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry III CE, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998;393:537–44.
213. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. Massive gene decay in the leprosy bacillus. Nature. 2001;409:1007–11.
214. Ainsa JA, Perez E, Pelicic V, Berthet FX, Gicquel B, Martin C. Aminoglycoside 2′-N-acetyltransferase genes are universally present in mycobacteria: characterization of the aac(2′)-Ic gene from Mycobacterium tuberculosis and the aac(2′)-Id gene from Mycobacterium smegmatis. Mol Microbiol. 1997;24:431–41.
215. Allen BW, Mitchison DA, Chan YC, Yew WW, Allan WG, Girling DJ. Amikacin in the treatment of pulmonary tuberculosis. Tubercle. 1983;64:111–8.
216. Tsukamura M, Mizuno S. Cross-resistant relationships among the aminoglucoside antibiotics in Mycobacterium tuberculosis. J Gen Microbiol. 1975;88:269–74.
217. Prammananan T, Sander P, Brown BA, Frischkorn K, Onyi GO, Zhang Y, Bottger EC, Wallace Jr RJ. A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in Mycobacterium abscessus and Mycobacterium chelonae. J Infect Dis. 1998;177:1573–81.
218. Alangaden GJ, Kreiswirth BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL, Manavathu EK, Lerner SA. Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1998;42:1295–7.
219. Cooksey RC, Morlock GP, McQueen A, Glickman SE, Crawford JT. Characterization of streptomycin resistance mechanisms among Mycobacterium tuberculosis isolates from patients in New York City. Antimicrob Agents Chemother. 1996;40:1186–8.
220. Finken M, Kirschner P, Meier A, Wrede A, Bottger EC. Molecular basis of streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations

within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol. 1993;9:1239–46.
221. Honore N, Cole ST. Streptomycin resistance in mycobacteria. Antimicrob Agents Chemother. 1994;38:238–42.
222. Kenney TJ, Churchward G. Cloning and sequence analysis of the rpsL and rpsG genes of Mycobacterium smegmatis and characterization of mutations causing resistance to streptomycin. J Bacteriol. 1994;176:6153–6.
223. Nair J, Rouse DA, Bai GH, Morris SL. The rpsL gene and streptomycin resistance in single and multiple drug-resistant strains of Mycobacterium tuberculosis. Mol Microbiol. 1993;10:521–7.
224. Sreevatsan S, Pan X, Stockbauer KE, Williams DL, Kreiswirth BN, Musser JM. Characterization of rpsL and rrs mutations in streptomycin-resistant Mycobacterium tuberculosis isolates from diverse geographic localities. Antimicrob Agents Chemother. 1996;40:1024–6.
225. Douglass J, Steyn LM. A ribosomal gene mutation in streptomycin-resistant Mycobacterium tuberculosis isolates. J Infect Dis. 1993;167:1505–6.
226. Reeves AZ, Campbell PJ, Sultana R, Malik S, Murray M, Plikaytis BB, Shinnick TM, Posey JE. Aminoglycoside cross-resistance in Mycobacterium tuberculosis due to mutations in the 5′ untranslated region of whiB7. Antimicrob Agents Chemother. 2013;57:1857–65.
227. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol Microbiol. 2007;63:1096–106.
228. Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations related to streptomycin resistance in clinical isolates of Mycobacterium tuberculosis and possible involvement of efflux mechanism. Antimicrob Agents Chemother. 2008;52:2947–9.
229. Via LE, Cho SN, Hwang S, Bang H, Park SK, Kang HS, Jeon D, Min SY, Oh T, Kim Y, Kim YM, Rajan V, Wong SY, Shamputa IC, Carroll M, Goldfeder L, Lee SA, Holland SM, Eum S, Lee H, Barry III CE. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in Mycobacterium tuberculosis isolates from South Korean Patients with drug-resistant tuberculosis. J Clin Microbiol. 2010;48:402–11.
230. Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HI, Barry III CE. Mutations in gidB confer low-level streptomycin resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2011;55:2515–22.
231. Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with Mycobacterium tuberculosis resistance to amikacin, kanamycin and capreomycin: a systematic review. PLoS One. 2012;7, e33275.
232. Johansen SK, Maus CE, Plikaytis BB, Douthwaite S. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2′-O-methylations in 16S and 23S rRNAs. Mol Cell. 2006;23:173–82.
233. Caminero JA, Sotgiu G, Zumla A, Migliori GB. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. Lancet Infect Dis. 2010;10:621–9.
234. Jacobs MR. Fluoroquinolones as chemotherapeutics against mycobacterial infections. Curr Pharm Des. 2004;10:3213–20.
235. Garcia-Rodriguez JA, Gomez Garcia AC. In-vitro activities of quinolones against mycobacteria. J Antimicrob Chemother. 1993;32:797–808.
236. Jacobs MR. Activity of quinolones against mycobacteria. Drugs. 1999;58 Suppl 2:19–22.
237. Klemens SP, Sharpe CA, Rogge MC, Cynamon MH. Activity of levofloxacin in a murine model of tuberculosis. Antimicrob Agents Chemother. 1994;38:1476–9.
238. Lounis N, Ji B, Truffot-Pernot C, Grosset J. Which aminoglycoside or fluoroquinolone is more active against Mycobacterium tuberculosis in mice? Antimicrob Agents Chemother. 1997;41:607–10.
239. Yoshimatsu T, Nuermberger E, Tyagi S, Chaisson R, Bishai W, Grosset J. Bactericidal activity of increasing daily and weekly doses of moxifloxacin in murine tuberculosis. Antimicrob Agents Chemother. 2002;46:1875–9.
240. Ginsburg AS, Grosset JH, Bishai WR. Fluoroquinolones, tuberculosis, and resistance. Lancet Infect Dis. 2003;3:432–42.
241. Ji B, Lounis N, Maslo C, Truffot-Pernot C, Bonnafous P, Grosset J. In vitro and in vivo activities of moxifloxacin and clinafloxacin against Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1998;42:2066–9.
242. Miyazaki E, Miyazaki M, Chen JM, Chaisson RE, Bishai WR. Moxifloxacin (BAY12-8039), a new 8-methoxyquinolone, is active in a mouse model of tuberculosis. Antimicrob Agents Chemother. 1999;43:85–9.
243. Gosling RD, Uiso LO, Sam NE, Bongard E, Kanduma EG, Nyindo M, Morris RW, Gillespie SH. The bactericidal activity of moxifloxacin in patients with pulmonary tuberculosis. Am J Respir Crit Care Med. 2003;168:1342–5.
244. Johnson JL, Hadad DJ, Boom WH, Daley CL, Peloquin CA, Eisenach KD, Jankus DD, Debanne SM, Charlebois ED, Maciel E, Palaci M, Dietze R. Early and extended early bactericidal activity of levofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. Int J Tuberc Lung Dis. 2006;10:605–12.
245. Pletz MW, De Roux A, Roth A, Neumann KH, Mauch H, Lode H. Early bactericidal activity of moxifloxacin in treatment of pulmonary tuberculosis: a prospective, randomized study. Antimicrob Agents Chemother. 2004;48:780–2.
246. Paramasivan CN, Sulochana S, Kubendiran G, Venkatesan P, Mitchison DA. Bactericidal action of gatifloxacin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2005;49:627–31.
247. Nuermberger EL, Yoshimatsu T, Tyagi S, O'Brien RJ, Vernon AN, Chaisson RE, Bishai WR, Grosset JH. Moxifloxacin-containing regimen greatly reduces time to culture conversion in murine tuberculosis. Am J Respir Crit Care Med. 2004;169:421–6.
248. Nuermberger EL, Yoshimatsu T, Tyagi S, Williams K, Rosenthal I, O'Brien RJ, Vernon AA, Chaisson RE, Bishai WR, Grosset JH. Moxifloxacin-containing regimens of reduced duration produce a stable cure in murine tuberculosis. Am J Respir Crit Care Med. 2004;170:1131–4.
249. Ginsburg AS, Hooper N, Parrish N, Dooley KE, Dorman SE, Booth J, Diener-West M, Merz WG, Bishai WR, Sterling TR. Fluoroquinolone resistance in patients with newly diagnosed tuberculosis. Clin Infect Dis. 2003;37:1448–52.
250. Ginsburg AS, Woolwine SC, Hooper N, Benjamin Jr WH, Bishai WR, Dorman SE, Sterling TR. The rapid development of fluoroquinolone resistance in M. tuberculosis. N Engl J Med. 2003;349:1977–8.
251. Bozeman L, Burman W, Metchock B, Welch L, Weiner M. Fluoroquinolone susceptibility among Mycobacterium tuberculosis isolates from the United States and Canada. Clin Infect Dis. 2005;40:386–91.
252. Drlica K, Malik M. Fluoroquinolones: action and resistance. Curr Top Med Chem. 2003;3:249–82.
253. Alangaden GJ, Manavathu EK, Vakulenko SB, Zvonok NM, Lerner SA. Characterization of fluoroquinolone-resistant mutant strains of Mycobacterium tuberculosis selected in the laboratory and isolated from patients. Antimicrob Agents Chemother. 1995;39:1700–3.
254. Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, Cole ST, Jacobs Jr WR, Telenti A. Cloning and

nucleotide sequence of Mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother. 1994;38:773–80.
255. Xu C, Kreiswirth BN, Sreevatsan S, Musser JM, Drlica K. Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J Infect Dis. 1996;174:1127–30.
256. Cambau E, Sougakoff W, Besson M, Truffot-Pernot C, Grosset J, Jarlier V. Selection of a gyrA mutant of Mycobacterium tuberculosis resistant to fluoroquinolones during treatment with ofloxacin. J Infect Dis. 1994;170:479–83.
257. Kocagoz T, Hackbarth CJ, Unsal I, Rosenberg EY, Nikaido H, Chambers HF. Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of Mycobacterium tuberculosis H37Ra. Antimicrob Agents Chemother. 1996;40:1768–74.
258. Zhou J, Dong Y, Zhao X, Lee S, Amin A, Ramaswamy S, Domagala J, Musser JM, Drlica K. Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations. J Infect Dis. 2000;182:517–25.
259. Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S, Ramasoota P. Mutations in the gyrA and gyrB genes of fluoroquinolone-resistant Mycobacterium tuberculosis from TB patients in Thailand. Southeast Asian J Trop Med Public Health. 2005;36 Suppl 4:228–37.
260. Pantel A, Petrella S, Veziris N, Brossier F, Bastian S, Jarlier V, Mayer C, Aubry A. Extending the definition of the GyrB quinolone resistance-determining region in Mycobacterium tuberculosis DNA gyrase for assessing fluoroquinolone resistance in M. tuberculosis. Antimicrob Agents Chemother. 2012;56:1990–6.
261. Malik S, Willby M, Sikes D, Tsodikov OV, Posey JE. New insights into fluoroquinolone resistance in Mycobacterium tuberculosis: functional genetic analysis of gyrA and gyrB mutations. PLoS One. 2012;7, e39754.
262. Liu J, Takiff HE, Nikaido H. Active efflux of fluoroquinolones in Mycobacterium smegmatis mediated by LfrA, a multidrug efflux pump. J Bacteriol. 1996;178:3791–5.
263. Takiff HE, Cimino M, Musso MC, Weisbrod T, Martinez R, Delgado MB, Salazar L, Bloom BR, Jacobs Jr WR. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in Mycobacterium smegmatis. Proc Natl Acad Sci U S A. 1996;93:362–6.
264. Bateman A, Murzin AG, Teichmann SA. Structure and distribution of pentapeptide repeats in bacteria. Protein Sci. 1998;7:1477–80.
265. Montero C, Mateu G, Rodriguez R, Takiff H. Intrinsic resistance of Mycobacterium smegmatis to fluoroquinolones may be influenced by new pentapeptide protein MfpA. Antimicrob Agents Chemother. 2001;45:3387–92.
266. Hegde SS, Vetting MW, Roderick SL, Mitchenall LA, Maxwell A, Takiff HE, Blanchard JS. A fluoroquinolone resistance protein from Mycobacterium tuberculosis that mimics DNA. Science. 2005;308:1480–3.
267. Bhatt K, Banerjee SK, Chakraborti PK. Evidence that phosphate specific transporter is amplified in a fluoroquinolone resistant Mycobacterium smegmatis. Eur J Biochem. 2000;267:4028–32.
268. Dautzenberg B, Truffot C, Legris S, Meyohas MC, Berlie HC, Mercat A, Chevret S, Grosset J. Activity of clarithromycin against Mycobacterium avium infection in patients with the acquired immune deficiency syndrome. A controlled clinical trial. Am Rev Respir Dis. 1991;144:564–9.
269. Chaisson RE, Benson CA, Dube MP, Heifets LB, Korvick JA, Elkin S, Smith T, Craft JC, Sattler FR. Clarithromycin therapy for bacteremic Mycobacterium avium complex disease. A randomized, double-blind, dose-ranging study in patients with AIDS. AIDS Clinical Trials Group Protocol 157 Study Team. Ann Intern Med. 1994;121:905–11.
270. Young LS, Wiviott L, Wu M, Kolonoski P, Bolan R, Inderlied CB. Azithromycin for treatment of Mycobacterium avium-intracellulare complex infection in patients with AIDS. Lancet. 1991;338:1107–9.
271. Ward TT, Rimland D, Kauffman C, Huycke M, Evans TG, Heifets L. Randomized, open-label trial of azithromycin plus ethambutol vs. clarithromycin plus ethambutol as therapy for Mycobacterium avium complex bacteremia in patients with human immunodeficiency virus infection. Veterans Affairs HIV Research Consortium. Clin Infect Dis. 1998;27:1278–85.
272. Dunne M, Fessel J, Kumar P, Dickenson G, Keiser P, Boulos M, Mogyros M, White Jr AC, Cahn P, O'Connor M, Lewi D, Green S, Tilles J, Hicks C, Bissett J, Schneider MM, Benner R. A randomized, double-blind trial comparing azithromycin and clarithromycin in the treatment of disseminated Mycobacterium avium infection in patients with human immunodeficiency virus. Clin Infect Dis. 2000;31:1245–52.
273. Chaisson RE, Keiser P, Pierce M, Fessel WJ, Ruskin J, Lahart C, Benson CA, Meek K, Siepman N, Craft JC. Clarithromycin and ethambutol with or without clofazimine for the treatment of bacteremic Mycobacterium avium complex disease in patients with HIV infection. Aids. 1997;11:311–7.
274. Piscitelli SC, Danziger LH, Rodvold KA. Clarithromycin and azithromycin: new macrolide antibiotics. Clin Pharm. 1992;11:137–52.
275. Bermudez LE, Yamazaki Y. Effects of macrolides and ketolides on mycobacterial infections. Curr Pharm Des. 2004;10:3221–8.
276. Buriankova K, Doucet-Populaire F, Dorson O, Gondran A, Ghnassia JC, Weiser J, Pernodet JL. Molecular basis of intrinsic macrolide resistance in the Mycobacterium tuberculosis complex. Antimicrob Agents Chemother. 2004;48:143–50.
277. Madsen CT, Jakobsen L, Buriankova K, Doucet-Populaire F, Pernodet JL, Douthwaite S. Methyltransferase Erm(37) slips on rRNA to confer atypical resistance in Mycobacterium tuberculosis. J Biol Chem. 2005;280:38942–7.
278. Andini N, Nash KA. Intrinsic macrolide resistance of the Mycobacterium tuberculosis complex is inducible. Antimicrob Agents Chemother. 2006;50:2560–2.
279. Philalay JS, Palermo CO, Hauge KA, Rustad TR, Cangelosi GA. Genes required for intrinsic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother. 2004;48:3412–8.
280. Ji B, Lounis N, Truffot-Pernot C, Grosset J. Selection of resistant mutants of Mycobacterium avium in beige mice by clarithromycin monotherapy. Antimicrob Agents Chemother. 1992;36:2839–40.
281. Doucet-Populaire F, Truffot-Pernot C, Grosset J, Jarlier V. Acquired resistance in Mycobacterium avium complex strains isolated from AIDS patients and beige mice during treatment with clarithromycin. J Antimicrob Chemother. 1995;36:129–36.
282. Meier A, Kirschner P, Springer B, Steingrube VA, Brown BA, Wallace Jr RJ, Bottger EC. Identification of mutations in 23S rRNA gene of clarithromycin-resistant Mycobacterium intracellulare. Antimicrob Agents Chemother. 1994;38:381–4.
283. Skinner R, Cundliffe E, Schmidt FJ. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. J Biol Chem. 1983;258:12702–6.
284. Nash KA, Inderlied CB. Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with disseminated disease. Antimicrob Agents Chemother. 1995;39:2625–30.
285. Thiermann S, Munzinger J, Bodmer T. Comparison of phenotypic and genotypic methods for the detection of clarithromycin resistance in Mycobacterium avium. J Antimicrob Chemother. 2002;49:679–81.
286. Wallace Jr RJ, Meier A, Brown BA, Zhang Y, Sander P, Onyi GO, Bottger EC. Genetic basis for clarithromycin resistance among isolates of Mycobacterium chelonae and Mycobacterium abscessus. Antimicrob Agents Chemother. 1996;40:1676–81.

287. Inagaki T, Yagi T, Ichikawa K, Nakagawa T, Moriyama M, Uchiya K, Nikai T, Ogawa K. Evaluation of a rapid detection method of clarithromycin resistance genes in Mycobacterium avium complex isolates. J Antimicrob Chemother. 2011;66:722–9.
288. Jamal MA, Maeda S, Nakata N, Kai M, Fukuchi K, Kashiwabara Y. Molecular basis of clarithromycin-resistance in Mycobacterium avium intracellulare complex. Tuber Lung Dis. 2000;80:1–4.
289. Morlock GP, Metchock B, Sikes D, Crawford JT, Cooksey RC. ethA, inhA, and katG loci of ethionamide-resistant clinical Mycobacterium tuberculosis isolates. Antimicrob Agents Chemother. 2003;47:3799–805.
290. DeBarber AE, Mdluli K, Bosman M, Bekker LG, Barry III CE. Ethionamide activation and sensitivity in multidrug-resistant Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 2000;97:9677–82.
291. Saribas Z, Kocagoz T, Alp A, Gunalp A. Rapid detection of rifampin resistance in Mycobacterium tuberculosis isolates by heteroduplex analysis and determination of rifamycin cross-resistance in rifampin-resistant isolates. J Clin Microbiol. 2003;41:816–8.
292. Uzun M, Erturan Z, Ang O. Investigation of cross-resistance between rifampin and rifabutin in Mycobacterium tuberculosis complex strains. Int J Tuberc Lung Dis. 2002;6:164–5.
293. Senol G, Erbaycu A, Ozsoz A. Incidence of cross resistance between rifampicin and rifabutin in Mycobacterium tuberculosis strains in Izmir, Turkey. J Chemother. 2005;17:380–4.
294. Yamada T, Masuda K, Mizuguchi Y, Suga K. Altered ribosomes in antibiotic-resistant mutants of Mycobacterium smegmatis. Antimicrob Agents Chemother. 1976;9:817–23.
295. Yamada T, Mizugichi Y, Nierhaus KH, Wittmann HG. Resistance to viomycin conferred by RNA of either ribosomal subunit. Nature. 1978;275:460–1.
296. Ng VH, Cox JS, Sousa AO, MacMicking JD, McKinney JD. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Mol Microbiol. 2004;52:1291–302.
297. Cohen T, Becerra MC, Murray MB. Isoniazid resistance and the future of drug-resistant tuberculosis. Microb Drug Resist. 2004;10:280–5.
298. Agerton TB, Valway SE, Blinkhorn RJ, Shilkret KL, Reves R, Schluter WW, Gore B, Pozsik CJ, Plikaytis BB, Woodley C, Onorato IM. Spread of strain W, a highly drug-resistant strain of Mycobacterium tuberculosis, across the United States. Clin Infect Dis. 1999;29:85–92. discussion 93-5.
299. Mukherjee JS, Rich ML, Socci AR, Joseph JK, Viru FA, Shin SS, Furin JJ, Becerra MC, Barry DJ, Kim JY, Bayona J, Farmer P, Smith Fawzi MC, Seung KJ. Programmes and principles in treatment of multidrug-resistant tuberculosis. Lancet. 2004;363:474–81.
300. Sharma SK, Mohan A. Multidrug-resistant tuberculosis: a menace that threatens to destabilize tuberculosis control. Chest. 2006;130:261–72.
301. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant Mycobacterium tuberculosis. Int J Tuberc Lung Dis. 2009;13:1456–66.
302. Dutta NK, Karakousis PC. Tuberculosis chemotherapy: present situation, possible solutions, and progress towards a TB-free world. Indian J Med Microbiol. 2012;30:261–3.
303. Grosset JH, Singer TG, Bishai WR. New drugs for the treatment of tuberculosis: hope and reality. Int J Tuberc Lung Dis. 2012;16:1005–14.
304. Diacon AH, Dawson R, Hanekom M, Narunsky K, Venter A, Hittel N, Geiter LJ, Wells CD, Paccaly AJ, Donald PR. Early bactericidal activity of delamanid (OPC-67683) in smear-positive pulmonary tuberculosis patients. Int J Tuberc Lung Dis. 2011;15:949–54.
305. Diacon AH, Dawson R, von Groote-Bidlingmaier F, Symons G, Venter A, Donald PR, van Niekerk C, Everitt D, Winter H, Becker P, Mendel CM, Spigelman MK. 14-day bactericidal activity of PA-824, bedaquiline, pyrazinamide, and moxifloxacin combinations: a randomised trial. Lancet. 2012;380:986–93.
306. Diacon AH, Donald PR, Pym A, Grobusch M, Patientia RF, Mahanyele R, Bantubani N, Narasimooloo R, De Marez T, van Heeswijk R, Lounis N, Meyvisch P, Andries K, McNeeley DF. Randomized pilot trial of eight weeks of bedaquiline (TMC207) treatment for multidrug-resistant tuberculosis: long-term outcome, tolerability, and effect on emergence of drug resistance. Antimicrob Agents Chemother. 2012;56:3271–6.
307. Sq109. Tuberculosis (Edinb). 2008;88:159–61.
308. Cattaneo D, Orlando G, Cordier L. Linezolid for extensively drug-resistant tuberculosis. N Engl J Med. 2013;368:291.
309. Williams K, Minkowski A, Amoabeng O, Peloquin CA, Taylor D, Andries K, Wallis RS, Mdluli KE, Nuermberger EL. Sterilizing activities of novel combinations lacking first- and second-line drugs in a murine model of tuberculosis. Antimicrob Agents Chemother. 2012;56:3114–20.
310. Makarov V, Manina G, Mikusova K, Mollmann U, Ryabova O, Saint-Joanis B, Dhar N, Pasca MR, Buroni S, Lucarelli AP, Milano A, De Rossi E, Belanova M, Bobovska A, Dianiskova P, Kordulakova J, Sala C, Fullam E, Schneider P, McKinney JD, Brodin P, Christophe T, Waddell S, Butcher P, Albrethsen J, Rosenkrands I, Brosch R, Nandi V, Bharath S, Gaonkar S, Shandil RK, Balasubramanian V, Balganesh T, Tyagi S, Grosset J, Riccardi G, Cole ST. Benzothiazinones kill Mycobacterium tuberculosis by blocking arabinan synthesis. Science. 2009;324:801–4.
311. Amaral L, Udwadia Z, Abbate E, van Soolingen D. The added effect of thioridazine in the treatment of drug-resistant tuberculosis. Int J Tuberc Lung Dis. 2012;16:1706–8. author reply 1708-9.
312. Dey T, Brigden G, Cox H, Shubber Z, Cooke G, Ford N. Outcomes of clofazimine for the treatment of drug-resistant tuberculosis: a systematic review and meta-analysis. J Antimicrob Chemother. 2013;68:284–93.
313. Dutta NK, Pinn ML, Zhao M, Rudek MA, Karakousis PC. Thioridazine lacks bactericidal activity in an animal model of extracellular tuberculosis. J Antimicrob Chemother. 2013;68(6):1327–30.
314. Dutta NK, Alsultan A, Gniadek TJ, Belchis DA, Pinn ML, Mdluli KE, Nuermberger EL, Peloquin CA, Karakousis PC. Potent rifamycin-sparing regimen cures guinea pig tuberculosis as rapidly as the standard regimen. Antimicrob Agents Chemother. 2013;57:3910–6.
315. Rist N, Grumbach F, Libermann D. Experiments on the antituberculous activity of alpha-ethylthioisonicotinamide. Am Rev Tuberc. 1959;79:1–5.
316. Quemard A, Laneelle G, Lacave C. Mycolic acid synthesis: a target for ethionamide in mycobacteria? Antimicrob Agents Chemother. 1992;36:1316–21.
317. Baulard AR, Betts JC, Engohang-Ndong J, Quan S, McAdam RA, Brennan PJ, Locht C, Besra GS. Activation of the pro-drug ethionamide is regulated in mycobacteria. J Biol Chem. 2000;275:28326–31.
318. Vannelli TA, Dykman A, Ortiz de Montellano PR. The antituberculosis drug ethionamide is activated by a flavoprotein monooxygenase. J Biol Chem. 2002;277:12824–9.
319. Wang F, Langley R, Gulten G, Dover LG, Besra GS, Jacobs Jr WR, Sacchettini JC. Mechanism of thioamide drug action against tuberculosis and leprosy. J Exp Med. 2007;204:73–8.
320. Vilcheze C, Av-Gay Y, Attarian R, Liu Z, Hazbon MH, Colangeli R, Chen B, Liu W, Alland D, Sacchettini JC, Jacobs Jr WR. Mycothiol biosynthesis is essential for ethionamide susceptibility in Mycobacterium tuberculosis. Mol Microbiol. 2008;69:1316–29.

321. Akbergenov R, Shcherbakov D, Matt T, Duscha S, Meyer M, Wilson DN, Bottger EC. Molecular basis for the selectivity of antituberculosis compounds capreomycin and viomycin. Antimicrob Agents Chemother. 2011;55:4712–7.
322. Taniguchi H, Chang B, Abe C, Nikaido Y, Mizuguchi Y, Yoshida SI. Molecular analysis of kanamycin and viomycin resistance in Mycobacterium smegmatis by use of the conjugation system. J Bacteriol. 1997;179:4795–801.
323. Engstrom A, Perskvist N, Werngren J, Hoffner SE, Jureen P. Comparison of clinical isolates and in vitro selected mutants reveals that tlyA is not a sensitive genetic marker for capreomycin resistance in Mycobacterium tuberculosis. J Antimicrob Chemother. 2011;66:1247–54.
324. Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F, Rigouts L. High level of cross-resistance between kanamycin, amikacin, and capreomycin among Mycobacterium tuberculosis isolates from Georgia and a close relation with mutations in the rrs gene. Antimicrob Agents Chemother. 2009;53:5064–8.
325. Shcherbakov D, Akbergenov R, Matt T, Sander P, Andersson DI, Bottger EC. Directed mutagenesis of Mycobacterium smegmatis 16S rRNA to reconstruct the in-vivo evolution of aminoglycoside resistance in Mycobacterium tuberculosis. Mol Microbiol. 2010;77(4):830–40.
326. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Med. 2009;6, e2.
327. Caceres NE, Harris NB, Wellehan JF, Feng Z, Kapur V, Barletta RG. Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in Mycobacterium smegmatis. J Bacteriol. 1997;179:5046–55.
328. Feng Z, Barletta RG. Roles of Mycobacterium smegmatis D-alanine: D-alanine ligase and D-alanine racemase in the mechanisms of action of and resistance to the peptidoglycan inhibitor D-cycloserine. Antimicrob Agents Chemother. 2003;47:283–91.
329. Chacon O, Feng Z, Harris NB, Caceres NE, Adams LG, Barletta RG. Mycobacterium smegmatis D-alanine racemase mutants are not dependent on D-alanine for growth. Antimicrob Agents Chemother. 2002;46:47–54.
330. Russell RR. Mapping of a D-cycloserine resistance locus in escherichia coli K-12. J Bacteriol. 1972;111:622–4.
331. Peteroy M, Severin A, Zhao F, Rosner D, Lopatin U, Scherman H, Belanger A, Harvey B, Hatfull GF, Brennan PJ, Connell ND. Characterization of a Mycobacterium smegmatis mutant that is simultaneously resistant to D-cycloserine and vancomycin. Antimicrob Agents Chemother. 2000;44:1701–4.
332. Lehmann J. para-Aminosalicylic acid in the treatment of tuberculosis. Lancet. 1946;i:15–6.
333. Lehmann J. Twenty years afterward historical notes on the discovery of the antituberculosis effect of para-aminosalicylic acid (Pas) and the first clinical trials. Am Rev Respir Dis. 1964;90:953–6.
334. TREATMENT of pulmonary tuberculosis with streptomycin and para-aminosalicylic acid; a Medical Research Council investigation. Br Med J. 1950;2:1073–85.
335. Rengarajan J, Sassetti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol Microbiol. 2004;53:275–82.
336. Leung KL, Yip CW, Yeung YL, Wong KL, Chan WY, Chan MY, Kam KM. Usefulness of resistant gene markers for predicting treatment outcome on second-line anti-tuberculosis drugs. J Appl Microbiol. 2010;109:2087–94.
337. Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, Anderson SW, Towell JA, Yuan Y, McMurray DN, Kreiswirth BN, Barry CE, Baker WR. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature. 2000;405:962–6.
338. Lenaerts AJ, Gruppo V, Marietta KS, Johnson CM, Driscoll DK, Tompkins NM, Rose JD, Reynolds RC, Orme IM. Preclinical testing of the nitroimidazopyran PA-824 for activity against Mycobacterium tuberculosis in a series of in vitro and in vivo models. Antimicrob Agents Chemother. 2005;49:2294–301.
339. Tyagi S, Nuermberger E, Yoshimatsu T, Williams K, Rosenthal I, Lounis N, Bishai W, Grosset J. Bactericidal activity of the nitroimidazopyran PA-824 in a murine model of tuberculosis. Antimicrob Agents Chemother. 2005;49:2289–93.
340. Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE, Daniels L, Dick T, Pang SS, Barry III CE. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 2006;103:431–6.
341. Singh R, Manjunatha U, Boshoff HI, Ha YH, Niyomrattanakit P, Ledwidge R, Dowd CS, Lee IY, Kim P, Zhang L, Kang S, Keller TH, Jiricek J, Barry III CE. PA-824 kills nonreplicating Mycobacterium tuberculosis by intracellular NO release. Science. 2008;322:1392–5.
342. Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, Shimokawa Y, Komatsu M. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. PLoS Med. 2006;3, e466.
343. Petrella S, Cambau E, Chauffour A, Andries K, Jarlier V, Sougakoff W. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. Antimicrob Agents Chemother. 2006;50:2853–6.
344. Huitric E, Verhasselt P, Koul A, Andries K, Hoffner S, Andersson DI. Rates and mechanisms of resistance development in Mycobacterium tuberculosis to a novel diarylquinoline ATP synthase inhibitor. Antimicrob Agents Chemother. 2010;54:1022–8.
345. Jia L, Coward L, Gorman GS, Noker PE, Tomaszewski JE. Pharmacoproteomic effects of isoniazid, ethambutol, and N-geranyl-N′-(2-adamantyl)ethane-1,2-diamine (SQ109) on Mycobacterium tuberculosis H37Rv. J Pharmacol Exp Ther. 2005;315:905–11.
346. Hillemann D, Rusch-Gerdes S, Richter E. In vitro-selected linezolid-resistant Mycobacterium tuberculosis mutants. Antimicrob Agents Chemother. 2008;52:800–1.
347. Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, Royo G. Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid. Chemotherapy. 2007;53:397–401.
348. Sander P, Belova L, Kidan YG, Pfister P, Mankin AS, Bottger EC. Ribosomal and non-ribosomal resistance to oxazolidinones: species-specific idiosyncrasy of ribosomal alterations. Mol Microbiol. 2002;46:1295–304.
349. Wallis RS, Jakubiec W, Kumar V, Bedarida G, Silvia A, Paige D, Zhu T, Mitton-Fry M, Ladutko L, Campbell S, Miller PF. Biomarker-assisted dose selection for safety and efficacy in early development of PNU-100480 for tuberculosis. Antimicrob Agents Chemother. 2011;55:567–74.
350. Pasca MR, Degiacomi G, Ribeiro AL, Zara F, De Mori P, Heym B, Mirrione M, Brerra R, Pagani L, Pucillo L, Troupioti P, Makarov V, Cole ST, Riccardi G. Clinical isolates of Mycobacterium tuberculosis in four European hospitals are uniformly susceptible to benzothiazinones. Antimicrob Agents Chemother. 2010;54:1616–8.
351. Yano T, Kassovska-Bratinova S, Teh JS, Winkler J, Sullivan K, Isaacs A, Schechter NM, Rubin H. Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species. J Biol Chem. 2011;286:10276–87.
352. Xu J, Lu Y, Fu L, Zhu H, Wang B, Mdluli K, Upton AM, Jin H, Zheng M, Zhao W, Li P. In vitro and in vivo activity of clofazimine

against Mycobacterium tuberculosis persisters. Int J Tuberc Lung Dis. 2012;16:1119–25.
353. Dutta NK, Mazumdar K, Dastidar SG, Karakousis PC, Amaral L. New patentable use of an old neuroleptic compound thioridazine to combat tuberculosis: a gene regulation perspective. Recent Pat Antiinfect Drug Discov. 2011;6:128–38.
354. Dutta NK, Mehra S, Kaushal D. A Mycobacterium tuberculosis sigma factor network responds to cell-envelope damage by the promising anti-mycobacterial thioridazine. PLoS One. 2010;5, e10069.
355. Weinstein EA, Yano T, Li LS, Avarbock D, Avarbock A, Helm D, McColm AA, Duncan K, Lonsdale JT, Rubin H. Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. Proc Natl Acad Sci U S A. 2005;102: 4548–53.

Part IV

Fungal Drug Resistance: Mechanisms

Amphotericin B: Polyene Resistance Mechanisms

26

Matthew McCarthy, Elizabeth M. O'Shaughnessy, and Thomas J. Walsh

1 Introduction

Amphotericin B is a polyene antimicrobial agent that is used to treat invasive fungal infections. It is active against most pathogenic yeasts and molds found in humans and is the only effective treatment for some mycoses. Although uncommon, resistance to amphotericin B is increasing and can be categorized into three main categories: primary or intrinsic, acquired, and clinical resistance. Herein, we provide an overview of polyene resistance mechanisms as well as strategies to overcome resistance.

The polyene antibiotics represent a class of biologically active fungal metabolites isolated from the genus Streptomyces, an aerobic actinomycete obtained from soil [1]. While more than 100 polyene antibiotics have been described, amphotericin B and nystatin are the two agents most commonly used to treat fungal and some protozoal infections in humans. Amphotericin B is active against most pathogenic fungi in humans, and for over 40 years has been the cornerstone of therapy for critically ill patients with invasive fungal infections. Nystatin is generally used to treat mucosal *Candida* infections.

Resistance to amphotericin B is uncommon but it is increasing in the context of emerging pathogens, such as *Candida lusitaniae*, *Candida guilliermondii*, as well as species of *Aspergillus*, *Fusarium*, *Scedosporium*, and *Trichosporon*. Non-*albicans* candidemia now accounts for 30–60 % of all episodes of candidemia [2]. In a multicenter surveillance study between 2004 and 2008 in the USA, 54 % of 2019 bloodstream isolates represented non-albicans *Candida* spp. and 46 % represented *C. albicans* [3], 26 % of all cases of candidemia were due to *Candida glabrata*, followed by *Candida parapsilosis* (16 %), and *Candida tropicalis* (8 %).

Resistance can be categorized into three main categories: primary or intrinsic, acquired, and clinical resistance. Intrinsic or primary resistance occurs without exposure to antifungal agents. Acquired or secondary resistance develops during treatment, and often occurs as a result of one or several genetic mutations [4]. Intrinsic resistance to amphotericin B is rare among pathogenic fungi infecting humans, and acquired resistance during therapy is even less common [5, 6]. Although polyene resistance has not been a major clinical problem to date, polyene-resistant fungi and protozoa continue to be reported. Identification of a particular pathogen to the species level helps to predict possible polyene resistance, and can be extremely important to help guide the choice of antifungal therapy. Clinical resistance, i.e., failure of antifungal therapy, is multifactorial, and depends on a variety of factors, such as the immune status of the host, pharmacokinetics of the antifungal agent, and the species of infecting fungus. In many instances, resistance to amphotericin B may not be related to the mean inhibitory concentrations (MIC), but to failure of the antifungal agent to penetrate into infected tissue [7].

It must be emphasized that the true rate of amphotericin B resistance is not known [8–10]. Detection of resistance can be technically difficult, and the limitations of the Clinical and Laboratory Standards Institute (CLSI) reference methodology for detecting AMB resistance are well documented [11–13]. Prior studies have suggested that minimum fungicidal concentration (MFC) may be a better measure for detecting fungicidal activity in vivo and in vitro [14, 15]. The

M. McCarthy, M.D.
Transplantation-Oncology Infectious Diseases Program, Weill Cornell Center, New York, NY, USA

E.M. O'Shaughnessy, M.D.
Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA

T.J. Walsh, M.D. (✉)
Department of Pediatrics, Transplantation-Oncology Infectious Diseases Program, Weill Cornell Medical Center, New York, NY, USA
e-mail: thw2003@med.cornell.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_26

Clinical Laboratory Standards Institute (CLSI) has developed a standardized broth dilution methodology for in vitro susceptibility testing of Candida species against amphotericin B, flucytosine, fluconazole, and itraconazole [16]. The mean inhibitory concentration (MIC) of amphotericin B can vary depending upon the test format, type of media, and the fungal species being tested. This method cannot always distinguish between amphotericin B-susceptible and amphotericin B-resistant isolates due to the narrow range of MIC values that are generated. Limitations with the current methodologies have precluded the establishment of interpretative MIC breakpoints for amphotericin B for yeasts and molds. Antibiotic medium-3 and E-test strips have been reported to enhance detection of fungal strains with diminished susceptibility to amphotericin B, because a broader range of MIC values can be generated [17, 18]. An evaluation of different in vitro susceptibility test formats for amphotericin B against *Candida* spp.—i.e., broth microdilution using different media, E-test, and MFC—did not generate results that correlated with therapeutic success or failure [19].

There is a narrow range of MIC values (0.06 ~ 2 μg/mL) for amphotericin B against *Candida* species; therefore, a one-dilution shift in a breakpoint can greatly alter how susceptibility or resistance is reported [20]. *Candida* spp. with MIC >1 μg/mL are considered resistant to amphotericin B [16]. Time-kill assays show that the time required for fungicidal activity for amphotericin B is species dependent. In a recent study, the time required for fungicidal activity was fastest for *C. albicans*, and the time increased, respectively, for the following species: *C. lusitaniae, C. glabrata, C. parapsilosis, C. krusei,* and *C. tropicalis* [21].

1.1 Epidemiology of Polyene Resistance

Amphotericin B resistance is rare, but has been reported in some *Candida* species, as well as *Trichosporon, Aspergillus, Scedosporium, Fusarium*, and the protozoa, Leishmania species [9, 10]. Although amphotericin B-resistant strains of *C. albicans* (defined as an MIC ≥2 μg/mL) have been reported, amphotericin B resistance is more common in non-albicans species [22]. Resistance is seen in a small but significant percentage of *Candida* species: *C. lusitaniae* (5–20 %), *C. rugosa* (5–20 %), *C. krusei* (10–15 %), and *C. guilliermondii* (5–10 %) [2]. Ostrosky-Zeichner et al. reported a 2–3 % rate of resistance to amphotericin B (defined as MIC >1.0 μg/mL) in *C. parapsilosis* and *C. krusei* isolates in a surveillance study of *Candida* bloodstream isolates in the USA [23]. *C. lusitaniae* and *C. guilliermondii* are known for inherent or rapid acquisition of amphotericin B resistance [2, 24, 25]. *C. glabrata* and *C. krusei* are generally susceptible to amphotericin B, but they tend to have higher MICs than *C. albicans*. A small proportion of isolates of both species have been found to be resistant to amphotericin B with MIC ≥2 μg/mL, including one isolate of *C. glabrata* harboring a missense mutation of the *ERG6* gene, which affects sterol content in the plasma membrane [26, 27]. Pfaller et al. reported *C. glabrata* with amphotericin B MIC ≥2 μg/mL in less than 1 % of US isolates and in 4.4 % of European isolates [28]. *Trichosporon* species, for example *T. asahii* (formerly *T. beigelii*), are generally resistant to amphotericin B; isolates may be inhibited, but are not killed by achievable serum levels of amphotericin B [29].

An in vitro susceptibility study of 100 *Aspergillus* species against amphotericin B demonstrated that 67 % of the isolates had an MIC ≥2 μg/mL, and 90 % had an MIC ≤4 μg/mL [15]. *A. fumigatus*, *A. flavus*, *A. nidulans*, and *A. niger* are generally susceptible to amphotericin B, but resistance has been reported [9]. *A. terreus* (MIC range: 1 to >4 μg/mL) is inherently resistant to amphotericin B, possibly due to elevated catalase production, protecting the organism from oxidative damage that has been implicated in amphotericin B action [30, 31].

Scedosporium apiospermum (MIC range: 1 to >16 μg/mL), *Pseudallescheria boydii* (MIC range: 1 to ≥16 μg/mL), some strains of *Sporothrix schenckii* (MIC range: 0.5–4 μg/mL), and some *Fusarium* species (MIC range: 1 to >4 μg/mL) have variable resistance to amphotericin B [9, 32–34]. *Scedosporium prolificans* (MIC range: 4>16 μg/mL) is another emerging infectious dematiaceous mold that is usually resistant to amphotericin B [35, 36]. The dimorphic fungi *Histoplasma capsulatum*, *Coccidioides* spp., and *Blastomyces dermatitidis* are generally susceptible to amphotericin B, and have MIC values that range from 0.5 to 1.0 μg/mL. Zygomycetes are typically susceptible to amphotericin B (MIC range: 0.5–2.0 μg/mL) [33, 37–39]. Resistance to amphotericin B has also been reported in the protozoan parasite *Leishmania donovani*, a causative agent in Leishmaniasis or kala azar. It has been suggested that resistance is possibly due to an upregulated thiol metabolic pathway as well as altered ATP-binding cassette transporters and membrane composition [10].

1.2 Emergence of Polyene Resistance

Emergence of resistance during amphotericin B therapy is an uncommon phenomenon [9]. The fungal pathogen may acquire resistance, or the patient may become infected with a different species intrinsically resistant to amphotericin B. Amphotericin B is often used as empiric therapy for neutropenic fever, and yeast isolates from patients undergoing myelosuppressive chemotherapy or hematopoietic stem cell transplantation have been reported to have significantly higher MICs to amphotericin B than colonizing isolates from immunocompetent patients [40–43]. Dannaoui et al. investigated the emergence of antifun-

gal resistance in 200 sequential isolates of *A. fumigatus* from immunocompromised patients on antifungal therapy, and found that resistance was rare [44].

Strains of *C. albicans* acquiring resistance to amphotericin B or amphotericin B plus azoles have been isolated from patients receiving treatment with these antifungals [45, 46]. Nolte et al. reported two cases of candidemia in leukemia patients, which were caused by fluconazole and amphotericin B-resistant isolates of *C. albicans*. The patients had received empiric therapy with both of these antifungals [45]. A cluster of cases of *C. rugosa* candidemia, reported from Brazil, were refractory to amphotericin B therapy and associated with a high mortality; two patients had received prior therapy with amphotericin B [47].

An association between in vitro-decreased susceptibility to amphotericin B in *Candida* species isolated from severely immunocompromised patients with candidemia and subsequent poor clinical outcome has been reported. Bloodstream isolates of *C. albicans* with MIC >0.8 μg/mL were associated with a high mortality in severely immunocompromised patients [42]. Infection with polyene-resistant isolates of *C. lusitaniae* and *C. guilliermondii* has been described in patients who received amphotericin B therapy [46, 48]. Cross-resistance to azoles and polyenes has been reported in *Candida glabrata* vaginal isolates in a case of recurrent vaginitis as well as *Cryptococcus neoformans* in HIV-infected patients following several courses of azoles, or azoles plus amphotericin B [49, 50]. For *Aspergillus* spp. and other molds, there is a little data on the ability of MICs to predict clinical outcome. In a study of 29 patients with hematological malignancies, infected with *A. flavus* 41 % [12], *A. fumigatus* 28 % [8], and *A. terreus* 31 % [9], infection with an *Aspergillus* species with MIC ≥2 μg/mL was associated with a high mortality rate. All patients infected with *A. terreus* (MIC ≥2 μg/mL) died [51].

2 Mechanism of Action of Amphotericin B

The mechanisms of action and toxicity of amphotericin B have been recently reviewed in a detailed report by Loo and colleagues [52]. Amphotericin B acts mainly at the plasma membrane, and impairs membrane barrier function. Susceptibility to polyenes depends on membrane structure, including sterols and other components such as phospholipids [52]. Sterols are essential components of eukaryotic cells, and ergosterol is the principal sterol in the fungal cell membrane. Similar to mammalian cholesterol, ergosterol serves as a bio-regulator of membrane fluidity, and of membrane integrity and permeability. Ergosterol also has a role in active growth phases of fungal cells [53]. Amphotericin B and nystatin bind to ergosterol present in the cell membrane of susceptible fungi, and also bind to cholesterol in human cells, but they bind more avidly to ergosterol-containing membranes than to cholesterol-containing membranes [54]. Amphotericin B has toxic effects on mammalian cells. It has been shown that in the presence of serum, amphotericin B binding is not limited to membrane binding, but also to binding with low-density lipoprotein (LDL) receptors. These toxic effects may be due to its capacity to modify or weaken the structure of LDLs by an oxidative process [55].

Ergosterol and its biosynthetic pathway are the two major targets for polyene antibiotics. The antifungal effects of amphotericin B are believed to be by two primary mechanisms: an increase in permeation by binding sterols in cellular membranes, and a pro-oxidant effect causing oxidative damage in target fungal cells. The type and quality of sterols in fungal cell membranes also influence the level of interaction between the cells and polyenes. The interaction leads to increased cell permeability, and sometimes to cell death. The latter effect does not always follow changes in cell permeability, and is probably affected by a separate process involving oxidative damage [56, 57].

2.1 Interaction with Sterols in the Fungal Cell Membrane

The most widely accepted model for the mechanism of action of amphotericin B was proposed in the early 1970s [58–60]. Interaction of amphotericin B with sterols causes the formation of transmembrane pores or channels that cause disruption of normal membrane function. Polyene antibiotics were one of the first model systems used to study transmembrane ionic channel structures. Amphotericin B binds to membrane ergosterol, which results in the production of aqueous pores. These pores consist of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols forming the staves in a barrel-like structure, with a hydrophilic interior and a hydrophobic exterior. The hydrophilic channel has a diameter of approximately 8 Å [59–61].

The formation of membrane pores or channels causes altered membrane permeability and leakage of potassium ions, and of other vital cytoplasmic components, leading to membrane disruption, and possible fungal cell death. To replace potassium loss, a subsequent transfer of hydrogen ions from the environment follows. The subsequent inflow of protons causes acidification of the fungal cytoplasm, which results in precipitation of cytoplasmic components [62].

Leakage of potassium ions does not always result in fungal cell killing [63]. In yeasts, increased permeability to small ions has been observed at low concentrations of amphotericin B (0.02–0.1 μg/mL), and cell lysis and death were observed at higher concentrations of the drug [64, 65]. Previous studies on *Saccharomyces cerevisiae* demonstrated

that low concentrations of amphotericin B and nystatin and other polyenes caused leakage of potassium ions, and, at high concentrations, caused fungal cell death and red cell hemolysis [66]. Different types of channels are formed with selectivity for different ions, and the type and number of channels formed have been shown to be critically dependent upon the concentration of polyene [67, 68].

In *Candida* species, the dose of amphotericin B needed to cause leakage of ions (Na+, K+, Cl−, Ca++, and Mg++) from the cell membrane is lower than that required to cause cell death, which led to the theory of two separate types of resistance mechanisms [56, 63, 68]. There is experimental evidence that amphotericin B has a number of mechanisms of cell disruption. Recent studies on artificial membranes have demonstrated that sterols do promote, but may not be necessary to produce, highly cationic selective amphotericin B channels [69]. Osmotic stress has been shown to sensitize sterol-free phospholipid bilayers to the action of amphotericin B, and to enhance the formation of amphotericin B channels in sterol-free egg phosphatidylcholine membranes [70].

2.2 Oxidative Damage to the Fungal Cell Membrane

Membrane permeability changes and membrane disturbances may explain the fungistatic effects of amphotericin B, but does not explain its lethal effects. Evidence from several studies has shown that killing of fungi and lysis of red cells can result from oxidative damage by amphotericin B [71, 72]. Amphotericin B-induced oxidative stress on fungal cells may be as important a factor as channel formation in causing cell disruption. Cell membrane damage is due to the formation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, that results in membrane disruption and cell death through membrane lipid peroxidation [73]. Defense against oxidative damage is involved in the resistance to amphotericin B [72]. Andrews et al. found that antioxidants, e.g., catalase, enhanced the antifungal activity of amphotericin B, and postulated that this effect resulted from protection of the amphotericin B molecule from auto-oxidation, thus enhancing or prolonging the drug's action. In the absence of oxygen, amphotericin B may act as an antioxidant, and therefore as a chain terminator of the peroxidation process, and it may partially protect the fungus against phagocytosis [74].

There is some experimental evidence that amphotericin B may act as an antioxidant. The presence of seven conjugated double bonds in amphotericin B suggests that it is prone to auto-oxidation. This tendency to auto-oxidation suggests that amphotericin B could also act like an antioxidant, possibly at low oxygen tensions [75].

3 Mechanisms of Resistance

Resistance to polyenes has developed slowly over time, because the interaction of amphotericin B with the plasma membrane is complex, and multiple changes may be required to prevent disruption of the cell membrane [53, 76]. Mechanisms of resistance to polyenes include alterations in membrane sterols, defense mechanisms against oxidative damage, defects in ergosterol biosynthetic genes, factors such as fatty acid composition of the cell membrane, and alterations in sterol-to-phospholipid ratio. Additionally, the existing ergosterol structure may be reoriented or masked—for example, by sequestration within phagocytes—leading to steric interference between the polyene and ergosterol [76]. The growth phase of the fungal cell and changes in cell wall structure may be involved in polyene resistance. Amphotericin B resistance may also be mediated by increased catalase activity, with decreasing susceptibility to oxidative damage [31].

Much of the knowledge of the mechanisms of resistance to polyenes in fungal species has come from studies on mutant isolates of *Saccharomyces cerevisiae*, *Candida*, and *Aspergillus* species generated by exposure to mutagenic agents, or serially passaged in media containing increasing amounts of the polyene [77]. The majority of the amphotericin B-resistant yeast characterized so far has quantitative or qualitative alterations in the sterol composition of their cell membranes [78, 79]. Efflux mechanisms have not been described to be involved in the development of polyene resistance. The large molecule volume of amphotericin B and its derivatives may inhibit its use as a substrate for efflux pumps in the fungal cell [80].

3.1 Polyene Resistance in Experimentally Induced Mutants and Clinical Isolates

Alterations in the sterol content of the plasma membrane occur in different ways—for example, the total ergosterol content of the fungal cell can be decreased without concomitant changes in the sterol composition. Some or all of the polyene-binding sterols may be replaced by sterol intermediates, such as fecosterol or episterol, which bind polyenes less well [81].

Genetic alterations in the ergosterol biosynthetic pathway or ERG genes have been shown to decrease sensitivity to polyenes and azoles. A limited number of studies have addressed the genetic basis of polyene resistance. The ERG3 gene from *S. cerevisiae* has been cloned. The ERG3 gene encodes Δ 5, 6 sterol desaturase, which is required for ergosterol biosynthesis. It may not, however, be essential for cell viability [82]. Micro-array analysis of experimentally induced *C. albicans* mutants (resistant to amphotericin B and fluconazole) and a wild strain showed that 134

genes were expressed. Cell stress genes and ERG5, ERG6, and ERG25 were found to be upregulated when differences in the expression of the ERG genes were compared with the wild-type strain. The mutant strains accumulated sterol intermediates such as lanosterol and eburicol, which have a reduced affinity for amphotericin B [83].

Studies in *C. albicans* and *S. cerevisiae* have shown that the ERG6 gene is not essential for viability, but is important for the production of ergosterol and for sensitivity to polyenes [84]. The ERG6 gene encodes sterol methyl transferase activity, and ERG6 mutants have altered membrane permeability. Artificially induced ERG11 mutants of *C. albicans* and S. *cerevisiae* have been described to be resistant to amphotericin B, and to accumulate sterol intermediates [85]. Young et al. investigated genetic alterations in the ergosterol biosynthetic pathway of *C. lusitaniae*. An ERG6 mutant strain of *C. lusitaniae* was designed to investigate amphotericin B resistance in this species. Amphotericin B-resistant isolates of *C. lusitaniae* were found to have increased levels of ERG6 transcript, as well as reduced ergosterol content. Further transcript analysis showed that expression of the ERG3 gene, which encodes C-5 sterol desaturase, was reduced in two of the amphotericin B-resistant isolates. These findings demonstrate that mutation or altered expression of ergosterol biosynthetic genes can result in resistance to amphotericin B in *C. lusitaniae* [24].

Several precursors of ergosterol have been identified as the major sterols in nystatin-resistant mutants of *S. cerevisiae*. A mutant strain resistant to low levels of nystatin was found to contain a 5,6 dihydroergosterol, an immediate precursor of ergosterol [86, 87]. *S. cerevisiae* mutants with mutations in the ergosterol synthetic genes, ERG4, ERG6, and ERG3, were shown to lack ergosterol, and were resistant to polyenes [82]. Sterols have been shown to be absent in membranes of amphotericin B-resistant *Leishmania donovani* promastigotes [88]. Mutant strains of *A. fennelliae* resistant to polyenes had a decreased amount of ergosterol content compared to wild-type strains, and contained metabolic blocks for a dehydrogenation and a reduction step in the biosynthesis of ergosterol [52]. In an animal model of *A. terreus* infection, strains with the highest MIC and minimum lethal concentration (MLC) were found to have the lowest ergosterol content [29].

Based on an analysis of sterol composition, some clinical isolates of *C. albicans* may be defective in ERG2 or ERG3 genes [89–91]. For example, some *C. albicans* isolates resistant to azoles and polyenes were found to have a low ergosterol content, associated with a defect in the ERG3 gene [45, 89]. Other amphotericin B-resistant *Candida* isolates were unable to form amphotericin B-generated pores in the cell membrane [90]. A clinical isolate of *C. glabrata* with decreased susceptibility to polyenes demonstrated lack of ergosterol with a buildup of late sterol intermediates, suggesting a defect in the final step in the ergosterol pathway. Sequencing of ERG 11, ERG 6, ERG 5, and ERG4 revealed a unique missense mutation in ERG6, leading to an amino acid substitution in the corresponding protein [27]. Evaluation of a number of polyene-resistant *Candida* species showed that incrementally more resistant isolates possessed principal sterols arising from blockage of the biosynthesis of ergosterol at successively earlier stages. Cultures of *Candida* spp. possessing Δ8-sterols were more resistant to polyenes than those possessing Δ7-sterols, which, in turn, were more resistant than those possessing Δ5,7-sterols [92]. In a hematopoietic stem cell transplantation population, polyene resistance was found in 55 *Candida* isolates (*C. albicans, C. tropicalis*, and *C. glabrata*) from six neutropenic patients, and resistance in these isolates was associated with loss or reduction of ergosterol in the cell membrane [22].

Clinical isolates and mutant strains of *C. albicans* cross-resistant to azoles and polyene have been shown to accumulate sterol intermediates in the cytoplasmic membrane due to a decrease in 5,6 desaturase activity. The altered membrane sterol pattern may provide a common basis for the dual resistance, by preventing polyene binding and by reducing azole inhibition of ergosterol synthesis [78, 93]. Resistance to amphotericin B and azoles in clinical isolates of *Candida* was found to be related to the accumulation of sterol intermediates, 3-β-ergosta-7,22-dienol and 3-β-ergosta-8-dienol, which was associated with a defect in ERG3 that encodes the Δ5,6 desaturase [45]. Kelly et al. compared the susceptibility and sterol pattern of two *Cryptococcus neoformans* isolates (pre- and posttreatment) from an AIDS patient who failed antifungal therapy. These authors observed a correlation between resistance to amphotericin B and the sterol pattern in the cell membrane. The resistant, posttreatment isolate had a defect in the Δ8,7-sterol isomerase, leading to accumulation of ergosta-5, 8,22-dienol, ergosta-8,22-dienol, fecosterol, and ergosta-8-enol. Ergosterol accounted for only 4 % of the sterols in the resistant isolates, compared to 75 % in the pretreatment isolates [43].

3.2 Resistance to Oxidation

Defense against oxidative damage is involved in the resistance of *C. albicans* cells to the lethal effects of amphotericin B. Increased levels of intracellular or extracellular catalase, as well as incubation under hypoxic conditions, have been shown to reduce the lethal effects of amphotericin B on *C. albicans* cells and protoplasts [72]. Amphotericin B-induced leakage of potassium was not hindered under hypoxic conditions or in the presence of catalase [56].

Further studies on amphotericin B-resistant strains of *C. albicans* demonstrated that these strains were significantly less sensitive to hydrogen peroxide. In the presence of amphotericin B, these resistant strains produced significantly more intracellular and extracellular catalase than controls [72]. Catalases are antioxidants, and therefore can remove hydrogen peroxide, a source of hydroxyl radicals, and thus ameliorate oxidative damage. Resistance to amphotericin B may arise from the ability of strains to cope more efficiently with the oxidative stress initiated by amphotericin B through increased catalase activity, as has been proposed in *A. terreus* [31].

3.3 Biofilm Formation

Candida spp. produce biofilms on biological and inert surfaces. The resistance of *Candida* biofilms to antifungal drugs has been previously documented. The mechanisms by which *Candida* biofilms are resistant are not well understood. One possible resistance mechanism is related to the slow growth rate of biofilm cells [94]. Lipid formulations of amphotericin B and echinocandins appear to be more active than triazoles (voriconazole, ravuconazole), fluconazole, and nystatin in experimental *Candida albicans* and *Candida parapsilosis* biofilms [91]. Histone deacetylase (HDA) inhibitors have been shown to enhance the activity of amphotericin B for biofilms of *C. albicans, C. parapsilosis*, and *C. krusei* but their use remains investigational [95].

3.4 Fatty Acid Composition

Alteration of sterol content and/or composition is not sufficient to explain polyene resistance. Previous work has shown that the type of sterols and phospholipids in cellular membranes was important in polyene resistance, but did not adequately explain resistance [96]. Some polyene-resistant mutants of *C. albicans* have been shown to have altered fatty acid compositions. Pierce et al. measured the phospholipid composition of sensitive and mutant strains of *C. albicans*, and noted a slightly higher proportion of saturated fatty acids in the resistant mutants, compared with the sensitive strains. The proportion of long-chain fatty acids was similar [96]. Broughton et al. designed, by nitrous acid mutagenesis, amphotericin B-resistant mutants of *C. albicans* that were similar in sterol to the wild type. When the fatty acid composition was examined, there were no significant differences among the major fatty acids compared to the wild type. The authors suggested that an increase in membrane fluidity might confer resistance to amphotericin B. Changes in membrane fluidity were associated with changes in membrane permeability and in cell growth characteristics [97].

3.5 Cell Wall Alterations

Cell wall components may affect the interaction of polyenes with the cytoplasmic membrane. Several authors have observed that some cell wall constituents were involved in the sensitivity or resistance of cells to amphotericin B; for example, low chitin content is associated with increased resistance to amphotericin B in *C. albicans*, Kluyveromyces spp., and *Schizosaccharomyces* spp. [98, 99]. Chitin, an aminopolysaccharide, is an essential structural component of the cell wall, and is usually present in small quantities. Bahmed et al. described two amphotericin B-resistant mutant strains of Kluyveromyces. The mutants had an increased amount of chitin in their cell walls. In both mutants, chitinase activity was significantly reduced in comparison with that of the wild-type strain, but no significant change in the chitin synthase enzymes could be detected [99]. The precise relationship between amphotericin B resistance and cell wall chitin content remains to be demonstrated.

Hammond et al. demonstrated that polyene resistance in *C. albicans* may be partly determined by binding factors in the cell wall [100]. Alterations in the cell wall components of mycelia were shown to lead to resistance in an *A. flavus* mutant. Chemical analysis of the cell wall showed that the level of glucans was higher in resistant mycelia, compared to wild-type amphotericin B-susceptible strains [51]. The precise role of glucans in the cell wall in inhibiting amphotericin B access to ergosterol and in contributing to resistance is poorly understood.

3.6 Yeast Cell Cycle

Ergosterol plays an essential role in the yeast cell cycle. Sterol-starved yeast cells undergo G1-phase arrest, and this can be reversed by adding exogenous ergosterol [84]. A study of polyene susceptibility in exponential- and stationary-phase *Candida* cells demonstrated that stationary-phase cells were more resistant than cells in the exponential phase [101]. This observation may be associated with reduced chitin synthase activity in the stationary growth phase [98].

4 Conclusions

Polyenes, particularly amphotericin B and its lipid formulations, are drugs of choice for the treatment of a wide range of invasive mycoses. Correlation between polyene resistance in vitro and clinical outcome has been difficult to demonstrate due to host and laboratory factors. With increased use and availability of different classes of antifungal agents, it is anticipated that there will be an increasing number and variety of fungal species resistant to these agents. Continued

efforts to study the mechanisms of antifungal resistance, and the development of experimental systems to study resistance mechanisms, will be important components of a strategy to limit the emergence of polyene and other antifungal drug resistance in the future.

Strategies to overcome polyene resistance would include modifications of existing drugs, development of new classes of antifungal agents, and new treatment strategies, such as combination antifungal therapy. Combination antifungal therapy has been shown to be synergistic in some animal models [102]. Combinations of echinocandins with azoles or amphotericin B appear to be promising regimens in the clinical setting [103, 104]. Optimization of dosing regimens of currently available antifungal drugs for specific infections is important, as is monitoring of antifungal susceptibility patterns and distribution of fungal species [105, 106]. Refinement of current in vitro susceptibility testing, establishment of breakpoints, development of molecular tests for detection of resistance, and establishment of clinical databases to complement information gained from clinical trials are additional important strategies to better understand polyene resistance.

Disclaimer The views expressed are those of the author(s) and do not reflect the official policy or position of the FDA or the US Government.

References

1. Donovick R, Gold W, Pagano JF, Stout HA. Amphotericins A and B, antifungal antibiotics produced by a streptomycete. I. In vitro studies. Antibiot Annu. 1955;3:579–86.
2. Krcmery V, Barnes AJ. Non-albicans Candida spp. causing fungaemia: pathogenicity and antifungal resistance. J Hosp Infect. 2002;50:243–60.
3. Horn DL, Neofytos D, Anaissie EJ, et al. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin Infect Dis. 2009;48(12):1695.
4. Masia Canuto M, Gutierrez Rodero F. Antifungal drug resistance to azoles and polyenes. Lancet Infect Dis. 2002;2:550–63.
5. Sanglard D, Odds FC. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis. 2002;2:73–85.
6. Vincent BM, Lancaster AK, Scherz-Shouval R, et al. Fitness trade-offs restrict the evolution of resistance to amphotericin B. PLoS Biol. 2013;11(10), e1001692.
7. Paterson PJ, Seaton S, Prentice HG, Kibbler CC. Treatment failure in invasive aspergillosis: susceptibility of deep tissue isolates following treatment with amphotericin B. J Antimicrob Chemother. 2003;52(5):873–6.
8. Moore CB, Sayers N, Mosquera J, Slaven J, Denning DW. Antifungal drug resistance in Aspergillus. J Infect. 2000;41:203–20.
9. Ellis D. Amphotericin B: spectrum and resistance. J Antimicrob Chemother. 2002;49 Suppl 1:7–10.
10. Purkait B, Kumar A, Nandi N, et al. Mechanism of amphotericin B resistance in clinical isolates of Leishmania donovani. Antimicrob Agents Chemother. 2012;56(2):1031–41. Epub 2011 Nov 28.
11. Alexander BD, Byrne TC, Smith KL, et al. Comparative evaluation of Etest and sensititre yeastone panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing Candida susceptibility to seven antifungal agents. J Clin Microbiol. 2007;45(3):698–706. Epub 2007 Jan 3.
12. Warnock DW, Arthington-Skaggs BA, Li RK. Antifungal drug susceptibility testing and resistance in Aspergillus. Drug Resist Updat. 1999;2:326–34.
13. Rex JH, Walsh TJ, Nettleman M, et al. Need for alternative trial designs and evaluation strategies for therapeutic studies of invasive mycoses. Clin Infect Dis. 2001;33:95–106.
14. Ernst EJ, Yodoi K, Roling EE, Klepser ME. Rates and extents of antifungal activities of amphotericin B, flucytosine, fluconazole, and voriconazole against Candida lusitaniae determined by microdilution, Etest, and time-kill methods. Antimicrob Agents Chemother. 2002;46:578–81.
15. Denning DW, Hanson LH, Perlman AM, Stevens DA. In vitro susceptibility and synergy studies of Aspergillus species to conventional and new agents. Diagn Microbiol Infect Dis. 1992;15:21–34.
16. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. NCCLS document M27-A2. National Committee for Clinical Laboratory Standards, Wayne, PA; 2002.
17. Rex JH, Cooper Jr CR, Merz WG, Galgiani JN, Anaissie EJ. Detection of amphotericin B-resistant Candida isolates in a brothbased system. Antimicrob Agents Chemother. 1995;39:906–9.
18. Wanger A, Mills K, Nelson PW, Rex JH. Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant Candida isolates. Antimicrob Agents Chemother. 1995;39:2520–2.
19. Park BJ, Arthington-Skaggs BA, Rana AH, et al. Evaluation of amphotericin B interpretive breakpoints for Candida bloodstream isolates by correlation with therapeutic outcome. Antimicrob Agents and Chemother. 2006;50:1287–92.
20. Nguyen MH, Clancy CJ, Yu VL, et al. Do in vitro susceptibility data predict the microbiologic response to amphotericin B? Results of a prospective study of patients with Candida fungemia. J Infect Dis. 1998;177:425–30.
21. Canton E, Peman J, Gobernado M, Viudes A, Espinel-Ingroff A. Patterns of amphotericin B killing kinetics against seven Candida species. Antimicrob Agents Chemother. 2004;48:2477–82.
22. Collin B, Clancy CJ, Nguyen MH. Antifungal resistance in non-albicans Candida species. Drug Resist Updat. 1999;2:9–14.
23. Ostrosky-Zeichner L, Rex JH, Pappas PG, et al. Antifungal susceptibility survey of 2,000 bloodstream Candida isolates in the United States. Antimicrob Agents Chemother. 2003;47:3149–54.
24. Young LY, Hull CM, Heitman J. Disruption of ergosterol biosynthesis confers resistance to amphotericin B in Candida lusitaniae. Antimicrob Agents Chemother. 2003;47:2717–24.
25. Dick JD, Rosengard BR, Merz WG, Stuart RK, Hutchins GM, Saral R. Fatal disseminated candidiasis due to amphotericin-B-resistant Candida guilliermondii. Ann Intern Med. 1985;102:67–8.
26. Rex JH, Walsh TJ, Sobel JD, et al. Practice guidelines for the treatment of candidiasis. Clin Infect Dis. 2000;30:662–78.
27. Vandeputte P, Tronchin G, Bergès T, et al. Reduced susceptibility to polyenes associated with a Missense mutation in the *ERG6* gene in a clinical isolate of *Candida glabrata* with pseudohyphal growth. Antimicrob Agents Chemother. 2007;51(3):982–90.
28. Pfaller MA, Messer SA, Boyken L, Tendolkar S, Hollis RJ, Diekema DJ. Geographic variation in the susceptibilities of invasive isolates of Candida glabrata to seven systemically active antifungal agents: a global assessment from the ARTEMIS Antifungal Surveillance Program conducted in 2001 and 2002. J Clin Microbiol. 2004;42:3142–6.

29. Walsh TJ, Melcher GP, Rinaldi MG, et al. Trichosporon beigelii, an emerging pathogen resistant to amphotericin B. J Clin Microbiol. 1990;28:1616–22.
30. Walsh TJ, Petraitis V, Petraitiene R, et al. Experimental pulmonary aspergillosis due to Aspergillus terreus: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J Infect Dis. 2003;188:305–19.
31. Blum G, Hörtnagl C, Jukic E, et al. New insight into amphotericin B resistance in Aspergillus terreus. Antimicrob Agents Chemother. 2013;57(4):1583–8.
32. Arikan S, Lozano-Chiu M, Paetznick V, Nangia S, Rex JH. Microdilution susceptibility testing of amphotericin B, itraconazole, and voriconazole against clinical isolates of Aspergillus and Fusarium species. J Clin Microbiol. 1999;37:3946–51.
33. Espinel-Ingroff A, Bartlett M, Bowden R, et al. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. J Clin Microbiol. 1997;35:139–43.
34. Torres HA, Raad II, Kontoyiannis DP, et al. Infections caused by Fusarium species. J Chemother. 2003;15 Suppl 2:28–35.
35. Gil-Lamaignere C, Roilides E, Maloukou A, Georgopoulou I, Petrikkos G, Walsh TJ. Amphotericin B lipid complex exerts additive antifungal activity in combination with polymorphonuclear leucocytes against Scedosporium prolificans and Scedosporium apiospermum. J Antimicrob Chemother. 2002;50:1027–30.
36. Berenguer J, Rodriguez-Tudela JL, Richard C, et al. Deep infections caused by Scedosporium prolificans. A report on 16 cases in Spain and a review of the literature. Scedosporium Prolificans Spanish Study Group. Medicine (Baltimore). 1997;76:256–65.
37. Li RK, Ciblak MA, Nordoff N, Pasarell L, Warnock DW, McGinnis MR. In vitro activities of voriconazole, itraconazole, and amphotericin B against Blastomyces dermatitidis, Coccidioides immitis, and Histoplasma capsulatum. Antimicrob Agents Chemother. 2000;44:1734–6.
38. Espinel-Ingroff A, Dawson K, Pfaller M, et al. Comparative and collaborative evaluation of standardization of antifungal susceptibility testing for filamentous fungi. Antimicrob Agents Chemother. 1995;39:314–9.
39. Dannaoui E, Meletiadis J, Mouton JW, Meis JF, Verweij PE. In vitro susceptibilities of zygomycetes to conventional and new antifungals. J Antimicrob Chemother. 2003;51:45–52.
40. Walsh TJ, Groll AH. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. Transpl Infect Dis. 1999;1:247–61.
41. Boutati EI, Anaissie EJ. Fusarium, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implications for management. Blood. 1997;90:999–1008.
42. Powderly WG, Kobayashi GS, Herzig GP, Medoff G. Amphotericin B-resistant yeast infection in severely immunocompromised patients. Am J Med. 1988;84:826–32.
43. Kelly SL, Lamb DC, Taylor M, Corran AJ, Baldwin BC, Powderly WG. Resistance to amphotericin B associated with defective sterol delta 8 → 7 isomerase in a Cryptococcus neoformans strain from an AIDS patient. FEMS Microbiol Lett. 1994;122:39–42.
44. Dannaoui E, Meletiadis J, Tortorano AM, et al. Susceptibility testing of sequential isolates of Aspergillus fumigatus recovered from treated patients. J Med Microbiol. 2004;53:129–34.
45. Nolte FS, Parkinson T, Falconer DJ, et al. Isolation and characterization of fluconazole- and amphotericin B-resistant Candida albicans from blood of two patients with leukemia. Antimicrob Agents Chemother. 1997;41:196–9.
46. Krcmery Jr V, Oravcova E, Spanik S, et al. Nosocomial breakthrough fungaemia during antifungal prophylaxis or empirical antifungal therapy in 41 cancer patients receiving antineoplastic chemotherapy: analysis of aetiology risk factors and outcome. J Antimicrob Chemother. 1998;41:373–80.
47. Colombo AL, Melo AS, Crespo Rosas RF, et al. Outbreak of Candida rugosa candidemia: an emerging pathogen that may be refractory to amphotericin B therapy. Diagn Microbiol Infect Dis. 2003;46(4):253–7.
48. Kovacicova G, Hanzen J, Pisarcikova M, et al. Nosocomial fungemia due to amphotericin B-resistant Candida spp. in three pediatric patients after previous neurosurgery for brain tumors. J Infect Chemother. 2001;7:45–8.
49. Khan ZU, Ahmad S, Al-Obaid I, et al. Emergence of resistance to amphotericin B and triazoles in Candida glabrata vaginal isolates in a case of recurrent vaginitis. J Chemother. 2008;20(4):488–91.
50. Joseph-Horne T, Hollomon D, Loeffler RS, Kelly SL. Cross-resistance to polyene and azole drugs in Cryptococcus neoformans. Antimicrob Agents Chemother. 1995;39:1526–9.
51. Lass-Florl C, Kofler G, Kropshofer G, et al. In-vitro testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis. J Antimicrob Chemother. 1998;42:497–502.
52. Loo AS, Muhsin SA, Walsh TJ. Toxicokinetic and mechanistic basis for the safety and tolerability of liposomal amphotericin B. Expert Opin Drug Saf. 2013;12:881–95.
53. Kim SJ, Kwon-Chung KJ, Milne GW, Prescott B. Polyene-resistant mutants of Aspergillus fennelliae: identification of sterols. Antimicrob Agents Chemother. 1974;6:405–10.
54. Brajtburg J, Powderly WG, Kobayashi GS, Medoff G. Amphotericin B: current understanding of mechanisms of action. Antimicrob Agents Chemother. 1990;34:183–8.
55. Barwicz J, Gruda I, Tancr inverted question markede P. A kinetic study of the oxidation effects of amphotericin B on human low-density lipoproteins. FEBS Lett. 2000;465:83–6.
56. Sokol-Anderson M, Sligh Jr JE, Elberg S, et al. Role of cell defense against oxidative damage in the resistance of Candida albicans to the killing effect of amphotericin B. Antimicrob Agents Chemother. 1988;32:702–5.
57. Kumar R, Shukla PK. Amphotericin B resistance leads to enhanced proteinase and phospholipase activity and reduced germ tube formation in Candida albicans. Fungal Biol. 2010;114(2–3):189–97.
58. Athar MA, Winner HI. The development of resistance by Candida species to polyene antibiotics in vitro. J Med Microbiol. 1971;4:505–17.
59. de Kruijff B, Demel RA. Polyene antibiotic-sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes. 3. Molecular structure of the polyene antibiotic-cholesterol complexes. Biochim Biophys Acta. 1974;339:57–70.
60. Hamilton-Miller JM. Fungal sterols and the mode of action of the polyene antibiotics. Adv Appl Microbiol. 1974;17:109–34.
61. Holz RW, Vanden Bossche H, Dromer F, et al. The effects of the polyene antibiotics nystatin and amphotericin B on thin lipid membranes. Ann N Y Acad Sci. 1974;235:469–79.
62. St Georgiev V. Membrane transporters and antifungal drug resistance. Curr Drug Targets. 2000;1:261–84.
63. HsuChen CC, Feingold DS. Two types of resistance to polyene antibiotics in Candida albicans. Nature. 1974;251:656–9.
64. Brajtburg J, Medoff G, Kobayashi GS, Elberg S, Finegold C. Permeabilizing and hemolytic action of large and small polyene antibiotics on human erythrocytes. Antimicrob Agents Chemother. 1980;18:586–92.
65. Brajtburg J, Medoff G, Kobayashi GS, Elberg S. Influence of extracellular K+ or Mg 2+ on the stages of the antifungal effects of amphotericin B and filipin. Antimicrob Agents Chemother. 1980;18:593–7.
66. Kotler-Brajtburg J, Medoff G, Kobayashi GS, et al. Classification of polyene antibiotics according to chemical structure and biological effects. Antimicrob Agents Chemother. 1979;15:716–22.
67. Hartsel S, Bolard J. Amphotericin B: new life for an old drug. Trends Pharmacol Sci. 1996;17:445–9.

68. Hartsel SC, Benz SK, Ayenew W, Bolard J. Na+, K+ and Cl− selectivity of the permeability pathways induced through sterol-containing membrane vesicles by amphotericin B and other polyene antibiotics. Eur Biophys J. 1994;23:125–32.
69. Ruckwardt T, Scott A, Scott J, Mikulecky P, Hartsel SC. Lipid and stress dependence of amphotericin B ion selective channels in sterol-free membranes. Biochim Biophys Acta. 1998;1372:283–8.
70. Wolf BD, Hartsel SC. Osmotic stress sensitizes sterol-free phospholipid bilayers to the action of Amphotericin B. Biochim Biophys Acta. 1995;1238:156–62.
71. Brajtburg J, Elberg S, Schwartz DR, et al. Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. Antimicrob Agents Chemother. 1985;27:172–6.
72. Anderson JB, Sirjusingh C, Syed N, et al. Gene expression and evolution of antifungal drug resistance. Antimicrob Agents Chemother. 2009;53(5):1931–6.
73. Lamy-Freund MT, Ferreira VF, Schreier S. Mechanism of inactivation of the polyene antibiotic amphotericin B. Evidence for radical formation in the process of autooxidation. J Antibiot (Tokyo). 1985;38:753–7.
74. Andrews FA, Sarosi GA, Beggs WH. Enhancement of amphotericin B activity by a series of compounds related to phenolic antioxidants. J Antimicrob Chemother. 1979;5:173–7.
75. Osaka K, Ritov VB, Bernardo JF, et al. Amphotericin B protects cis-parinaric acid against peroxyl radical-induced oxidation: amphotericin B as an antioxidant. Antimicrob Agents Chemother. 1997;41:743–7.
76. Vanden Bossche H, Marichal P, Odds FC. Molecular mechanisms of drug resistance in fungi. Trends Microbiol. 1994;2:393–400.
77. Manavathu EK, Alangaden GJ, Chandrasekar PH. In-vitro isolation and antifungal susceptibility of amphotericin B-resistant mutants of Aspergillus fumigatus. J Antimicrob Chemother. 1998;41:615–9.
78. Eddouzi J, Parker JE, Vale-Silva LA, et al. Molecular mechanisms of drug resistance in clinical Candida species isolated from Tunisian hospitals. Antimicrob Agents Chemother. 2013;57(7):3182–93.
79. Subden RE, Safe L, Morris DC, Brown RG, Safe S. Eburicol, lichesterol, ergosterol, and obtusifoliol from polyene antibiotic-resistant mutants of Candida albicans. Can J Microbiol. 1977;23:751–4.
80. Slisz M, Cybulska B, Grzybowska J, et al. The mechanism of overcoming multidrug resistance (MDR) of fungi by amphotericin B and its derivatives. J Antibiot (Tokyo). 2007;60:436–46.
81. Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev. 1999;12:501–17.
82. Arthington BA, Hoskins J, Skatrud PL, et al. Nucleotide sequence of the gene encoding yeast C-8 sterol isomerase. Gene. 1991;107:173–4.
83. Barker KS, Crisp S, Wiederhold N, et al. Genome-wide expression profiling reveals genes associated with amphotericin B and fluconazole resistance in experimentally induced antifungal resistant isolates of Candida albicans. J Antimicrob Chemother. 2004;54(2):376–85.
84. Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M. The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol Cell Biol. 1989;9:3447–56.
85. Sanglard D, Ischer F, Parkinson T, Falconer D, Bille J. Candida albicans mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother. 2003;47:2404–12.
86. Fryberg M, Oehlschlager AC, Unrau AM. Sterol biosynthesis in antibiotic-resistant yeast: nystatin. Arch Biochem Biophys. 1974;160:83–9.
87. Barton DH, Corrie JE, Bard M, Woods RA. Biosynthesis of terpenes and steroids. IX. The sterols of some mutant yeasts and their relationship to the biosynthesis of ergosterol. J Chem Soc [Perkin 1]. 1974;11:1326–33.
88. Pourshafie M, Morand S, Virion A, Rakotomanga M, Dupuy C, Loiseau PM. Cloning of S-adenosyl-L-methionine: C-24-Deltasterol-methyltransferase (ERG6) from Leishmania donovaniand characterization of mRNAs in wild-type and amphotericin B-Resistant promastigotes. Antimicrob Agents Chemother. 2004;48:2409–14.
89. Kelly SL, Lamb DC, Kelly DE, et al. Resistance to fluconazole and amphotericin in Candida albicans from AIDS patients. Lancet. 1996;348:1523–4.
90. Haynes MP, Chong PL, Buckley HR, Pieringer RA. Fluorescence studies on the molecular action of amphotericin B on susceptible and resistant fungal cells. Biochemistry. 1996;35:7983–92.
91. Kuhn DM, George T, Chandra J, et al. Antifungal susceptibility of Candida biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother. 2002;46:1773–80.
92. Fryberg M, Oehlschlager AC, Unrau AM, Lomb M, Avruch L. Sterol biosynthesis in antibiotic sensitive and resistant Candida. Arch Biochem Biophys. 1976;173:171–7.
93. Hitchcock CA, Russell NJ, Barrett-Bee KJ. Sterols in Candida albicans mutants resistant to polyene or azole antifungals, and of a double mutant C. albicans 6.4. Crit Rev Microbiol. 1987;15:111–5.
94. Baillie GS, Douglas LJ. Effect of growth rate on resistance of Candida albicans biofilms to antifungal drugs. Antimicrob Agents Chemother. 1998;42:1900–5.
95. Al-Dhaheri RS, Douglas LJ. Apoptosis in Candida biofilms exposed to amphotericin B. J Med Microbiol. 2010;59(Pt 2):149–57.
96. Pierce AM, Pierce Jr HD, Unrau AM, Oehlschlager AC. Lipid composition and polyene antibiotic resistance of Candida albicans mutants. Can J Biochem. 1978;56:135–42.
97. Broughton MC, Bard M, Lees ND. Polyene resistance in ergosterol producing strains of Candida albicans. Mycoses. 1991; 34:75–83.
98. Bahmed K, Bonaly R, Coulon J. Relation between cell wall chitin content and susceptibility to amphotericin B in Kluyveromyces, Candida and Schizosaccharomyces species. Res Microbiol. 2003;154:215–22.
99. Bahmed K, Bonaly R, Wathier M, Pucci B, Coulon J. Change of cell wall chitin content in amphotericin B resistant Kluyveromyces strains. FEMS Microbiol Lett. 2002;216:99–103.
100. Hammond SM, Kliger BN, Lambert PA. Differential effects of monovalent and divalent ions upon the mode of action of the polyene antibiotic candicidin. J Appl Bacteriol. 1976;41:59–68.
101. Gale EF, Ingram J, Kerridge D, Notario V, Wayman F. Reduction of amphotericin resistance in stationary phase cultures of Candida albicans by treatment with enzymes. J Gen Microbiol. 1980;117:383–91.
102. Petraitis V, Petraitiene R, Sarafandi AA, et al. Combination therapy in treatment of experimental pulmonary aspergillosis: synergistic interaction between an antifungal triazole and an echinocandin. J Infect Dis. 2003;187:1834–43.
103. Caillot D, Thiébaut A, Herbrecht R, et al. Liposomal amphotericin B in combination with caspofungin for invasive aspergillosis in patients with hematologic malignancies. Cancer. 2007; 110(12):2740–6.
104. Groll AH, Walsh TJ. Antifungal chemotherapy: advances and perspectives. Swiss Med Wkly. 2002;132:303–11.
105. Groll AH, Piscitelli SC, Walsh TJ. Antifungal pharmacodynamics: concentration-effect relationships in vitro and in vivo. Pharmacotherapy. 2001;21:133S–48.
106. Klepser ME. Antifungal resistance among Candida species. Pharmacotherapy. 2001;21:124S–32.

Fungal Drug Resistance: Azoles

27

Jose L. Lopez-Ribot, Nathan P. Wiederhold, and Thomas F. Patterson

1 Introduction

Azole antifungal agents are widely used in the clinical arena to treat a variety of fungal infections. The azoles inhibit fungal lanosterol demethylase, a key fungal enzyme in the ergosterol biosynthetic pathway, leading to an altered membrane physiology and, most frequently, a fungistatic effect. However, the development of resistance to azole derivatives has become an increasingly important problem affecting the management of patients suffering from fungal infections. At the molecular level the most common mechanisms responsible for resistance to azoles are alterations in the target abundance and in drug affinity, reduced intracellular levels due to activation of multidrug efflux pumps, and formation of biofilms. In recent years, new information on genetic factors regulating these mechanisms as well as on fungal stress response pathways have provided additional insights into the development of azole resistance.

J.L. Lopez-Ribot, Pharm.D., Ph.D.
Department of Biology and South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio, San Antonio, TX, USA

N.P. Wiederhold, Pharm.D.
Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

T.F. Patterson, M.D., F.A.C.P., F.I.D.S.A. (✉)
Division of Infectious Diseases, Department of Medicine, University of Texas Health Science, Center at San Antonio and Audie Murphy Division, South Texas Veterans Health Care System, San Antonio, TX, USA
e-mail: patterson@uthscsa.edu

2 Azole Antifungal Agents: History, Mode of Action, and Clinical Utility

Azole derivatives represent one of the major groups of antifungal drugs used in clinical practice to treat fungal infections in humans, including skin and vaginal infections in the general population and more serious life-threatening invasive mycoses in severely immunocompromised patients. Although this class of antifungal agents was originally developed in the 1960s and 1970s, the first available azole derivative for the oral treatment of systemic fungal infections, ketoconazole, an imidazole, was released in the early 1980s. A few years later the introduction of the first-generation triazoles, such as fluconazole and itraconazole, constituted a major advance in the treatment of fungal infections and quickly became the drugs of choice for the treatment of a number of fungal infections, particularly candidiasis [1, 2]. The "new-generation" triazoles, including voriconazole, posaconazole, and most recently isavuconazole, are welcome additions to the limited arsenal of antifungal agents, mainly due to their increased potency and broader spectrum of action [1, 2].

The mode of action of azole derivatives is by binding to and inhibiting lanosterol 14-α demethylase (Cyp51p or Erg11p), a cytochrome P450 enzyme responsible for the C-14 demethylation of lanosterol, thus blocking ergosterol biosynthesis (the major membrane sterol of fungi) and leading to a fungistatic effect in the majority of cases [3]. The unhindered nitrogen of the imidazole or triazole ring of azole antifungal agents binds to the heme iron of Erg11p as a sixth ligand, thus inhibiting the enzymatic reaction. The remainder of the azole molecule binds to the apoprotein in a manner that is dependent upon the individual molecular structure of each azole derivative [3]. The exact conformation of the active site differs between fungal species and amongst the many mammalian P450 mono-oxygenases. The precise nature of the interaction between each azole molecule and each kind of P450 therefore determines the extent of the inhibitory effect of each azole antifungal agent in different

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_27

fungal species (meaning that some fungi could be intrinsically resistant to a given azole derivative). Inhibition of 14α-demethylase by azoles leads to the depletion of ergosterol that is a major bioregulator of fungal cytoplasm membrane fluidity, and to asymmetry and accumulation of aberrant sterol precursors, including 14α-demethylated sterols, resulting in the formation of a plasma membrane with altered structure and function and the arrest of fungal growth. Azoles may also inhibit another cytochrome P450 responsible for sterol Δ^{22}-desaturation (Erg5), a later step in ergosterol biosynthesis [4].

Because of the different characteristics in their activity, pharmacodynamics, pharmacokinetics, and safety profiles, each of the different azole derivatives has found utility in different clinical settings [2, 5]. In general, as a class, azole antifungals have a broad spectrum of activity, including activity against *Candida* species, *Cryptococcus neoformans*, dimorphic fungi, and molds. For example, fluconazole has broad clinical efficacy for mucosal candidiasis (vaginal and oropharyngeal) and was also considered as a first choice for the prophylaxis and treatment of invasive candidiasis in neutropenic and non-neutropenic patients. Fluconazole is also active against *C. neoformans* and some of the causative agents of endemic mycoses. However, fluconazole is not active against *Aspergillus* and other molds, and some *Candida* species (namely *C. krusei* and *C. glabrata*) show decreased susceptibility or are intrinsically resistant to fluconazole. Itraconazole displays potent activity against *Candida* and *Aspergillus spp*., dimorphic and dematiaceous fungi [6]. Voriconazole has been shown to be superior to amphotericin B deoxycholate in the primary treatment of invasive aspergillosis, and as such it is recommended for the primary treatment of invasive aspergillosis in most patients [7, 8]. Posaconazole—and isavuconazole, which has similar spectrum of activity—displays potent activity and an expanded spectrum of activity, including Mucorales infections [9–11].

3 Resistance to Azole Antifungal Agents

3.1 General Considerations and Definitions

Reports on resistance to azole antifungal agents were rare until the late 1980s. However, development of resistance to the current clinically used azole antifungal agents has become an increasing problem. This is particularly true in patients requiring long-term treatment and in those receiving antifungal prophylaxis [12–14]. Thus, azole resistance has been frequently described in patients with AIDS and mucosal candidiasis (particularly in the era prior to highly active antiretroviral therapy, HAART), oral candidiasis, and less frequently in invasive infections. Resistance can be stable or transient, in response to azole treatment [15]. In addition, there is a growing awareness of the changing epidemiology of fungal infections, with a shift towards species that are intrinsically resistant to the most commonly used antifungal agents (namely fluconazole) [16, 17]. Microbiological resistance is defined as a decrease in antifungal drug susceptibility that can be measured in vitro by appropriate laboratory methods. This highlights the importance of the development of standardized methods for antifungal drug susceptibility testing in the last two decades which are considered major milestones in the field of medical mycology. By performing these techniques, a distinction between a susceptible and a resistant fungal isolate can be made according to a threshold drug susceptibility value (i.e., the breakpoint MIC, for minimal inhibitory concentration) which could potentially, and should ideally, predict the success or failure of a given antifungal regimen. However, clinically refractory disease (clinical resistance) may result not only from microbiological resistance, but also from the complexity of host/fungus interactions, normally in a debilitated patient. As a result, with a few exceptions, it has been difficult to correlate the in vitro and in vivo susceptibility data [18, 19].

Primary resistance occurs in organisms never exposed in that host to a given drug. This intrinsic resistance is displayed by all or almost all isolates of one species to a certain drug and it is predictive of clinical failure. Examples are the resistance of *C. krusei* and *A. fumigatus* to fluconazole. In contrast, secondary resistance (also defined as acquired resistance) develops only after exposure of the organism to the drug. The archetypical example of secondary resistance is the development of fluconazole resistance in *C. albicans* strains isolated longitudinally from HIV-infected patients with oropharyngeal candidiasis under long-term treatment with this drug [13, 14].

3.2 Molecular Mechanisms of Azole Resistance

At the molecular level different mechanisms contribute to the resistance against azole antifungal agents (reviewed in [20–22]). These mechanisms include modification of the antifungal target (in the case of azoles lanosterol demethylase, the product of the *ERG11* gene), decreased drug accumulation inside the fungal cells due to the overexpression of multidrug drug efflux pumps, and other alterations in sterol biosynthesis. Deficiency in the uptake of some azole derivatives could also contribute to resistance [23]. Biofilm formation by different fungi leads to high-level azole resistance [24, 25]. Although initial studies were mostly performed in *C. albicans* due to the unique opportunity to analyze a series of matched susceptible and resistant isolates recovered sequentially from the same patient [22, 26–32], subsequent

studies in other pathogenic fungi such as *C. glabrata*, *A. fumigatus*, and *C. neoformans* support these observations [33–40]. In most instances resistance to azoles is a multifactorial process involving several mechanisms. Moreover, cross-resistance within the azole class of antifungal agents is not uncommon, and is becoming an important issue [41, 42].

3.2.1 Alterations in the Target Enzyme

Alterations in the target enzyme (lanosterol 14-α-demethylase), including point mutations and overexpression, lead to decreased susceptibilities to azole drugs, which may also lead to cross-resistance to other azole derivatives. Pathogenic fungi can overcome the inhibition of azoles by increasing the content of the target enzyme molecules, either by gene amplification or by overexpressing the corresponding gene (*ERG11/CYP51*). This results in increased target abundance that requires higher intracellular azole concentrations to complex all the enzyme molecules present in the cells [20, 22]. *ERG11* overexpression is relatively common in *C. albicans* clinical isolates, with two main mechanisms responsible for this overexpression. The first occurs because of the formation of an isochromosome with two copies of the left arm of chromosome 5 (i(5 L)), in which *ERG11* resides, or by duplication of the entire chromosome, leading to gene amplification [43, 44]. The second mechanism involves activating mutations in the gene encoding the transcription factor Upc2, which results in the overexpression of most *ERG* genes, including *ERG11* [45–47]. Overexpression of *ERG11* has also been described in other *Candida* species, and similar to *C. albicans*, Upc2A is a key regulator of ergosterol biosynthesis and is essential for azole resistance in *C. glabrata* [48]. In *A. fumigatus*, overexpression of Cyp51A has been reported, which was associated with a mutation in the CCAAT-binding transcription factor complex subunit HapE [49, 50]. Also, heteroresistance in *C. neoformans* appears to involve chromosomal aneuploidies leading to an increase in copy number of *ERG11* [51].

Point mutations in the gene encoding the target enzyme for azoles (*ERG11/CYP51*) result in amino acid substitutions leading to decreased affinity for azole derivatives. In the case of *C. albicans ERG11* alleles from azole-resistant isolates were sequenced and compared to alleles of matched azole-susceptible isolates. While some *ERG11* alleles contain a single mutation responsible for azole resistance, other *ERG11* alleles were found to contain several mutations with potential additive effects [27, 29, 31, 52–54]. Importantly, some of these mutations have been repeatedly identified by different groups in different geographical locations, and these mutations may represent "hot spots" for the development of azole resistance. Remarkably, most of these substitutions are present in domains that are highly conserved in lanosterol demethylases across fungi suggesting the importance of these residues for function maintenance through evolution. According to molecular modeling of *C. albicans* lanosterol demethylase these regions correspond to important functional domains of the enzyme in its interaction with the heme moiety at its active site and at another region believed to play a role in the entry of the substrate in the substrate pocket [55, 56]. Interestingly some of the new-generation azoles, due to differences in the way they interact with Erg11p, may be more insensitive to alterations in the target enzyme. For example posaconazole is active against *C. albicans* isolates that have mutations in their *ERG11* genes causing resistance to other azole derivatives, and multiple (up to five) mutations in *ERG11* were required to confer decreased susceptibility to posaconazole [53]. A limited number of *ERG11* mutations associated with the development of azole resistance have also been reported in *C. neoformans* [38, 57]. Point mutations in the *CYP51A* gene, as well as tandem repeats in the promoter region of this gene, represent the main mechanisms of azole resistance found in clinical isolates of *Aspergillus fumigatus* cultured from patients who have failed therapy [58–60]. The location of the point mutations and consequent amino acid substitutions affect the azoles differently, thus resulting in different resistance patterns. Some point mutations lead to pan-azole resistance, while others may specifically affect individual agents. Point mutations may affect azoles that are structurally alike in a similar fashion, as point mutations that affect itraconazole also appear to affect posaconazole, while those that lead to voriconazole resistance may also affect isavuconazole [60–63]. Tandem repeats in the promoter region of the *CYP51A* gene, which lead to its increased expression, have also been found in association with these point mutations [64]. The tandem repeats in the promoter region associated with single-point mutations *CYP51A* that have been isolated from patients and associated with clinical failure include TR_{34}/L98H and TR_{46}/Y121F/T289A [58, 65]. Interestingly, studies have reported the recovery of isolates with two mechanisms of resistance in azole-naïve patients as well as from environmental samples collected in Europe, Asia, Africa, and Australia, including areas where azole fungicides are used in agriculture [60, 61, 66–69]. These findings have led to concern that the presence of azoles in the environment may be driving resistance to azoles used to treat patients with invasive aspergillosis.

Mutations in other genes in the ergosterol biosynthetic pathway have also been linked to azole resistance. To illustrate this point, in azole-sensitive *C. albicans* clinical isolates treated with azoles, 14-methyl-3,6-diol accumulates and leads to a fungistatic effect, whereas in sterol $\Delta^{(5,6)}$ desaturase mutants (due to mutations in the gene *ERG3*), its precursor, 14-methylfecosterol, accumulates, which can support growth of the fungal cell. Interestingly, a consequence of this mechanism is that it causes cross-resistance to amphotericin B, due to the fact that ergosterol is absent from cell membranes [70–72].

3.2.2 Increased Drug Efflux

A second major mechanism leading to azole resistance is by prevention of accumulation of sufficient effective concentrations of the azole antifungal agent in the fungal cells as a consequence of enhanced drug efflux. This mechanism is mediated by two types of multidrug efflux systems, the major facilitator superfamily and the ATP-binding cassette superfamily (ABC transporters) [20–22].

The major facilitators contain a transmembrane pore but use proton motive force as their energy source. Up to 95 major facilitators from 17 different families have been predicted from the *C. albicans* genome sequence; however only 1, *MDR1*, has been described to play a role specifically in fluconazole resistance (but not resistance to other azole derivatives). Although expression of the *C. albicans* gene *FLU1* (for fluconazole resistance) encoding another major facilitator in *S. cerevisiae* confers fluconazole-specific efflux, its overexpression has not yet been described as a cause of azole resistance in *C. albicans* clinical isolates [73]. In *A. fumigatus*, *AfuMDR3* encodes major facilitator whose upregulation has been linked to itraconazole resistance [74].

ABC transporters, which have been associated with drug resistance in a variety of eukaryotic cells, include a membrane pore composed of transmembrane segments and two ATP-binding cassettes on the cytosolic side of the membrane which provide the energy source for the pump [75, 76]. *C. albicans* is predicted to contain 28 proteins of several classes of ABC transporters [77]; of these *CDR1* (for *Candida* drug resistance) and *CDR2*, both members of the pleiotropic drug resistance (PDR) class, play a predominant role in azole drug resistance. In *C. glabrata* the preponderant mechanism of azole resistance is the constitutive upregulated expression of genes encoding ABC transporters (*CgCDR1*, *CgCDR2*/*PDH1*, and *CgSNQ2*) [20]. Other genes encoding ABC transporters from *Candida dubliniensis* (*CdCDR1* and *CdCDR2*), *Candida krusei* (ABC1 and 2), *C. tropicalis* (*CDR1* homologue), and *C. neoformans* (*CnAFR1*, for "antifungal resistance 1") have been reported to be upregulated in azole-resistant clinical isolates of these different *Candida* species (reviewed in [20]). In *A. fumigatus*, *AfuMDR1*, most recently renamed *CDR1B*, remains the only gene encoding an ABC transporter that has been directly implicated in azole resistance [78]; however other genes coding for ABC transporters (including *AfuMDR2*, and five genes designated *abcA–E*) have been described to be upregulated in clinical isolates and also in response to azole exposure [33, 40, 74, 79].

More recently a series of studies have provided new insights into genetic factors regulating this mechanism and have identified several key regulators of multidrug transporters in *C. albicans* and other fungal species [20, 80]. *TAC1* (for transcriptional activator of *CDR* genes), a member of the Zn_2–Cys_6 transcription factor family, was the first major transcription factor important for regulating efflux activity in *C. albicans* [81]. *TAC1* is critical for the up-regulation of *CDR1* and *CDR2* both in azole-resistant clinical isolates and after drug exposure, with gain-of-function mutations in different domains of *TAC1* leading to *CDR1* and *CDR2* upregulation [81]. Regarding regulation of major facilitators, another Zn_2–Cys_6 transcription factor, Mrr1 (for multidrug resistance regulator 1), represents the main regulator of *MDR1*, and several gain-of-function mutations in *MRR1* have been reported to cause constitutive upregulation of *MDR1* in *C. albicans* [82]. Similar to *ERG11,* both *MRR1* and *TAC1* reside on the left arm of chromosome 5; thus multiple genomic alterations and in particular the formation of the isochromosome i(5 L) in *C. albicans* increase their copy number leading to azole resistance [83, 84]. In *C. glabrata* ABC-transporter regulation is similar to *S. cerevisiae*, and high-level azole resistance is mostly due to gain-of-function mutations in the gene encoding the transcription factor Pdr1, resulting in upregulation of genes encoding the multidrug resistance ABC transporters Cdr1, Pdh1, and Snq2 [85].

3.2.3 Cellular Stress Responses Mediated by Hsp90 and Related Factors

Fungi have evolved a highly sophisticated stress response circuitry that enables them to cope with diverse environmental stresses [86], and these same signal transduction pathways can be used by fungal cells to respond to the membrane stress induced by treatment with azole antifungal drugs [86]. The highly conserved and essential molecular chaperone Hsp90 regulates a complex cellular circuitry in fungi (and other eukaryotes) by stabilizing numerous client proteins, many of which are key regulators of cellular signaling [86]. Inhibition of Hsp90 reduces tolerance of *Candida* species against azoles and blocks the evolution of azole resistance [86]. In fact, Hsp90 pharmacological inhibitors of Hsp90 increase the efficacy of azole drugs against resistant *Candida* isolates, both in vitro and in vivo [20, 86]. Calcineurin and Mkc1 represent the key client proteins by which Hsp90 regulates the emergence and maintenance of azole resistance in fungi. The protein phosphatase calcineurin is required to survive the cell membrane stress induced by azoles [87–89]. Hsp90 physically interacts with the catalytic subunit of calcineurin, keeping it stable and poised for activation. Hsp90 also stabilizes Mkc1, the terminal mitogen-activated protein kinase of the cell wall integrity pathway, and modulates additional responses to the stress induced by exposure to azole derivatives [20, 90].

3.2.4 Acquisition of Resistance Through Multiple Mechanisms, Prevalence, and Heterogeneity of Molecular Mechanisms of Resistance

The multiplicity of resistance mechanisms to azole antifungals represents a set of biological tools that enables fungal

cells to develop resistance. The emergence of resistance often occurs through stepwise increases over time, frequently involving multiple mechanisms as documented in longitudinal studies analyzing sequential clinical isolates recovered from patients during the course of infection. Some studies have also investigated the prevalence and relative frequency of resistance mechanisms in a large number of azole-resistant isolates. To illustrate this point, in one of these studies [29], most of the *C. albicans* isolates resistant to fluconazole presented a combination of mechanisms including upregulation of efflux transporters and point mutations in the *ERG11* alleles. In 85 % of resistant isolates a major mechanism of resistance was the upregulation of multidrug resistance of both families (ABC transporters and major facilitators). Also, almost 60 % of patients presented *C. albicans* isolates harboring point mutations in their *ERG11* genes leading to enzymes with decreased affinity for fluconazole. Overall, 75 % of the azole-resistant isolates showed combined resistance mechanisms. All the isolates that showed cross-resistance against multiple azoles presented increase in *CDR* mRNA. Only one isolate overexpressed *ERG11* genes without concomitant upregulation of *CDR* and *MDR* genes, and only two resistant isolates presented point mutations in *ERG11* genes as mechanism of resistance not associated with upregulation of efflux pumps. Overall these studies point to the complexity of the distribution of the molecular mechanisms of azole drug resistance.

An often-overlooked and underappreciated phenomenon, particularly in early studies, is the fact that different fungal subpopulations may exist that respond and evolve differently under antifungal drug pressure, providing an additional level of complexity in the molecular mechanisms of azole resistance. Earlier studies on molecular mechanisms of azole resistance in oropharyngeal candidiasis were limited due to the fact that only a single isolate from each episode was available for study, but recovery and analyses of multiple isolates from the same episode in some subsequent studies allowed a comprehensive assessment of the epidemiology of resistance in OPC. In the case of *C. albicans*, it was demonstrated that despite mostly a clonal origin, different subpopulations exhibited distinct resistance mechanisms, including concomitant presence and absence of functional point mutations in *ERG11* genes and different patterns of expression of genes encoding multidrug efflux pumps [91, 92]. Genomic instability with multiple potential genomic alterations, including loss of heterozygosity, increase of chromosome copy number, and aneuploidy [43, 44], impact azole resistance and add a second layer of complexity to this phenomenon. All together these observations are indicative of microevolution of fungal populations under azole antifungal pressure, as it was also demonstrated by Cowen and colleagues in a study of development of azole resistance in experimental populations of *C. albicans* [93, 94], and indicate that different fungal subpopulations may coexist at a given time in the same patient and may develop resistance through different mechanisms [91, 92].

3.2.5 Biofilm Resistance

A significant proportion of all human microbial infections, including mycoses, involve biofilm formation [24] and several groups have demonstrated that biofilm formation by different fungi, including *Candida*, *Aspergillus*, and *Cryptococcus* spp., leads to dramatically increased levels of resistance to azole derivatives (reviewed in [24]). This resistance is multifactorial, and includes mechanisms that are specific to the biofilm lifestyle. Mechanisms contributing to azole resistance in fungal biofilms include (a) the increased numbers of fungal cells within the biofilm [95]; (b) subpopulations of "persister" cells that develop tolerance to azoles [96]; (c) activation of efflux systems, which may occur physiologically as a means to facilitate the removal of waste products but may concomitantly result in increased efflux of azole antifungals [97]; (d) changes in the membrane sterols of cells within the biofilms [98]; (e) cellular stress responses mostly orchestrated by Hsp90 and the calcineurin pathway [99]; and (f) a role for the biofilm matrix due to azole binding by exopolymeric components (mostly β-glucans) and presence of extracellular DNA [100–102]. For further information and an in-depth description of mechanisms of azole resistance in fungal biofilms readers are referred to some excellent reviews on this topic [25, 103, 104].

4 Conclusions

Azoles are an important class of antifungal drugs that have found widespread utility in the clinical practice for the treatment of fungal infections. However, with their increasing usage, emergence of resistance has become a problem, especially in patients requiring long-term treatment and those receiving azole prophylaxis. Also, azole use has had a tremendous impact in the epidemiology of fungal infections. At the molecular level, the main mechanisms responsible for azole resistance are alterations in the target enzyme and increased efflux of drug. Very often resistance is multifactorial, and combinations of different mechanisms are operative in a high proportion of resistant isolates. Development of azole resistance is controlled by a complex regulatory network, with core transcription factors involved in the regulation of key genes which play a major role in resistance. In addition to these mechanisms, different signal transduction pathways enable fungal cells to mount an efficient response against the stress induced by exposure to azoles. Fungal biofilms display high-level resistance against azole derivatives.

References

1. Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FC, Rex JH. An insight into the antifungal pipeline: selected new molecules and beyond. Nat Rev Drug Discov. 2010;9:719–27.
2. Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. Clin Microbiol Rev. 1999;12:40–79.
3. Hitchcock CA. Cytochrome P-450-dependent 14 alpha-sterol demethylase of Candida albicans and its interaction with azole antifungals. Biochem Soc Trans. 1991;19:782–7.
4. Skaggs BA, Alexander JF, Pierson CA, Schweitzer KS, Chun KT, Koegel C, Barbuch R, Bard M. Cloning and characterization of the Saccharomyces cerevisiae C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. Gene. 1996;169:105–9.
5. Graybill JR. The future of antifungal therapy. Clin Infect Dis. 1996;22 Suppl 2:S166–78.
6. Winston DJ, Maziarz RT, Chandrasekar PH, Lazarus HM, Goldman M, Blumer JL, Leitz GJ, Territo MC. Intravenous and oral itraconazole versus intravenous and oral fluconazole for long-term antifungal prophylaxis in allogeneic hematopoietic stem-cell transplant recipients. A multicenter, randomized trial. Ann Intern Med. 2003;138:705–13.
7. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, Kern WV, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B, Invasive Fungal Infections Group of the European Organisation for Research and Treatment of Cancer and the Global Aspergillus Study Group. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med. 2002;347:408–15.
8. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik JA, Wingard JR, Patterson TF, Infectious Diseases Society of America. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis. 2008;46:327–60.
9. Spellberg B, Ibrahim AS. Recent advances in the treatment of mucormycosis. Curr Infect Dis Rep. 2010;12:423–9.
10. Sun QN, Fothergill AW, McCarthy DI, Rinaldi MG, Graybill JR. In vitro activities of posaconazole, itraconazole, voriconazole, amphotericin B, and fluconazole against 37 clinical isolates of zygomycetes. Antimicrob Agents Chemother. 2002;46:1581–2.
11. Sun QN, Najvar LK, Bocanegra R, Loebenberg D, Graybill JR. In vivo activity of posaconazole against Mucor spp. in an immunosuppressed-mouse model. Antimicrob Agents Chemother. 2002;46:2310–2.
12. Epstein JB, Ransier A, Lunn R, Chin E, Jacobson JJ, Le N, Reece D. Prophylaxis of candidiasis in patients with leukemia and bone marrow transplants. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1996;81:291–6.
13. Redding S, Smith J, Farinacci G, Rinaldi M, Fothergill A, Rhine-Chalberg J, Pfaller M. Resistance of Candida albicans to fluconazole during treatment of oropharyngeal candidiasis in a patient with AIDS: documentation by in vitro susceptibility testing and DNA subtype analysis. Clin Infect Dis. 1994;18:240–2.
14. Revankar SG, Kirkpatrick WR, McAtee RK, Dib OP, Fothergill AW, Redding SW, Rinaldi MG, Patterson TF. Detection and significance of fluconazole resistance in oropharyngeal candidiasis in human immunodeficiency virus-infected patients. J Infect Dis. 1996;174:821–7.
15. Marr KA, Lyons CN, Rustad TR, Bowden RA, White TC. Rapid, transient fluconazole resistance in Candida albicans is associated with increased mRNA levels of CDR. Antimicrob Agents Chemother. 1998;42:2584–9.
16. Marr KA. Invasive Candida infections: the changing epidemiology. Oncology. 2004;18:9–14.
17. Nucci M. Emerging moulds: Fusarium, Scedosporium and Zygomycetes in transplant recipients. Curr Opin Infect Dis. 2003;16:607–12.
18. Rex JH, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum MA, Lancaster M, Odds FC, Rinaldi MG, Walsh TJ, Barry AL. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and candida infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. Clin Infect Dis. 1997;24:235–47.
19. Viudes A, Canton E, Peman J, Lopez-Ribot JL, Gobernado M. Correlation between in vitro susceptibility to antifungal drugs and the clinical evolution of patients with candidiasis and cryptococcosis. Rev Esp Quimioter. 2002;15:32–42.
20. Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. Mechanisms of antifungal drug resistance. Cold Spring Harb Perspect Med. 2014;5(7):a019752.
21. Sanglard D, Odds FC. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis. 2002;2:73–85.
22. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev. 1998;11:382–402.
23. Mansfield BE, Oltean HN, Oliver BG, Hoot SJ, Leyde SE, Hedstrom L, White TC. Azole drugs are imported by facilitated diffusion in Candida albicans and other pathogenic fungi. PLoS Pathog. 2010;6, e1001126.
24. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. Our current understanding of fungal biofilms. Crit Rev Microbiol. 2009;35:340–55.
25. Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. Int J Microbiol. 2012;2012:528521.
26. Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhauser J. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical Candida albicans strains. Antimicrob Agents Chemother. 1998;42:3065–72.
27. Loffler J, Kelly SL, Hebart H, Schumacher U, Lass-Florl C, Einsele H. Molecular analysis of cyp51 from fluconazole-resistant Candida albicans strains. FEMS Microbiol Lett. 1997;151:263–8.
28. Lopez-Ribot JL, McAtee RK, Lee LN, Kirkpatrick WR, White TC, Sanglard D, Patterson TF. Distinct patterns of gene expression associated with development of fluconazole resistance in serial Candida albicans isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob Agents Chemother. 1998;42:2932–7.
29. Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D, Patterson TF. Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother. 2001;45:2676–84.
30. Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J. Mechanisms of resistance to azole antifungal agents in Candida albicans isolates from AIDS patients involve specific multidrug transporters. Antimicrob Agents Chemother. 1995;39:2378–86.
31. White TC. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol

14alpha demethylase in Candida albicans. Antimicrob Agents Chemother. 1997;41:1488–94.
32. White TC. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother. 1997;41:1482–7.
33. da Silva Ferreira ME, Capellaro JL, dos Reis ME, Malavazi I, Perlin D, Park S, Anderson JB, Colombo AL, Arthington-Skaggs BA, Goldman MH, Goldman GH. In vitro evolution of itraconazole resistance in Aspergillus fumigatus involves multiple mechanisms of resistance. Antimicrob Agents Chemother. 2004;48:4405–13.
34. Garcia-Effron G, Mellado E, Gomez-Lopez A, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Differences in interactions between azole drugs related to modifications in the 14-alpha sterol demethylase gene (cyp51A) of Aspergillus fumigatus. Antimicrob Agents Chemother. 2005;49:2119–21.
35. Perea S, Lopez-Ribot JL, Wickes BL, Kirkpatrick WR, Dib OP, Bachmann SP, Keller SM, Martinez M, Patterson TF. Molecular mechanisms of fluconazole resistance in Candida dubliniensis isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob Agents Chemother. 2002;46:1695–703.
36. Posteraro B, Sanguinetti M, Sanglard D, La Sorda M, Boccia S, Romano L, Morace G, Fadda G. Identification and characterization of a Cryptococcus neoformans ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole. Mol Microbiol. 2003;47:357–71.
37. Redding SW, Kirkpatrick WR, Saville S, Coco BJ, White W, Fothergill A, Rinaldi M, Eng T, Patterson TF, Lopez-Ribot J. Multiple patterns of resistance to fluconazole in Candida glabrata isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. J Clin Microbiol. 2003;41:619–22.
38. Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-Estrella M, Davel G, Rodriguez-Tudela JL. G484S amino acid substitution in lanosterol 14-alpha demethylase (ERG11) is related to fluconazole resistance in a recurrent Cryptococcus neoformans clinical isolate. Antimicrob Agents Chemother. 2003;47:3653–6.
39. Sanglard D, Ischer F, Calabrese D, Majcherczyk PA, Bille J. The ATP binding cassette transporter gene CgCDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob Agents Chemother. 1999;43:2753–65.
40. Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW. Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol. 2002;36:199–206.
41. Muller FM, Weig M, Peter J, Walsh TJ. Azole cross-resistance to ketoconazole, fluconazole, itraconazole and voriconazole in clinical Candida albicans isolates from HIV-infected children with oropharyngeal candidosis. J Antimicrob Chemother. 2000;46:338–40.
42. Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Diekema DJ. Cross-resistance between fluconazole and ravuconazole and the use of fluconazole as a surrogate marker to predict susceptibility and resistance to ravuconazole among 12,796 clinical isolates of Candida spp. J Clin Microbiol. 2004;42:3137–41.
43. Selmecki A, Bergmann S, Berman J. Comparative genome hybridization reveals widespread aneuploidy in Candida albicans laboratory strains. Mol Microbiol. 2005;55:1553–65.
44. Selmecki A, Forche A, Berman J. Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science. 2006;313:367–70.
45. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhauser J, Rogers PD. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryot Cell. 2012;11:1289–99.
46. MacPherson S, Akache B, Weber S, De Deken X, Raymond M, Turcotte B. Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother. 2005;49:1745–52.
47. Silver PM, Oliver BG, White TC. Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell. 2004;3:1391–7.
48. Whaley SG, Caudle KE, Vermitsky JP, Chadwick SG, Toner G, Barker KS, Gygax SE, Rogers PD. UPC2A is required for high-level azole antifungal resistance in Candida glabrata. Antimicrob Agents Chemother. 2014;58:4543–54.
49. Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Moller N, Khan H, Melchers WJ, Verweij PE. Development of azole resistance in Aspergillus fumigatus during azole therapy associated with change in virulence. PLoS One. 2010;5, e10080.
50. Camps SM, Dutilh BE, Arendrup MC, Rijs AJ, Snelders E, Huynen MA, Verweij PE, Melchers WJ. Discovery of a HapE mutation that causes azole resistance in Aspergillus fumigatus through whole genome sequencing and sexual crossing. PLoS One. 2012;7, e50034.
51. Sionov E, Lee H, Chang YC, Kwon-Chung KJ. Cryptococcus neoformans overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes. PLoS Pathog. 2010;6, e1000848.
52. Favre B, Didmon M, Ryder NS. Multiple amino acid substitutions in lanosterol 14alpha-demethylase contribute to azole resistance in Candida albicans. Microbiology. 1999;145(Pt 10):2715–25.
53. Li X, Brown N, Chau AS, Lopez-Ribot JL, Ruesga MT, Quindos G, Mendrick CA, Hare RS, Loebenberg D, DiDomenico B, McNicholas PM. Changes in susceptibility to posaconazole in clinical isolates of Candida albicans. J Antimicrob Chemother. 2004;53:74–80.
54. Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant Candida albicans clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother. 1998;42:241–53.
55. Podust LM, Poulos TL, Waterman MR. Crystal structure of cytochrome P450 14alpha -sterol demethylase (CYP51) from Mycobacterium tuberculosis in complex with azole inhibitors. Proc Natl Acad Sci U S A. 2001;98:3068–73.
56. Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RE, McNicholas PM. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from Aspergillus fumigatus and Candida albicans provide insights into posaconazole binding. Antimicrob Agents Chemother. 2004;48:568–74.
57. Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ. Identification of a Cryptococcus neoformans cytochrome P450 lanosterol 14alpha-demethylase (Erg11) residue critical for differential susceptibility between fluconazole/voriconazole and itraconazole/posaconazole. Antimicrob Agents Chemother. 2012;56:1162–9.
58. Vermeulen E, Lagrou K, Verweij PE. Azole resistance in Aspergillus fumigatus: a growing public health concern. Curr Opin Infect Dis. 2013;26:493–500.
59. Seyedmousavi S, Mouton JW, Melchers WJ, Bruggemann RJ, Verweij PE. The role of azoles in the management of azole-resistant aspergillosis: from the bench to the bedside. Drug Resist Updat. 2014;17:37–50.

60. Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. Frequency and evolution of Azole resistance in Aspergillus fumigatus associated with treatment failure. Emerg Infect Dis. 2009;15:1068–76.
61. Chowdhary A, Sharma C, Kathuria S, Hagen F, Meis JF. Azole-resistant Aspergillus fumigatus with the environmental TR46/Y121F/T289A mutation in India. J Antimicrob Chemother. 2014;69:555–7.
62. Chowdhary A, Sharma C, van den Boom M, Yntema JB, Hagen F, Verweij PE, Meis JF. Multi-azole-resistant Aspergillus fumigatus in the environment in Tanzania. J Antimicrob Chemother. 2014;69:2979–83.
63. Verweij PE, Howard SJ, Melchers WJ, Denning DW. Azole-resistance in Aspergillus: proposed nomenclature and breakpoints. Drug Resist Updat. 2009;12:141–7.
64. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, Rodriguez-Tudela JL. A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. Antimicrob Agents Chemother. 2007;51:1897–904.
65. Verweij PE, Mellado E, Melchers WJ. Multiple-triazole-resistant aspergillosis. N Engl J Med. 2007;356:1481–3.
66. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW. Azole antifungal resistance in Aspergillus fumigatus: 2008 and 2009. J Antimicrob Chemother. 2010;65:2116–8.
67. Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, Mellado E, Donders AR, Melchers WJ, Verweij PE. Emergence of azole resistance in Aspergillus fumigatus and spread of a single resistance mechanism. PLoS Med. 2008;5, e219.
68. Chowdhary A, Kathuria S, Xu J, Sharma C, Sundar G, Singh PK, Gaur SN, Hagen F, Klaassen CH, Meis JF. Clonal expansion and emergence of environmental multiple-triazole-resistant Aspergillus fumigatus strains carrying the TR(3)(4)/L98H mutations in the cyp51A gene in India. PLoS One. 2012;7, e52871.
69. Badali H, Vaezi A, Haghani I, Yazdanparast SA, Hedayati MT, Mousavi B, Ansari S, Hagen F, Meis JF, Chowdhary A. Environmental study of azole-resistant Aspergillus fumigatus with TR34/L98H mutations in the cyp51A gene in Iran. Mycoses. 2013;56:659–63.
70. Geber A, Hitchcock CA, Swartz JE, Pullen FS, Marsden KE, Kwon-Chung KJ, Bennett JE. Deletion of the Candida glabrata ERG3 and ERG11 genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. Antimicrob Agents Chemother. 1995;39:2708–17.
71. Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, Schumacher U, Einsele H. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett. 1997;400:80–2.
72. Sanglard D, Ischer F, Parkinson T, Falconer D, Bille J. Candida albicans mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother. 2003;47:2404–12.
73. Calabrese D, Bille J, Sanglard D. A novel multidrug efflux transporter gene of the major facilitator superfamily from Candida albicans (FLU1) conferring resistance to fluconazole. Microbiology. 2000;146(Pt 11):2743–54.
74. Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. Multiple resistance mechanisms among Aspergillus fumigatus mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother. 2003;47:1719–26.
75. Balzi E, Goffeau A. Genetics and biochemistry of yeast multidrug resistance. Biochim Biophys Acta. 1994;1187:152–62.
76. Balzi E, Goffeau A. Yeast multidrug resistance: the PDR network. J Bioenerg Biomembr. 1995;27:71–6.
77. Prasad R, Goffeau A. Yeast ATP-binding cassette transporters conferring multidrug resistance. Annu Rev Microbiol. 2012;66:39–63.
78. Fraczek MG, Bromley M, Buied A, Moore CB, Rajendran R, Rautemaa R, Ramage G, Denning DW, Bowyer P. The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in Aspergillus fumigatus. J Antimicrob Chemother. 2013;68:1486–96.
79. Tobin MB, Peery RB, Skatrud PL. Genes encoding multiple drug resistance-like proteins in Aspergillus fumigatus and Aspergillus flavus. Gene. 1997;200:11–23.
80. Sanglard D, Coste A, Ferrari S. Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. FEMS Yeast Res. 2009;9:1029–50.
81. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryot Cell. 2004;3:1639–52.
82. Schubert S, Rogers PD, Morschhauser J. Gain-of-function mutations in the transcription factor MRR1 are responsible for overexpression of the MDR1 efflux pump in fluconazole-resistant Candida dubliniensis strains. Antimicrob Agents Chemother. 2008;52:4274–80.
83. Coste A, Turner V, Ischer F, Morschhauser J, Forche A, Selmecki A, Berman J, Bille J, Sanglard D. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics. 2006;172:2139–56.
84. Dunkel N, Blass J, Rogers PD, Morschhauser J. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains. Mol Microbiol. 2008;69:827–40.
85. Ferrari S, Ischer F, Calabrese D, Posteraro B, Sanguinetti M, Fadda G, Rohde B, Bauser C, Bader O, Sanglard D. Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence. PLoS Pathog. 2009;5, e1000268.
86. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev. 2011;75:213–67.
87. Cruz MC, Goldstein AL, Blankenship JR, Del Poeta M, Davis D, Cardenas ME, Perfect JR, McCusker JH, Heitman J. Calcineurin is essential for survival during membrane stress in Candida albicans. EMBO J. 2002;21:546–59.
88. Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J. Calcineurin A of Candida albicans: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol Microbiol. 2003;48:959–76.
89. Steinbach WJ, Cramer Jr RA, Perfect BZ, Henn C, Nielsen K, Heitman J, Perfect JR. Calcineurin inhibition or mutation enhances cell wall inhibitors against Aspergillus fumigatus. Antimicrob Agents Chemother. 2007;51:2979–81.
90. Diezmann S, Michaut M, Shapiro RS, Bader GD, Cowen LE. Mapping the Hsp90 genetic interaction network in Candida albicans reveals environmental contingency and rewired circuitry. PLoS Genet. 2012;8, e1002562.
91. Lopez-Ribot JL, McAtee RK, Perea S, Kirkpatrick WR, Rinaldi MG, Patterson TF. Multiple resistant phenotypes of Candida albicans coexist during episodes of oropharyngeal candidiasis in human immunodeficiency virus-infected patients. Antimicrob Agents Chemother. 1999;43:1621–30.
92. Martinez M, Lopez-Ribot JL, Kirkpatrick WR, Bachmann SP, Perea S, Ruesga MT, Patterson TF. Heterogeneous mechanisms of azole resistance in Candida albicans clinical isolates from an HIV-

infected patient on continuous fluconazole therapy for oropharyngeal candidosis. J Antimicrob Chemother. 2002;49:515–24.
93. Cowen LE, Nantel A, Whiteway MS, Thomas DY, Tessier DC, Kohn LM, Anderson JB. Population genomics of drug resistance in Candida albicans. Proc Natl Acad Sci U S A. 2002;99:9284–9.
94. Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB, Kohn LM. Evolution of drug resistance in experimental populations of Candida albicans. J Bacteriol. 2000;182:1515–22.
95. Perumal P, Mekala S, Chaffin WL. Role for cell density in antifungal drug resistance in Candida albicans biofilms. Antimicrob Agents Chemother. 2007;51:2454–63.
96. LaFleur MD, Kumamoto CA, Lewis K. Candida albicans biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother. 2006;50:3839–46.
97. Ramage G, Bachmann S, Patterson TF, Wickes BL, Lopez-Ribot JL. Investigation of multidrug efflux pumps in relation to fluconazole resistance in Candida albicans biofilms. J Antimicrob Chemother. 2002;49:973–80.
98. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. Infect Immun. 2003;71:4333–40.
99. Robbins N, Uppuluri P, Nett J, Rajendran R, Ramage G, Lopez-Ribot JL, Andes D, Cowen LE. Hsp90 governs dispersion and drug resistance of fungal biofilms. PLoS Pathog. 2011;7, e1002257.
100. Martins M, Henriques M, Lopez-Ribot JL, Oliveira R. Addition of DNase improves the in vitro activity of antifungal drugs against Candida albicans biofilms. Mycoses. 2012;55:80–5.
101. Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, Oliveira R. Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. Mycopathologia. 2010;169:323–31.
102. Nett JE, Sanchez H, Cain MT, Andes DR. Genetic basis of Candida biofilm resistance due to drug-sequestering matrix glucan. J Infect Dis. 2010;202:171–5.
103. Mathe L, Van Dijck P. Recent insights into Candida albicans biofilm resistance mechanisms. Curr Genet. 2013;59:251–64.
104. Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of Candida biofilm drug resistance. Future Microbiol. 2013;8:1325–37.

Flucytosine Treatment and Resistance Mechanisms

28

Jyotsna Chandra and Mahmoud A. Ghannoum

1 Introduction

Flucytosine is a synthetic antimycotic compound which, after being taken up by susceptible fungal cells, is converted to 5-fluorouracil and subsequent intermediate metabolites that are responsible for inhibition of fungal DNA, RNA, and protein synthesis. Due to primary resistance of yeast, flucytosine is used mainly in combination. Flucytosine administered in combination with amphotericin B remains the standard of care for cryptococcal meningitis, and the drug continues to have a role in the treatment of *Candida* infections which are life threatening or in circumstances where drug penetration may be problematic. It is also used in combination to treat severe systemic mycoses, such as cryptococcosis, chromoblastomycosis, and aspergillosis. Recently, 5-FC has been combined with gene therapy approaches and used as a chemotherapeutic drug to inhibit tumor growth and in curing lesions. In this chapter we review the mechanism of action of flucytosine, its resistance mechanisms, as well as the utility of combining it with other antifungal agents. The available new data may lead to improved dosing practices and a "rebirth" of this agent as a useful adjunct in the treatment of serious fungal infections.

2 Background

Flucytosine (fluorocytosine; 5-FC) was originally synthesized in 1957 as an anticancer metabolite. Unlike 5-fluorouracil (5-FU), a closely related fluorinated pyrimidine, 5-FC did not exhibit antineoplastic activity but was subsequently found to possess antifungal activity and was used in 1968 to treat human cryptococcosis and candidiasis [1]. 5-FC administered in combination with amphotericin B (AmB) remains the standard of care for cryptococcal meningitis, and the drug continues to have a role in the treatment of *Candida* infections which are life threatening or in circumstances where drug penetration may be problematic [1]. Recently, 5-FC has been combined with gene therapy approaches and has been used as a chemotherapeutic drug in inhibiting multicell tumor spheroids [2] and in oncolytic effectiveness of MeV-based virotherapies [3]. It has also been shown that progressively growing lesions due to *Leishmania* can be completely cured by 2 weeks of treatment with 5-FC alone or in combination in vivo [4]. When taken up by susceptible fungal cells, 5-FC is converted to 5-FU, which is further converted to metabolites that inhibit fungal RNA and DNA synthesis. Monotherapy with 5-FC is limited because of the frequent development of resistance in many fungal species. Due to this primary resistance, 5-FC is used mainly in combination with other antifungals (primarily AmB). In combination with AmB, 5-FC can be used to treat severe systemic mycoses, such as cryptococcosis, candidosis, chromoblastomycosis, and aspergillosis. In addition, 5-FC has been investigated in combination with other agents also including fluconazole (FLU), ketoconazole (KTZ), itraconazole (ITRA), voriconazole (VORI), and echinocandins, e.g., micafungin (MICA), posaconazole (POSA), and caspofungin (CAS). The severe side effects of 5-FC include hepatotoxicity and bone marrow depression. In most patients, these side effects are concentration dependent, predictable, possibly avoidable with close monitoring to maintain 5-FC concentrations at <100 mg/L, and reversible with drug discontinuation or reduction of dose. 5-FC is well absorbed after oral administration, penetrates into body tissues well, and is excreted mainly by the kidneys.

J. Chandra, Ph.D. • M.A. Ghannoum, Ph.D. (✉)
Department of Dermatology, Center for Medical Mycology, University Hospitals Cleveland, Case Western Reserve University, 11100 Euclid Ave, Cleveland, OH 44106-5028, USA
e-mail: mag3@case.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_28

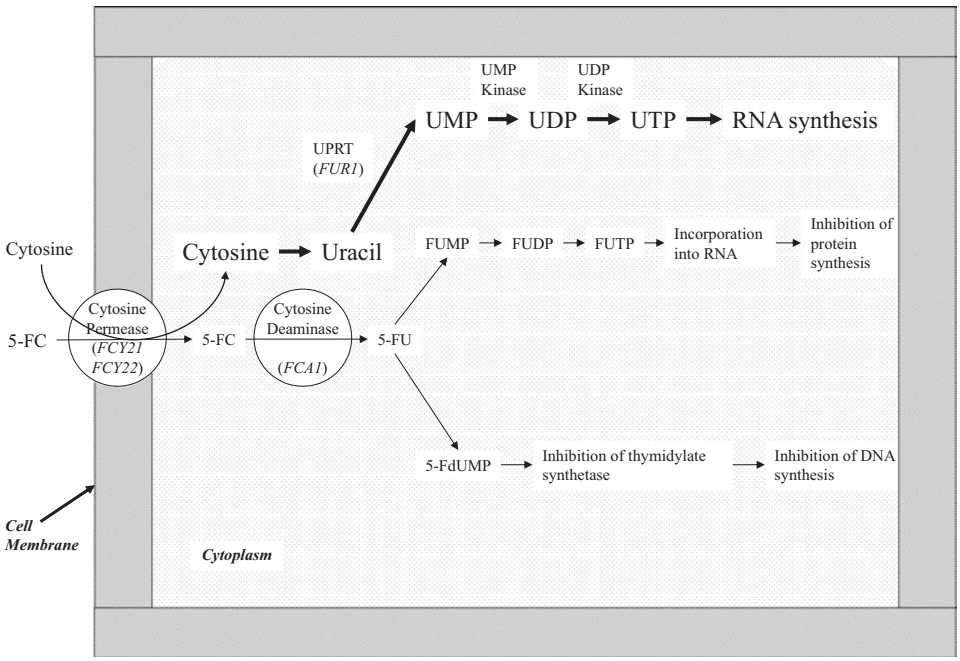


Fig. 28.1 Intracellular pathway and mechanism of action of 5-flucytosine. Abbreviations: 5-FC (5-flucytosine), 5-FU (5-fluorouracil), FUMP (5-fluorouridine monophosphate), FUDP (5-fluorouridine diphosphate), FUTP (5-fluorouridine triphosphate), FdUMP (5-fluorodeoxy-uridine monophosphate), UPRT (uracil phosphoribosyltransferase), UMP (uridine monophosphate), UDP (uridine diphosphate), UTP (uridine triphosphate). Genes *FCA1, FUR1, FCY21,* and *FCY22* encode for cytosine deaminase, UPRT, and two purine-cytosine permeases, respectively

3 Mechanism of Action

The antimycotic activity of 5-FC results from its rapid conversion to 5-fluorouracil (5-FU) by the enzyme cytosine deaminase, within susceptible fungal cells. There are two mechanisms involved by which 5-fluorouracil exerts its antifungal activity (Fig. 28.1). The first mechanism includes the conversion of 5-fluorouracil through 5-fluorouridine monophosphate (FUMP) and 5-fluorouridine diphosphate (FUDP) to 5-fluorouridine triphosphate (FUTP). FUTP is further incorporated into fungal RNA in place of uridylic acid; this alters the amino-acylation of tRNA, disturbs the amino acid pool, and inhibits protein synthesis. The second mechanism is the metabolic conversion of 5-FU to 5-fluorodeoxyuridine monophosphate (FdUMP) by uridine monophosphate pyrophosphorylase (Fig. 28.1). FdUMP is a potent inhibitor of thymidylate synthase, which is a key enzyme involved in DNA synthesis and nuclear division. Thus, 5-FC acts by interfering with pyrimidine metabolism and protein synthesis in the fungal cell, ultimately resulting in cell lysis and death.

4 5-Flucytocine in Combination

Monotherapy with 5-FC is limited because of the frequent development of resistance in many fungal species. Due to this primary resistance, 5-FC is used mainly in combination with other antifungals. In combination with AmB, 5-FC can be used to treat severe systemic mycoses, such as cryptococcosis, candidosis, chromoblastomycosis, and aspergillosis. All the combination studies are summarized in Table 28.1.

4.1 Double Combination

Pharmacokinetic/pharmacodynamic in animal models have suggested that the combination of AmB and 5-FC has an additive effect when treating disseminated candidiasis [5]. The additive fungicidal effect of 5-FC is neglectable when the AmB dose is high. Also 5-FC has higher tissue penetrance due to its small size and is thus recommended in combination with amphotericin B for treatment of a number of diseases including *Candida* endocarditis, *Candida* CNS infections, and ocular candidiasis [6, 7].

A recent study reported that combination of AmB and 5-FC can reduce the risk of dying from cryptococcal meningitis by 40 % compared to treatment with amphotericin B alone [8]. This study was a randomized, three-group trial of induction therapy for cryptococcal meningitis in patients with human immunodeficiency virus infection and showed that AmB and 5-FC combination was associated with significantly increased rates of yeast clearance from cerebrospinal fluid [8]. Patients received AmB for 4 weeks and 5-FC for 2 weeks and fewer deaths occurred by day 14 among patients receiving both AmB and 5-FC compared to patients receiving AmB alone (15 vs. 25 deaths by day 14) [8]. Another study by Bicanic et al. [9] involved 64 HIV-seropositive, antiretroviral therapy-naive patients who experienced their first episode of cryptococcal meningitis and were randomized to receive either (1) AmB,

Table 28.1 Combination table

Organism	Disease	Drug combination	Study	Drug effect	Reference
Cryptococcus	Meningitis	AmB + 5-FC	Clinical	Positive	[8]
Cryptococcus	Meningitis	AmB + 5-FC	Clinical	Positive	[9]
C. albicans	Spondylitis	Liposomal AmB + 5-FC	Clinical	Positive	[10]
C. parapsilosis	Candiduria (pyelonephritis)	CAS + 5-FC	Clinical	Positive	[11]
L. major	Lesions	GCV + 5-FC	Murine in vivo model	Completely cured by 2 weeks	[4]
Aspergillus	–	CAS + 5-FC + AmB/VORI	In vitro	Synergistic	[14]
C. albicans and C. neoformans	–	5-FC + AmB + FLU	In vitro	Combined effect depending on conc. of drug	[15]
C. neoformans	Meningitis	AmB colloidal dispersion + 5-FC + FLU	Murine in vivo model	Triple combination gave the greatest antifungal activity	[13]
Candida sp.	*Candida*-infected human platelet-fibrin clots	5-FC + MICA + VORI	Platelet-fibrin clots used as simulated endocardial vegetation model	5-FC + MICA were superior in fungal burden reduction than VORI	[16]
Cladophialophora bantiana	Disseminated infection	POSA + MICA + 5-FC	Murine model	Half of the animals survived when treatment extended for 30 days	[17]

0.7 mg/kg per day, plus 5-FC, 25 mg/kg four times per day (group 1; 30 patients), or (2) AmB, 1 mg/kg per day, plus 5-FC 25 mg/kg four times per day (group 2; 34 patients). Regimens were given for 2 weeks, followed by treatment with oral fluconazole. The early fungicidal activity, as determined by results of serial, quantitative cerebral spinal fluid cryptococcal cultures, was significantly greater for group 2 than for group 1. This case study showed that AmB (1 mg/kg per day) plus 5-FC is more rapidly fungicidal than is standard dose of 0.7 mg/day AmB plus 5-FC [9].

Candida spondylitis is rare, and most cases have been reported in adults. The most frequent symptom is localized pain [10]. Rapid diagnosis and treatment are important in order to prevent vertebral collapse. Recently, Storm et al. [10] published a case where a patient with *Candida* spondylitis failed 2 weeks of FLU combined with CAS. The infection relapsed despite 6 weeks of liposomal AmB followed by 2 months of FLU. They showed that 6-month therapy with high-dose liposomal AmB combined with 5-FC effectively cured the patient [10].

Candiduria may be a marker of serious fungal infections such as pyelonephritis. With the exception of FLU and 5-FC, antifungal drugs are not excreted into the urine as active drugs, making the management of infection due to fluconazole-resistant *Candida* difficult. Recently Garcia et al. [11] reported a case of recurrent *C. parapsilosis* candiduria in a kidney transplant recipient suffering from chronic ureteral obstruction requiring permanent ureteral catheterization. Attempts to remove the stent led to pyelonephritis episodes during which only *Candida* was isolated from the urine. Following several courses of azole-based therapy, the causative agent became resistant to FLU. In order to guarantee a constant antifungal action, CAS was infused in continuously. This treatment, combined with oral 5-FC, was well tolerated, where notably no medullar toxicity was noticed, and allowed a rapid (72 h) sterilization of the urine culture. This strategy may represent an interesting therapeutic alternative for the treatment of FLU-resistant symptomatic candiduria [11].

Recently, 5-FC in combination has been used to cure progressively growing lesions. Cutaneous leishmaniasis (CL) manifests as a localized self-healing lesion(s) that in rare cases develops to a nonhealing lesion. The nonhealing lesions are extremely difficult to treat with current therapies [12]. Despite ample evidence that development of an effective vaccine against leishmaniasis is possible there is still no vaccine available against any form of human leishmaniasis [12]. Davoudi et al. [4] developed a double-drug-sensitive strain of *Leishmania major* using advances in gene targeting technology by stably introducing into the chromosome a modified herpes simplex virus thymidine kinase gene (tk), conferring increased sensitivity to ganciclovir (GCV), and a *Saccharomyces cerevisiae* cytosine deaminase gene (cd), conferring sensitivity to 5-FC [4]. In vitro studies showed that the homozygous *L. major* (tk-cd+/+) promastigotes were killed by either drug alone, and together the drugs acted

synergistically. In vivo infection studies showed that progressively growing lesions in BALB/c mice, caused by *L. major* (tk-cd+/+), were completely cured by 2 weeks of treatment with GCV or 5-FC alone or in combination. Treated animals showed no signs of reoccurrence of infection for at least 4 months when the experiments were terminated [4]. In another study, Davoudi et al. [12] assessed the transgenic lmtkcd+/+ strain as a live vaccine model to determine the time necessary to develop a protective immune response. C57BL/6 mice were inoculated with the transgenic lmtkcd+/+ strain, and treated with a combination of GCV and 5-FC, at the time of inoculation (day 0) or at day 8 after inoculation. The rate of protection, parasite burden, and type of immune response were checked, and the results showed that complete protection is induced by inoculation of lmtkcd+/+ strain if treatment with GCV and 5-FC is initiated on day 8 post-inoculation [12].

Overall, these studies show that 5-FC can be used successfully in double combinations and has reduced the risk of severe infections/deaths caused due to cryptococcal meningitis or candidiasis.

4.2 Triple Combination

Studies with animals and in vitro studies have demonstrated that triple combinations with 5-FC plus AmB and FLU have significantly improved mycologic activity against meningitis caused by *C. neoformans* compared to the activity of AmB or FLU used alone [13].

Dannaoui et al. [14] used microdilution broth checkerboard techniques based on the National Committee for Clinical and Laboratory Institute Standards methodology to study triple-antifungal combinations against clinical isolates of *Aspergillus fumigatus* and *A. terreus*. The influences of the end-point definition (partial or complete inhibition) and the mode of reading (visually or spectrophotometrically) were determined. Interactions between antifungal drugs were also evaluated by agar diffusion tests. The triple combination of CAS with 5-FC and AmB was synergistic for all the isolates tested [14]. The triple combination of CAS with 5-FC and VORI was also mostly synergistic; but complex interactions were obtained for some isolates, with synergy or antagonism depending on the concentrations of CAS and VORI [14].

Earlier, Ghannoum et al. [15] studied three-drug regimens (AmB, FLU, and 5-FC) against three isolates each of *C. albicans* and *C. neoformans*. Using a microdilution plate technique, two-drug combinations against both *C. albicans* and *C. neoformans* were tested. Results of two-drug combinations against both *C. albicans* and *C. neoformans* showed that inhibition with AmB + FLU was greater than inhibition by either drug alone. At low concentrations of AmB, addition of 5-FC enhanced the growth inhibitory effect against *C. albicans*, but antagonism was noted at higher concentrations of AmB. Data for the three drug pairs (AmB + FLU; AmB + 5-FC; FLU + 5-FC) were presented as contour plots, which showed distinct upwards or downwards contour plots for *C. neoformans* and *C. albicans*. Results of the three-drug combinations for *C. neoformans* showed inhibition with AmB at varying concentrations of FLU and a single fixed dose of 5-FC. In the presence of 5-FC, the combined effects of AmB and FLU on the growth of *C. neoformans* remained indifferent; when the AmB concentration was greater than approximately 1–1.2 μg/mL, addition of 5-FC had no further effect on growth. These investigators suggested that the effects of a drug combination on in vitro fungal growth depend on the ratios and concentrations of the drugs used, as well as the fungal strains tested, apart from other differences related to variations in study design, pathogens, drug conditions, and regimens.

Diamond et al. [13] evaluated the antifungal efficacy of AmB colloidal dispersion (ABCD) combined with 5-FC with and without FLU in a murine model of cryptococcal meningitis. Meningitis was established in male BALB/c mice by intracerebral injection of *C. neoformans*. Treatment with 5-FC with or without FLU dissolved in the sole source of drinking water was started on day 2; animals were sacrificed on day 16, and the numbers of fungal colonies in the brain were quantified [13]. A survival rate of 100 % was achieved with ABCD plus 5-FC without FLU; however, the addition of FLU was required to prevent weight loss ($P < 0.00001$) and to achieve the maximum antifungal effect ($P < 0.00001$). The only region of dose combinations for which the 99 % confidence intervals were less than 100 colony-forming units (CFU/g) of brain was defined by ABCD at 5.0–7.5 mg/kg combined with 5-FC at 20–60 mg/kg/day and FLU at 30–40 mg/kg/day. The triple combination of ABCD plus 5-FC and FLU was necessary to achieve the greatest antifungal activity [13].

In vitro pharmacodynamic model (PDM) simulation of serum antifungal concentrations was used to predict the value of combination antifungal regimens against *Candida* sp. endocarditis [16]. Pai et al. [16] investigated the effects of combinations of 5-FC, MICA, and VORI against *Candida*-infected human platelet-fibrin clots, used as simulated endocardial vegetations (SEVs). Single clinical bloodstream isolates of *C. albicans, Candida glabrata, Candida parapsilosis,* and *Candida tropicalis* were used. All four isolates were susceptible to 5-FC and this drug was the most active against all *Candida* spp. except for *C. tropicalis*. The triple combination of 5-FC plus VORI plus MICA was no better than single or dual agents against any of the *Candida* spp. In general, 5-FC and MICA were superior in their rates and extents of fungal burden reduction compared to VORI against *Candida*-infected SEVs.

Marine et al. [17] tested 10-day courses of AMB, MICA, VORI, 5-FC, and POSA alone and in double or triple combinations in the treatment of disseminated infections caused by *Cladophialophora bantiana* in a murine model. Animals were monitored for survival for 40 days [17]. These investigators found that the triple combination of POSA+MICA+5-FC improved the survival with respect to both the control group and the component monotherapies, but all the animals died during the experiment. When treatment with this triple therapy was extended up to 30 days, half of the animals survived for at least 10 months. Combination therapy with the three drugs (POSA, MICA, and 5-FC) appears to be a promising option for the treatment of *C. bantiana* infections [17].

Jackson et al. [18] performed clinical studies examining the efficacy of FLU combined with 5-FC and/or short-course AmB in the treatment of cryptococcal meningitis in Africa. The primary endpoint was rate of clearance of infection (early fungicidal activity, EFA), while the secondary endpoints related to safety and mortality [18]. Forty patients (25 % with Glasgow Coma Scale <15) were analyzed. These investigators reported that EFA for the triple-combination arm was greater than both the AmB + FLU arm: -0.50 ± 0.15 log CFU/day vs. -0.38 ± 0.19 log CFUs/day ($P = 0.03$), and the FLU + 5-FC (-0.28 ± 0.17). Combined analysis across steps revealed that addition of 5-FC and AmB had significant, independent additive effects on EFA, with trends toward fewer early deaths with the addition of 5-FC (4/41 vs. 11/39, $P = 0.05$) and fewer deaths overall with the addition of AmB (13/39 vs. 20/40, $P = 0.1$) [18].

Overall, these in vitro and animal studies as well as clinical studies demonstrate the advantages of using double and/or triple combinations of 5-FC with other antifungals against susceptible fungi.

5 Resistance Mechanisms

Although 5-FC resistance mechanisms have been investigated and reviewed in depth [19, 20], new data using molecular techniques warrants a review of our current knowledge of 5-FC resistance mechanism. Two mechanisms of 5-FC resistance can be distinguished: (a) decreased cellular transport or uptake of 5-FC due to the loss of enzymatic activity (loss of permease activity) responsible for conversion to FUMP. The resistance due to decreased uptake is found in *S. cerevisiae* and *C. glabrata*; this mechanism does not seem to be important in *C. albicans* or *C. neoformans* [19, 20]. (b) Resistance of 5-FC may also result from increased synthesis of pyrimidines, which compete with the fluorinated antimetabolites of 5-FC and thus decrease its antimycotic activity [21]. Defective uridine monophosphate pyrophosphorylase is the most common type of acquired 5-FC resistance in fungal cells [22].

In a recent study, Song et al. [23] performed comparative transcriptome analysis by employing two-component system mutants (tco1Δ and tco2Δ) exhibiting low 5-FC susceptibility. A total of 177 5-FC-responsive genes were identified, and many of them were found to be regulated by *TCO1* or *TCO2* (e.g., APSES-like transcription factor), and Mbs1 (Mbp1- and Swi4-like protein 1). Expression analysis revealed that *MBS1* was regulated in response to 5-FC in a Tco2/Hog1-dependent manner. Moreover, deletion of *MBS1* resulted in increased susceptibility to 5-FC. Intriguingly, *MBS1* played pleiotropic roles in diverse cellular processes of *C. neoformans*, including ergosterol biosynthesis, genotoxic and oxidative stress responses, and melanin production [23].

Edlind et al. [24] showed that resistant mutants occurred at a relatively low frequency (2×10^{-7}) when *C. glabrata* cells were exposed to 1 μg/ml 5-FC (32-fold above the MIC, but less than 1/10 of typical serum levels) [24]. Three of six mutants characterized were 5-FU cross-resistant, suggesting a mutation downstream of the *FCY1* gene (cytosine deaminase), which was confirmed by sequence analysis of the *FUR1* gene (uracil phosphoribosyl transferase). The remaining three mutants had *FCY1* mutations. To ascertain the effects of 5-FC resistance mutations on enzyme function, mutants were isolated in ura3 strains. Three of seven mutants harbored *FCY1* mutations and failed to grow in uridine-free, cytosine-supplemented medium, consistent with inactive *FCY1*. The remainder grew in this medium and had wild-type *FCY1*; further analysis revealed these to be mutated in the *FCY2L* homolog of *S. cerevisiae FCY2* (purine-cytosine transporter). Based on this analysis, three 5-FC-resistant clinical isolates and mutations were identified in *FUR1* and *FCY1*.

In a separate study, Vandeputte et al. [25] conducted a study on laboratory mutants of *C. glabrata* obtained by exposure of a wild-type isolate to 5-FC. Based on their susceptibility to 5-fluorouracil (5-FU), two of these mutants were selected for further analysis of the molecular mechanisms of 5-FC resistance. One mutant, resistant to both compounds, exhibited a missense mutation in the gene coding the cytosine deaminase and a decrease in the expression level of the gene coding the uridine monophosphate pyrophosphorylase. The other mutant that showed a reduced susceptibility to 5-FC and 5-FU exhibited an overexpression of the genes coding the thymidylate synthase and a cytosine permease, associated with a missense mutation in the last gene. Thus, besides mutations in the *FUR1* gene which represent the most common cause of resistance to 5-FC, this study showed that other mechanisms also occur in *C. glabrata* [25].

Hope et al. [1] investigated primary resistance in *C. albicans* to 5-FC in 25 strains by identifying and sequencing the genes *FCA1, FUR1, FCY21*, and *FCY22,* which code for cytosine deaminase, uracil phosphoribosyltransferase (UPRT),

Table 28.2 Genes involved in flucytosine resistance mechanisms

Genes	Protein	Function	Reference
TCO1/TCO2/MBS1	Transcription factor Mbs1	Role in diverse cellular processes (ergosterol biosysnthesis, genotoxic and oxidative stress responses, and melanin production)	[23]
FCA1/FCY1	Cytosine deaminase	Converting 5-fluorocytosine to 5-fluorouracil	[1, 24, 26]
FUR1	Uracil phosphoribosyl transferase	Converting uracil to uridine monophosphate	[1, 24]
FCY2/FCY21/22	Purine cytosine permeases	Purine cytosine transporter	[1, 24, 26]
CDC21	Cdc21p	Nucleotide metabolism	[27]
E2F1	Transcription factor	Transcription	[27]
SGN1, RIM4, and *NOP1*	Sgn1p, Rim4p, Nop1p	RNA processing	[27]

and two purine-cytosine permeases, respectively. These proteins are involved in pyrimidine salvage and 5-FC metabolism. An association between a polymorphic nucleotide and resistance to 5-FC was found within *FUR1* where the substitution of cytidylate for thymidylate at nucleotide position 301 results in the replacement of arginine with cysteine at amino acid position 101 in UPRT. Isolates that are homozygous for this mutation display increased levels of resistance to 5-FC, whereas heterozygous isolates have reduced susceptibility. Three-dimensional protein modeling of UPRT suggests that the Arg101Cys mutation disturbs the quaternary structure of the enzyme, which is postulated to compromise optimal enzyme activity. A single resistant isolate, lacking the above polymorphism in *FUR1*, has a homozygous polymorphism in *FCA1* that results in a glycine-to-aspartate substitution at position 28 in cytosine deaminase [1].

Florent et al. [26] elucidated the molecular mechanisms of 5-FC resistance and 5-FC/FLU cross-resistance in 11 genetically and epidemiologically unrelated clinical isolates of *Candida lusitaniae*. They showed that the levels of transcription of the *FCY2* gene encoding purine-cytosine permease (PCP) in the isolates were similar to those in the wild-type strain. Nucleotide sequencing of the *FCY2* alleles revealed that 5-FC and 5-FC/FLU resistance could be correlated with a cytosine-to-thymine substitution at nucleotide 505 in the *FCY2* genes of seven clinical isolates, resulting in a nonsense mutation and in a putative nonfunctional truncated PCP of 168 amino acids. Reintroducing a *FCY2* wild-type allele at the *FCY2* locus of a ura3 auxotrophic strain derived from the clinical isolate CL38 *FCY2* (C505T) restored levels of susceptibility to antifungals comparable to those of the wild-type strains. In the remaining four isolates, a polymorphic nucleotide was found in *FCY1* where the nucleotide substitution T26C resulted in the amino acid replacement M9T in cytosine deaminase. Introducing this mutated allele into a 5-FC- and 5-FC/FLU-resistant *FCY1*Delta strain failed to restore antifungal susceptibility, while susceptibility was obtained by introducing a wild-type *FCY1* allele. A correlation between the fcy1 T26C mutation and both 5-FC and 5-FC/FLU resistances was found. This study demonstrated that only two genetic events occurred in 11 unrelated clinical isolates of *C. lusitaniae* to support 5-FC and 5-FC/FLU resistance: either the nonsense mutation C505T in the *FCY2* gene or the missense mutation T26C in the *FCY1* gene [26].

Zhao et al. [27] performed cDNA microarray analysis to identify global transcriptional profiles of drug-specific responses in the dermatophyte *Trichophyton rubrum* after exposure to 5-FC. cDNA microarray was constructed from the *T. rubrum*-expressed sequence tag (ESTs) database, the minimum inhibitory concentration (MIC) of 5-FC was determined, and microarray hybridization and data analysis were applied. A total of 474 genes were found differentially expressed, 196 showed an increase in expression and 278 showed a decrease in expression. Marked downregulation of genes involved in nucleotide metabolism (such as *CDC21*), transcription (such as *E2F1*), and RNA processing (such as *SGN1, RIM4,* and *NOP1*) was observed. Other genes involved in signal transduction, chaperones, inorganic ion transport, secondary metabolite biosynthesis, amino acid transport, lipid transport, and potential drug resistance mechanism were also affected by 5-FC [27]. Quantitative real-time RT-PCR of the selected genes confirmed the reliability of the microarray results. Taken together, these studies showed that several genes including *FUR1*, *FCY1*, *FCY2, FCY21*, and *FCY22* contribute to 5-FC resistance in fungal pathogens. The genes involved in resistance mechanisms are summarized in Table 28.2.

6 Conclusions

In summary, the emergence of new data demonstrating the current low level of yeast resistance to 5-FC and the favorable antifungal activity of 5-FC in combination with other

antifungal agents should renew the interest in this drug and could lead to a "rebirth" of this agent as a useful adjunct in combination studies.

References

1. Hope WW, Tabernero L, Denning DW, Anderson MJ. Molecular mechanisms of primary resistance to flucytosine in *Candida albicans*. Antimicrob Agents Chemother. 2004;48:4377–86.
2. Wang F, Zamora G, Sun CH, Trinidad A, Chun C, Kwon YJ, Berg K, Madsen SJ, Hirschberg H. Increased sensitivity of glioma cells to 5-fluorocytosine following photo-chemical internalization enhanced nonviral transfection of the cytosine deaminase suicide gene. J Neurooncol. 2014;118:29–37.
3. Yurttas C, Berchtold S, Malek NP, Bitzer M, Lauer UM. Pulsed versus continuous application of the prodrug 5-fluorocytosine to enhance the oncolytic effectiveness of a measles vaccine virus armed with a suicide gene. Hum Gene Ther Clin Dev. 2014;25:85–96.
4. Davoudi N, Tate CA, Warburton C, Murray A, Mahboudi F, McMaster WR. Development of a recombinant *Leishmania major* strain sensitive to ganciclovir and 5-fluorocytosine for use as a live vaccine challenge in clinical trials. Vaccine. 2005;23:1170–7.
5. Hope WW, Warn PA, Sharp A, Reed P, Keevil B, Louie A, Walsh TJ, Denning DW, Drusano GL. Optimization of the dosage of flucytosine in combination with amphotericin B for disseminated candidiasis: a pharmacodynamic rationale for reduced dosing. Antimicrob Agents Chemother. 2007;51:3760–2.
6. Pappas PG, Kauffman CA, Andes D, Benjamin Jr DK, Calandra TF, Edwards Jr JE, Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD, Infectious Diseases Society of America. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis. 2009;48:503–35.
7. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Florl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ, Group EFIS. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect. 2012;18 Suppl 7:19–37.
8. Day JN, Chau TT, Lalloo DG. Combination antifungal therapy for cryptococcal meningitis. N Engl J Med. 2013;368:2522–3.
9. Bicanic T, Wood R, Meintjes G, Rebe K, Brouwer A, Loyse A, Bekker LG, Jaffar S, Harrison T. High-dose amphotericin B with flucytosine for the treatment of cryptococcal meningitis in HIV-infected patients: a randomized trial. Clin Infect Dis. 2008;47:123–30.
10. Storm L, Lausch KR, Arendrup MC, Mortensen KL, Petersen E. Vertebral infection with *Candida albicans* failing caspofungin and fluconazole combination therapy but successfully treated with high dose liposomal amphotericin B and flucytosine. Med Mycol Case Rep. 2014;6:6–9.
11. Garcia H, Guitard J, Peltier J, Tligui M, Benbouzid S, Elhaj SA, Rondeau E, Hennequin C. Caspofungin irrigation through percutaneous calicostomy catheter combined with oral flucytosine to treat fluconazole-resistant symptomatic candiduria. J Mycol Med. 2015;25:87–90.
12. Davoudi N, Khamesipour A, Mahboudi F, McMaster WR. A dual drug sensitive L. major induces protection without lesion in C57BL/6 mice. PLoS Negl Trop Dis. 2014;8, e2785.
13. Diamond DM, Bauer M, Daniel BE, Leal MA, Johnson D, Williams BK, Thomas AM, Ding JC, Najvar L, Graybill JR, Larsen RA. Amphotericin B colloidal dispersion combined with flucytosine with or without fluconazole for treatment of murine cryptococcal meningitis. Antimicrob Agents Chemother. 1998;42:528–33.
14. Dannaoui E, Lortholary O, Dromer F. *In vitro* evaluation of double and triple combinations of antifungal drugs against *Aspergillus fumigatus* and *Aspergillus terreus*. Antimicrob Agents Chemother. 2004;48:970–8.
15. Ghannoum MA, Fu Y, Ibrahim AS, Mortara LA, Shafiq MC, Edwards Jr JE, Criddle RS. *In vitro* determination of optimal antifungal combinations against *Cryptococcus neoformans* and *Candida albicans*. Antimicrob Agents Chemother. 1995;39:2459–65.
16. Pai MP, Samples ML, Mercier RC, Spilde MN. Activities and ultrastructural effects of antifungal combinations against simulated *Candida* endocardial vegetations. Antimicrob Agents Chemother. 2008;52:2367–76.
17. Marine M, Pastor FJ, Guarro J. Combined antifungal therapy in a murine model of disseminated infection by *Cladophialophora bantiana*. Med Mycol. 2009;47:45–9.
18. Jackson AT, Nussbaum JC, Phulusa J, Namarika D, Chikasema M, Kanyemba C, Jarvis JN, Jaffar S, Hosseinipour MC, van der Horst C, Harrison TS. A phase II randomized controlled trial adding oral flucytosine to high-dose fluconazole, with short-course amphotericin B, for cryptococcal meningitis. AIDS. 2012;26:1363–70.
19. Jund R, Lacroute F. Genetic and physiological aspects of resistance to 5-fluoropyrimidines in *Saccharomyces cerevisiae*. J Bacteriol. 1970;102:607–15.
20. Whelan WL. The genetic basis of resistance to 5-fluorocytosine in *Candida* species and *Cryptococcus neoformans*. Crit Rev Microbiol. 1987;15:45–56.
21. Polak A. 5-Fluorocytosine—current status with special references to mode of action and drug resistance. Contrib Microbiol Immunol. 1977;4:158–67.
22. Francis P, Walsh TJ. Evolving role of flucytosine in immunocompromised patients: new insights into safety, pharmacokinetics, and antifungal therapy. Clin Infect Dis. 1992;15:1003–18.
23. Song MH, Lee JW, Kim MS, Yoon JK, White TC, Floyd A, Heitman J, Strain AK, Nielsen JN, Nielsen K, Bahn YS. A flucytosine-responsive Mbp1/Swi4-like protein, Mbs1, plays pleiotropic roles in antifungal drug resistance, stress response, and virulence of *Cryptococcus neoformans*. Eukaryot Cell. 2012;11:53–67.
24. Edlind TD, Katiyar SK. Mutational analysis of flucytosine resistance in *Candida glabrata*. Antimicrob Agents Chemother. 2010;54:4733–8.
25. Vandeputte P, Pineau L, Larcher G, Noel T, Brethes D, Chabasse D, Bouchara JP. Molecular mechanisms of resistance to 5-fluorocytosine in laboratory mutants of *Candida glabrata*. Mycopathologia. 2011;171:11–21.
26. Florent M, Noel T, Ruprich-Robert G, Da Silva B, Fitton-Ouhabi V, Chastin C, Papon N, Chapeland-Leclerc F. Nonsense and missense mutations in FCY2 and FCY1 genes are responsible for flucytosine resistance and flucytosine-fluconazole cross-resistance in clinical isolates of *Candida lusitaniae*. Antimicrob Agents Chemother. 2009;53:2982–90.
27. Zhao R, Bin W, Diao Y, Yang J, Liu T, Peng J, Jin Q. Global transcriptional profiles of *Trichophyton rubrum* in response to Flucytosine. Sci China C Life Sci. 2009;52:1173–85.

Echinocandin Resistance

29

David S. Perlin

1 Introduction

Fungal diseases cause life-threatening illnesses such as meningitis and pneumonias, chronic asthma, other respiratory diseases, and recurrent diseases like oral and vaginal thrush. Invasive fungal infections are a consequence of underlying health problems often associated with immunosuppression [1]. Fungal infections often carry high mortality and successful patient management requires antifungal therapy. Yet, treatment options remain extremely limited due to restricted classes of antifungal agents and by the emergence of prominent antifungal drug resistance. Currently registered antifungal drugs represented by polyenes and azoles, flucytosine, and echinocandins target the cell membrane, nucleic acid biosynthesis, and cell wall, respectively [2]. The latter and most recently approved class, the echinocandins, are now recommended as primary therapy for non-neutropenic patients with invasive candidiasis [3]. It is estimated that 60 % of candidemia patients now receive an echinocandin for treatment or prophylaxis [4]. As worldwide use of echinocandins broadens, clinical failures due to resistant organisms are a concern, especially among certain *Candida* species. The development of echinocandin resistance among most susceptible organisms like *Candida albicans* is an uncommon event. Yet, there is a disturbing trend of increased resistance among strains of *Candida glabrata*, which are frequently cross-resistant to azole drugs. Echinocandin resistance is acquired during therapy and its mechanism is firmly established to involve amino acid changes in "hot-spot" regions of the Fks subunits of the target glucan synthase. These changes significantly decrease the sensitivity of the enzyme to drug resulting in higher MIC values and reduced pharmacodynamic responses. Biological factors that promote selection of Fks-resistant strains involve complex cellular stress response pathways. The use of broth microdilution assays to assess susceptibility can be problematic with some drug- and species-related variability among clinical microbiology laboratories. Clinical factors promoting resistance include expanding use of echinocandins for therapy and prophylaxis, and localized reservoirs such as those in the gastrointestinal tract or intra-abdominal infections, which can seed emergence of resistant organisms. A basic understanding of the resistance mechanism, along with cellular and clinical factors promoting resistance, will promote better strategies to overcome and prevent echinocandin resistance.

2 Fungal Cell Walls and 1,3-β-D-Glucan

The cell wall of human fungal pathogens is essential for maintaining cell shape and rigidity. It consists primarily of an interwoven mesh of glucans, mannoproteins, and chitin. In yeasts like *Candida albicans*, branched fibrils of 1,3-β-D glucan form a network that acts as a scaffold for other macromolecules [5, 6]. Short 1-6-β-D-glucan chains establish bridges between linear 1,3-β-D glucan and cell wall proteins that coat the external surface of the cell wall. The majority of these proteins are heavily mannosylated through both *O*- and *N*-glycosidic linkages. Most cell wall proteins are covalently linked to the growing wall structure via 1-6-β-D-glucan. Chitin is found both below the network of 1,3-β-D glucan and as a linker between glucans. In other pathogenic fungi, including *Aspergillus fumigatus* and *Cryptococcus neoformans*, many of the same polysaccharides and mannoproteins are found in the cell wall, but the organization is somewhat different [7, 8] as polymers occur with other linkages between glucose units or sugars (e.g., galactomannan) [9]. When synthesis of a functional cell wall is reduced or eliminated, either by gene disruption or by inhibition with an antifungal inhibitor, cell growth is often adversely impacted leading to lysis and death.

D.S. Perlin (✉)
Public Health Research Institute, New Jersey Medical School, Rutgers, The State University of New Jersey, 225 Warren Street, New Brunswick, NJ 07103, USA
e-mail: perlinds@njms.rutgers.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_29

3 Glucan Synthase

The fungal-specific enzyme 1,3-β-D glucan synthase (GS) is responsible for the biosynthesis of the central cell wall building block 1,3-β-D glucan. The enzyme is a membrane-associated complex that uses UDP-glucose to synthesize a 1,3-β-D glucan polysaccharide product 60 to 80 glucose residues in length. The enzyme has been extensively studied in *S. cerevisiae* [10], although it has also been studied in other yeasts and molds including *Neurospora crassa*, *Aspergillus nidulans*, and *Aspergillus fumigatus*; *Schizosaccharomyces pombe*; various *Candida species*; and *Cryptococcus neoformans*. GS is minimally a heterodimer involving a large integral membrane protein, encoded by *FKS* genes, that catalyzes the biosynthesis of 1,3-β-D-glucan and Rho, a regulatory GTP-binding protein. The *FKS* and *RHO1* genes are conserved across numerous fungal genera. A high degree of homology among members of the *FKS* gene family aided cloning of paralogs from *C. albicans* [11, 12], *C. neoformans* [13], *A. fumigatus* [14], *Neurospora crassa* [15], *P. carinii* [16], and other fungi [10]. Conservation of *FKS* extends to the plant kingdom as well, where an *FKS* homolog is associated with synthesis of plant 1,3-β-D glucan (callose) in cotton and barley [17, 18]. Likewise, *RHO1* genes have been identified and characterized in *C. albicans* [19], *C. neoformans* [20], and *A. fumigatus* [14]. Most yeast have three *FKS* genes, *FKS1*, *FKS2*, and *FKS3*. The *FKS1* gene is essential in *C. albicans* [12, 13] and other *Candida* spp., while in *C. glabrata*, *FKS1* and *FKS2* are functionally redundant [21]. The *FKS3* gene is expressed at a very low level relative to the other genes and its role is uncertain [22]. The GS enzyme complex has not been crystallized but it can be studied in an enriched form by a product entrapment technique [23, 24], which has allowed an evaluation of its kinetic properties [25].

4 Glucan Synthase Inhibitors and Echinocandins

There are three structural classes that define natural product inhibitors of 1,3-β-D glucan synthesis [10]. The first class are the lipopeptides including echinocandins, aerothricin lipopeptidolactones, and arborcandins. A second class comprises the glycolipid papulacandins, and a third class, the terpenoids, are represented by enfumafungin, ascosteroside, arundifungin, and ergokonin A. All GS inhibitor classes are noncompetitive with the biosynthetic substrate UDP-glucose. Cells exposed to GS inhibitors distort and lyse due to changes in cell wall glucans [26–28]. Of the three GS inhibitor classes, the echinocandins are best studied. The echinocandins are cyclic hexapeptides with an amide-linked fatty acyl side chain [29]. An early striking feature of this class was the potent activity of echinocandins in animal infection models due to *C. albicans* [30] and *Pneumocystis jiroveci* [31]. This led to medicinal chemistry efforts at Merck, Eli Lilly, and Fujisawa (Astellas) and the development of current semisynthetic echinocandins caspofungin, anidulafungin, and micafungin, respectively [32]. The US Food and Drug Administration has approved echinocandin drugs for the treatment of esophageal and invasive candidiasis, including candidemia, empirical therapy in febrile neutropenic patients, and prophylaxis in patients undergoing hematopoietic stem cell transplantation (HSCT) [33, 34]. The first in-class drug caspofungin was also approved for salvage therapy for patients with invasive aspergillosis refractory to conventional therapy [35]. Echinocandin drugs show in vitro fungicidal activity against susceptible *Candida* spp. [36, 37], although they are fungistatic against molds where they alter morphology, cell wall composition, and organization [38, 39]. The echinocandins are largely inactive against invasive *Zygomycetes, Cryptococcus* spp., or *Fusarium* spp. As echinocandin drugs have a distinct mechanism of action specific for glucan synthase, they are highly effective against yeasts with reduced susceptibility to azoles, such as *C. glabrata* and *C. krusei* [40–42]; they are also active against some *Candida* biofilms [43–46]. The echinocandins have an excellent therapeutic index with a low potential for renal or hepatic toxicity or serious drug-drug interactions [47, 48].

5 Antifungal Spectrum and Breakpoints

The CLSI and EUCAST have established standardized microbroth dilution susceptibility tests for *Candida* and echinocandins, which show uniformly potent activity against most *Candida* species including *C. albicans*, *C. glabrata*, *Candida tropicalis*, and *Candida krusei* [49, 50]. The *C. parapsilosis* complex (*Candida parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*) and *C. guilliermondii* are notable exceptions displaying higher echinocandin antifungal MIC values relative to other highly susceptible *Candida* species [51–56]. Intrinsic reduced susceptibility has an unclear clinical significance, as patients infected with these strains are successfully treated with echinocandin drugs [57], although clinical response may vary with patient population [58–60]. The effect of echinocandins on filamentous fungi in vitro is less prominent with molds like *A. fumigatus* and other *Aspergillus* spp., showing reduced growth and altered hyphae morphology [39]. The multidrug-resistant pathogen *Aspergillus lentulus* is largely unresponsive to echinocandin action [61]. For *A. fumigatus*, the echinocandin-induced change in cell wall morphology correlates with exposure of masked epitopes (e.g., 1,3-β-D glucan), which promote a robust immune response contributing to in vivo efficacy [62]. Echinocandins show similar in vitro behavior with black molds such as *Alternaria* spp., and hyalohyphomycetes such as *Scedosporium apiospermum* [63]. In contrast, *Rhizopus oryzae* and other zygomycetes are

Table 29.1 EUCAST and CLSI antifungal breakpoints for major *Candida* species[a]

Antifungal agent	MIC breakpoint (mg/L)									
	Candida albicans		*Candida glabrata*		*Candida krusei*		*Candida parapsilosis*		*Candida tropicalis*	
	S	R	S	R	S	R	S	R	S	R
Anidulafungin										
EUCAST	0.03	0.03	0.06	0.06	0.06	0.06	0.002	4	0.06	0.06
CLSI	0.25	0.5	0.12	0.25	0.25	0.5	2	4	0.25	0.5
Caspofungin										
EUCAST	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND
CLSI	0.25	0.5	0.12	0.25	0.25	0.5	2	4	0.25	0.5
Micafungin										
EUCAST	0.016	0.016	0.03	0.03	IE[c]	IE	0.002	2	IE	IE
CLSI	0.25	0.5	0.06	0.125	0.25	0.5	2	4	0.25	0.5

[a]Adapted from Arendrup et al. [72]
[b]ND: Not determined due to significant inter-laboratory variation in MIC ranges
[c]IE: Insufficient evidence (IE) due to small number of cases

largely unaffected by caspofungin [64]. Micafungin is active against mycelial forms of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis* but it is less active against yeast-like forms [65]. Like *Aspergillus* species, dermatophytes *Trichophyton rubrum* and *Microsporum canis* show diminished growth and malformed hyphae in response to echinocandins [66]. Finally, the neurotropic pathogen *Cryptococcus neoformans* is unresponsive to echinocandins [67, 68]. However, in vitro susceptibility can be overcome by addition of the calcineurin inhibitor FK506 [69]. Epidemiologic cutoff values (ECVs) have been determined for echinocandins against the most clinically important yeasts and molds from numerous global surveillance studies verifying the potent behavior of these drugs [70, 71]. The CLSI and EUCAST have also established species- and drug-specific clinical breakpoints (CBP) for echinocandin drugs based on extensive pharmacokinetic, microbiological, enzyme kinetic, and clinical response data [72, 73] (Table 29.1). See section on "Standardized Testing for Resistance."

6 Epidemiology of Echinocandin Resistance

Candida species isolates resistant to echinocandin drugs were first reported in 2005 [74]. Their frequency remains relatively low at less than 2–3 % with *C. albicans* and most other *Candida* species [75–78]. Yet, consistent with the broader application of echinocandin therapy, high MIC clinical isolates associated with clinical failures are more commonly reported [22, 25, 79–89]. Despite these reports, echinocandin resistance among most *Candida* species has been largely unchanged in the past decade [90]. However, this is not the case for *C. glabrata*, where echinocandin resistance is rising and there is serious cause for concern since many isolates also display azole resistance [91–93], which greatly limits therapy. The SENTRY Antimicrobial Surveillance Program reported echinocandin resistance of 8.0–9.3 % among bloodstream isolates (BSI) of *C. glabrata* from 2006 to 2010 [92]. In a study of *C. glabrata* bloodstream isolates from Duke hospital spanning 10 years, echinocandin resistance of *C. glabrata* rose from 2 to 3 % in 2001–2006 to more than 13 % in 2009–2010 [91]. Resistance is not uniform, as a study involving 1380 isolates of *C. glabrata* collected between 2008 and 2013 from four US cities showed that 3.1–3.6 % of the isolates were resistant to the echinocandin drugs [93]. This is consistent with rates of 3.6 and 5.7 % from anidulafungin and caspofungin, respectively, obtained from regional data of *Candida* non-albicans strains at US medical centers over a 6-year period (2006–2011) [90]. Yet, echinocandin resistance among *C. glabrata* has also coincided with a nearly parallel rise in azole resistance resulting in multidrug-resistant strains (Fig. 29.1). In a recent study covering 1032 isolates, nearly all isolates containing an *FKS* mutation were resistant to at least one echinocandin and 36 % were also resistant to fluconazole [93]. The expanding use of echinocandin and azole prophylaxis in many healthcare centers has prompted an epidemiologic shift with *C. glabrata* emerging as the most dominant fungal bloodstream pathogen [94, 95]. The development of echinocandin resistance typically occurs after prolonged therapy (3–4 weeks or longer) [87]. Yet, it has been observed to emerge shortly after the start of therapy [88, 96]. Echinocandin resistance in molds is rarely encountered but it has been reported for *A. fumigatus* [97] and the inherently multidrug-resistant *A. lentulus* [61].

7 Mechanism of Acquired Resistance

Echinocandin resistance resulting in clinical failures due to high MIC isolates involves modification of the catalytic subunit of glucan synthase, which is encoded by genes *FKS1*

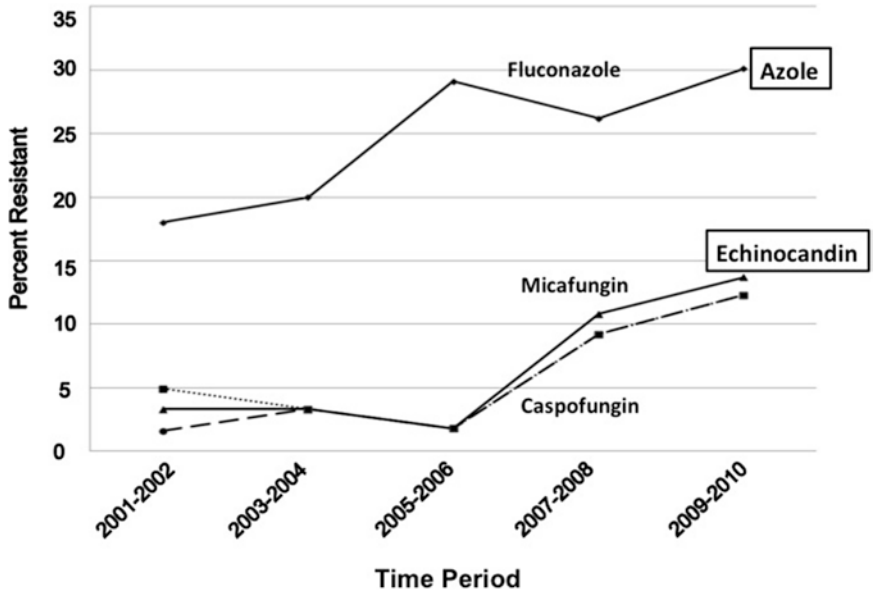


Fig. 29.1 Rise in antifungal resistance of *Candida glabrata* to azole (fluconazole) and echinocandin (anidulafungin, caspofungin, and micafungin) drugs from 2001 to 2010. Adapted from Alexander et al. [91]

Table 29.2 Amino acid substitutions in hot-spot regions of Fks subunits of glucan synthase associated with reduced echinocandin susceptibility[a,b]

	FKS1p				FKS2p			
	Hot spot 1		Hot spot 2		Hot spot 1		Hot spot 2	
	AA Pos							
C. albicans	641	FLTLSLRDP	1357	DWIRRYTL				
C. dubliniensis	641	FLTLSLRDP	1357	DWIRRYTL				
C. glabrata	625	FLILSLRDP	1340	DWVRRYTL	659	FLILSLRDP	1374	DWIRRYTL
C. kefyr	54*	FLTLSLRDP	769*	DWVRRYTL				
C. krusei	655	FLILSIRDP	1364	DWIRRYTL				
C. lusitaniae	634*	FLTLSLRDP	**	DWIRRYTL				
C. tropicalis	76*	FLTLSLRDP	792*	DWIRRYTL				
C. parapsilosis	652	FLTLSLRDA	1369	DWIRRYTL				
C. metapsilosis	104*	FLTLSLRDA	821*	DWIRRYTL				
C. orthopsilosis	39*	FLTLSLRDA	756*	DWVRRYTL				
C. guilliermondii	632	FMALSLRDP	1347	DWIRRYTL				
C. lipolytica	662	FLILSLRDP	1387	DWIRRCVL				
S. cerevisiae	639	FLVLSLRDP	1353	DWVRRYTL	658	FLILSLRDP	1372	DWVRRYTL

[a]Adapted from Arendrup and Perlin [98]
[b]Red: Strong resistance, difficult to treat; yellow: weak resistance, can be overcome with dosing; blue: natural polymorphism, elevated MIC but treatable; green: no effect on susceptibility

and/or *FKS2*. Echinocandin drugs are not substrates for multidrug transporters like azole drugs [42], and other cellular mechanisms conferring azole resistance do not affect echinocandin susceptibility. This has led to the recommendation of echinocandins as preferred therapy for infections involving azole-resistant strains of *Candida.* Echinocandin resistance is well characterized and known to be conferred by restricted mutations in two highly conserved "hot-spot" regions of the *FKS* genes [34] (Table 29.2). These *fks* mutations result in amino acid substitutions that induce elevated MIC values from 20- to 100-fold and reduced sensitivity of glucan synthase (IC_{50}) to drug by 50- to 3000-fold [22, 25, 99]. These less susceptible *fks* mutant strains respond poorly to echinocandin drugs in pharmacodynamic models of infection

[100–103], and the manifestation of characteristic *fks* mutations is associated with reduced clinical response [104–106]. The presence of an *FKS* mutation was found to be the only independent risk factor associated with echinocandin failure among *C. glabrata* isolates in a study of patients with invasive candidiasis [105]. The *FKS* resistance mechanism has been observed in many *Candida* species including *C. albicans, C. glabrata*, *C. tropicalis, C. krusei, C. kefyr, and C. lusitaniae* [96, 107, 108]. In all *Candida* species, except *C. glabrata*, mutations occur within two "hot-spot" regions of *FKS1*, encoding residues Phe641-Pro649 and Arg1361 (Table 29.2). In *C. albicans*, amino acid substitutions at Ser645 and Phe641 are the most abundant (Table 29.2). In *C. glabrata,* mutations occur in the homologous hot-spot regions of *FKS1 and FKS2* [22, 99], although mutations are observed within *FKS2* at twice the frequency of FKS1 [22, 34, 109]. Amino acid substitutions at Fks1 positions F625 and S629 and Fks2 positions F659 and S663 are most prominent inducing elevated MIC values (Table 29.2) [98]. In some cases, nonsense mutations and deletions are observed in *FKS1* or *FKS2* in *C. glabrata* [22, 98, 112]. Mutations in *FKS1* or *FKS2* can significantly alter the relative expression of their genes [21, 22], which can influence susceptibility. In *C. glabrata*, *FKS2* expression is calcineurin dependent and downregulated by FK506 [111], and echinocandin resistance conferred by mutations in *FKS2* are mitigated with FK506 [21]. A third hot-spot modification W695 (*S. cerevisiae*) was recently identified by in vitro selection [112], but it is not associated with clinical failures.

8 Biofilms

Biofilms also play a factor in resistance. They are one of the most important microbial communities encountered in nature, and they are well established to contribute to antifungal drug resistance [113]. It has been shown for echinocandin drugs that the extensive production of β-glucan within the extracellular glucan matrix helps sequester drugs by decreasing their concentration at the cell membrane surface [114]. Decreasing glucan productions, either by genetic or chemical means, increases the susceptibility to antifungal agents [115]. Genetic factors that regulate glucan formation promoting drug-sequestering biofilms include Rlm, Smi1, and glucan synthase Fks1 [115].

9 Acquired Resistance and Microbial Fitness

It is a well-established microbial paradigm that drug resistance often carries a fitness cost for microorganisms. The most prominent amino acid substitutions (e.g., Ser645 in *C. albicans*) in hot-spot regions of Fks subunits have been shown to decrease the catalytic efficiency for glucan biosynthesis [22, 25]. This reduced capacity for glucan production results in compensatory changes that alter cell wall morphology [116], which can reduce the fitness of such mutants. In *C. albicans*, reduced fitness has been observed for *fks* mutants in animal models [21, 22, 116]. The *fks* mutant strains compete weakly with their wild-type equivalents [116]. This reduced competition may account for the observation that resistance is with acquired during therapy and patient-patient transmission is not observed.

10 Cellular Stress and Drug Tolerance

The inhibition of glucan synthase following exposure of cells to an echinocandin drug induces significant cellular stress. In response, fungi activate a wide range of adaptive mechanisms that promote survival by helping protect against cell stress [117, 118]. These stress adaptation responses often result in drug-tolerant cells with elevated in vitro MIC values to echinocandins. Yet, they are not typically associated with clinical failures [119–121], as drug-exposed cells are less robust because glucan synthase is inhibited. Cell wall stress is sensed by receptors such Mtl2 and Wsc1, which induce stress tolerance involving cell wall integrity, protein kinace C (PKC), calcineurin-Crz1, and HOG [122, 123] interacting pathways. Hsp90 is an important protein that helps induce tolerance through its major client proteins calcineurin, along with its effector Crz1 [124–126]. Genetic or chemical impairment of Hsp90 function diminishes the ability of *C. albicans* and *C. glabrata* to develop tolerance in the presence of caspofungin [126, 127].

Chitin and glucans comprise the major structural components of the fungal cell wall and there is a prominent biosynthetic interdependence for both constituents [128]. Therefore, it is not surprising that echinocandin exposure results in compensatory increases in chitin synthesis to strengthen the cell wall and resistant drug action. Cell wall mutants with higher basal chitin contents are less susceptible to caspofungin [122, 123, 129, 130] and they confer reduced pharmacodynamics responses in animal model [131]. Paradoxical growth at very high drug levels has also been linked to prominent compensatory responses in chitin biosynthesis [132, 133]. Finally, defects in sphingolipid biosynthesis can differentially alter in drug-dependent fashion responses to echinocandin drugs. This mixed susceptibility phenotype is linked to interactions of the aliphatic tail of echinocandins and membrane sphingolipids [134, 135].

In general, tolerance pathways are insufficient to result in clinical drug failure. Yet, they are important for stabilizing cells in the presence of drug, and may account for stasis behavior of cells exposed to echinocandin drugs in animal

model systems [102]. Even though these cells are not sufficiently resistant to induce therapeutic failures, they are poised to develop higher level resistance, as the drug-tolerant state allows cells sufficient time to overcome drug action by forming stable *FKS* mutations. It is not entirely clear how this ultimately occurs, although it may involve defects in DNA repair. Genome plasticity, observed widely in *C. albicans* and *C. glabrata* in response to azole drugs [136, 137], may also emerge as a factor for echinocandin drugs [138].

11 Mechanisms of Inherent Reduced Susceptibility

Candida parapsilosis complex (*C. parapsilosis sensu stricto, Candida orthopsilosis, and Candida metapsilosis*) and *C. guilliermondii* are intrinsically less susceptible in vitro to echinocandin drugs (MIC 0.5–8 μg/mL) relative to other highly susceptible *Candida* species [70, 95, 139], which prompted the CLSI to adopt higher breakpoints [73]. The clinical significance of this reduced susceptibility is unclear since patients can be successfully treated with echinocandins at standard dosages [54–56]; however, clinical efficacy may vary with patient population [58–60]. The underlying molecular mechanism appears to be naturally occurring polymorphisms in *FKS* hot-spot regions, which confer reduced sensitivity of glucan synthase to drug [140]. In *C. parapsilosis* complex, a highly conserved Pro660 is converted to alanine at the distal edge of hot-spot 1. Enzyme kinetic inhibition studies demonstrated that glucan synthase from the *C. parapsilosis* group were 10- to 50-fold less to echinocandin drugs than from enzymes obtained from highly susceptible species like *C. albicans* [140]. Furthermore, an engineered lab strain and clinical isolates of *C. albicans* and *C. glabrata* strains containing amino acid substitutions at this position display comparable decreases in target enzyme sensitivity and increased MIC values [140]. An additional I1359V polymorphism is observed in hot-spot 2 of *C. orthopsilosis* and *S. cerevisiae*, which confers higher MIC values. *C. guilliermondii* shows several additional amino acid polymorphisms in HS1 [140], although their relative contribution to overall insensitivity is unclear.

Cryptococcus neoformans is inherently resistant to echinocandin drugs even though 1,3 glucan synthase is essential and appears fully inhibited by echinocandin drugs in vitro [141]. It has been suggested that capsular melanin may help protect but capsule-deficient strains are also unresponsive to drug [142]. Finally, *Aspergillus lentulus*, a sibling species of *A. fumigatus*, is inherently resistant to a wide range of antifungal drugs including the echinocandins. The mechanism of this resistant is unclear but appears to be independent of *FKS* mutations [143].

12 Serum Effects on Drug Action

The echinocandin drugs are highly serum protein bound (>98 %), which reduces their relative in vitro efficacy causing a shift in MIC [144–146]. The magnitude of the shift depends on the specific drugs with anidulafungin and micafungin showing a larger relative shift than caspofungin. A consequence of this shift in efficacy is that serum alters the relative fungicidal properties of the drugs, often resulting in fungistatic behavior against certain *Candida* species [147, 148]. The serum effects are more pronounced with mutant strains carrying *FKS* mutations [149].

13 Standardized Testing for Resistance

The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have established comparable standards for broth microdilution (BMD) antifungal susceptibility testing of echinocandins against *Candida* species [53, 150, 151]. The objective for susceptibility testing is to establish an in vitro assessment that differentiates infecting strains as either susceptible or likely to respond to therapy or as resistant with an enhanced probability to fail therapy. In the case of echinocandin drugs, it is essential to capture high MIC strains containing *FKS* mutations. Initially, the CLSI used clinical and microbiological data to establish a preliminary common clinical breakpoint (CBP) for all three echinocandins against *Candida* spp. [120]. However, resistant strains with *FKS* mutations were often misclassified by this CBP [25, 152]. In response, the CLSI revised the CBP based on pharmacokinetic, microbiological, enzyme kinetic, and clinical data and established new species- and drug-specific breakpoints that better accounted for strains containing *FKS* mutations [73] (Table 29.3). However, the lower CBPs presented a clinical microbiology testing challenge, as BMD testing using either CLSI and EUCAST failed to promote consistent inter-laboratory test results without major errors (misclassifying wild-type strains as resistant or *fks*-containing mutants as susceptible) between laboratory groups [153–154]. Disturbingly, there were wide modal ranges encountered with *C. glabrata* and caspofungin [153–155]. Consistent MIC results were obtained for micafungin and anidulafungin, and it was suggested that they could serve as testing surrogates for the class to assess resistance [98, 156, 157]. EUCAST has now established species-specific clinical breakpoints for micafungin against *C. albicans, C. glabrata,* and *C. parapsilosis* [72], and they have established breakpoints for anidulafungin to accommodate use of these compounds in some clinical situations [72, 158]. EUCAST has not set caspofungin breakpoints and does not currently

Table 29.3 Anidulafungin and micafungin ECVs for eight species of Candida*

Antifungal agent tested	Species	No. of isolates	MIC (μg/mL)		ECV (μg/mL)[a]		
			Range	Mode	≥95 %	≥97.5 %	≥99 %
Anidulafungin	*C. albicans*	8210	0.008–2	0.03	0.06	0.12	0.12
	C. glabrata	2680	0.008–4	0.06	0.12	0.12	0.25
	C. parapsilosis	3976	0.008–8	2	4	8	8
	C. tropicalis	2042	0.008–2	0.03	0.12	0.12	0.12
	C. krusei	322	0.008–2	0.06	0.12	0.25	0.25
	C. lusitaniae	234	0.008–1	0.25	1	1	1
	C. guilliermondii	222	0.03–4	1	4	8	8
	C. dubliniensis	131	0.015–4	0.03	0.12	0.12	0.12
Micafungin	*C. albicans*	7874	0.008–4	0.015	0.03	0.03	0.03
	C. glabrata	3102	0.008–4	0.015	0.03	0.03	0.03
	C. parapsilosis	3484	0.015–4	1	2	4	4
	C. tropicalis	1605	0.008–8	0.015	0.06	0.06	0.12
	C. kruse	617	0.015–1	0.06	0.25	0.25	0.25
	C. lusitaniae	258	0.008–≥16	0.25	0.5	0.5	1
	C. guilliermondii	234	0.015–8	0.5	2	2	4
	C. dubliniensis	117	0.008–8	0.06	0.12	0.12	0.12

[a]Adapted from Pfaller et al. [49]
Calculated ECVs comprising ≥95 %, ≥97.5 %, or ≥99 % of the statistically modeled MIC population

recommend caspofungin MIC testing for clinical decision making involving echinocandin drugs [72]. Epidemiological cutoff values (ECV or ECOFF), which define the upper limit of the wild-type MIC population in the absence of a known resistance mechanism [49, 159], have been defined for anidulafungin and micafungin against common *Candida* species (Table 29.3). The ECV does not replace the BP, but it provides additional information for clinical decision making when a BP is not available. Although the designation of NWT does not allow a clinician to determine whether a particular isolate will respond to a particular antifungal agent, it does allow for a more informed decision based on how wild-type organisms would likely respond to therapy.

Rather than seeking testing surrogates or special conditions for BMD to distinguish wild-type strains from resistant isolates containing an *FKS* hot-spot mutation, it has been suggested that direct molecular testing for resistance mutations may provide a reliable alternative [160]. Direct DNA sequencing or real-time probing with allele-specific molecular probes provides an easy and unequivocal assessment of the resistance potential. The presence of an *FKS* mutation is the most important independent risk factor in predicting echinocandin therapeutic responses among patients with invasive candidiasis [104, 105, 110], which is well supported by extensive pharmacodynamics, MIC, and biochemical data [161, 162]. One criticism of this approach is that molecular testing requires specific knowledge of known resistance mechanisms and an unknown mechanism would not be detected. Yet, this probability is sufficiently remote given the large body of current data. Molecular testing to directly identify mutant strains containing *FKS* mutations would eliminate the current controversy surrounding some susceptibility testing, which prevents an accurate determination of resistance.

14 Paradoxical Growth Effects

The "paradoxical effect" refers to the unusual behavior of echinocandin drugs in susceptibility testing assays to show strong growth inhibition at low and moderate levels of drugs and then loss of inhibition at supra high drug concentrations, well in excess of the MIC. First described by Stevens and colleagues, it is a commonly observed property of echinocandin drugs [163]. This behavior is largely conditional as paradoxical strains show normal susceptibility properties following culture. The mechanism responsible for paradoxical growth is unclear, but is unrelated to mutations in *FKS* [124, 164]. It is not due to antifungal degradation or instability. The drug-induced growth behavior is more consistent with adaptive stress responses, which can lead to reduced susceptibility. In one instance, a paradoxical *C. albicans* strain showed a 900 % increase in chitin content [133]. Consistent with changes in cell wall composition, remodeling is observed [165, 166]. The paradoxical effect is eliminated by serum, chitin synthase inhibitor nikkomycin Z, and calcineurin pathway inhibitors [167], and in *C. albicans* mutants that lack phosphatidylinositol-(4,5)-bisphosphate 5′-phosphatase [167]. Paradoxical behavior has been observed in a murine model of pulmonary aspergillosis [168] and in a patient with pulmonary aspergillosis [169]. Paradoxical growth in response to caspofungin in *Candida* species does not confer

survival advantage in a Drosophila or moth model of candidiasis [165, 170]. The clinical significance of the paradoxical growth remains unclear, as the drug levels necessary to induce it exceed normal human dosing levels.

15 Risk Factors for Resistance Emergence

The gastrointestinal (GI) tract is colonized with *Candida* species, often at very high burdens [171–178], which are in the form of a complex microbial biofilm [179]. Typically, drug penetration varies across the biofilm and drug concentrations in the glucan matrix are irregular [114]. This creates a drug exposure environment that can select for resistant variants, which may desorb from the biofilm and cause systemic infections. As biofilms are difficult to eradicate, they can form a resistance reservoir that seeds resistant infections. Similarly, intra-abdominal candidiasis occurs in 40 % or more of patients following repeated gastrointestinal surgery, GI perforation, or necrotizing pancreatitis [180]. The high burden of *Candida* in this protected space with poor drug penetration creates a strong selection for resistant variants. Prophylaxis is another potential source for resistance. Prior and repeated exposure to echinocandin drugs is a risk factor development of resistance. As the *FKS* resistance mechanism is a prominent risk factor for therapeutic failure [105], resistance emergence is directly linked prior to exposure [106, 181, 182]. Antifungal prophylaxis with an azole or echinocandin class drug is standard prevention in many clinical settings with immunosuppressed patients at high risk for development of invasive fungal infections. Echinocandin drugs have been used because they have favorable pharmacokinetics and safety profile, and they are active against azole-resistant yeasts and molds. Both micafungin and caspofungin have been successfully applied for this purpose in adults [183–186] and children [187]. Meta-analyses have confirmed that echinocandin prophylaxis reduces the incidence of invasive fungal infections greater than fluconazole or itraconazole [188, 189]. Micafungin is FDA approved for prophylaxis of *Candida* infections in patients undergoing hematopoietic SCT or expected to be neutropenic for at least 10 days [190] and the European Society of Clinical Microbiology and Infectious Diseases guidelines also recommend micafungin for prophylaxis against *Candida* infections in allogeneic HSCT adult and pediatric patients, as well as in pediatric patients with acute myeloid and recurrent leukemia [191]. A consequence of the expanding use of echinocandins for prophylaxis is that patient drug exposure is on the rise, which has implication for inducing higher rates of echinocandin drug resistance, especially among resistance-prone organisms like *C. glabrata*. Even more concerning is the high prevalence of multidrug-resistant *C. glabrata* isolates cross-resistant to both azole- and echinocandin-class drugs [91, 192–196]. The coevolution of azole and echinocandin multidrug resistance among *C. glabrata* is an alarming trend [91]. Breakthrough infections involving *C. albicans* are also reported in patients following transplantation who received micafungin prophylaxis [197]. It is not surprising that broadening patient exposure to echinocandin drugs would promote development of resistance. Echinocandin prophylaxis may continue to fuel an increase in the frequency of isolates that are resistant to multiple classes of antifungal drugs. Furthermore, prior antifungal exposure, especially with fluconazole, leads to genomic instability, which increases azole resistance [138] and may potentially predispose for enhanced mutations leading to *FKS*-mediated drug resistance.

16 Conclusions

Echinocandin resistance among *Candida* species is low but significant, especially among *C. glabrata* where high-frequency resistance is often associated with azole resistance resulting in multidrug-resistant strains. Characteristic mutations in hot-spot regions of *FKS* genes encoding glucan synthase remain the most significant factor responsible for resistant isolates that are refractory to therapy. However, in response to echinocandin action, cellular stress response pathways induce drug-adapted persister states, which can ultimately facilitate development of stable *FKS*-resistant genotypes. Host factors that promote resistance include biofilm formation within the gastrointestinal tract and intra-abdominal candidiasis. The widespread use of echinocandin prophylaxis needs to be monitored for its effects on promoting enhanced drug exposure and resistance emergence. Effective antibiotic stewardship is required, especially in certain settings where resistance is prominent. Finally, new drug- and species-specific breakpoints have resulted in testing challenges, which may require drug surrogates for the class, but it may be more prudent to transition to sequence-based evaluation of *FKS* genotypes as the new gold standard for resistance assessment for all echinocandin drugs.

Acknowledgments David S. Perlin is supported by grants from National Institutes of Health (AI069397 and AI109025) and Astellas Pharma.
Disclosures Dr. Perlin serves on scientific advisory boards for Merck, Astellas, Amplyx, Cidara,and Synexis and he receives grant support from Astellas, Cidara and Amplyx. He is an inventor in US patent 8,753,819 entitled “Assays for Resistance to Echinocandin-Class Drugs.”

References

1. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, et al. Hidden killers: human fungal infections. Sci Transl Med. 2012;4:165rv113.
2. Odds FC, Brown AJ, Gow NA. Antifungal agents: mechanisms of action. Trends Microbiol. 2003;11:272–9.
3. Pappas PG, Kauffman CA, Andes D, Benjamin Jr DK, Calandra TF, et al. Clinical practice guidelines for the management of

candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis. 2009;48:503–35.
4. Cleveland AA, Farley MM, Harrison LH, Stein B, Hollick R, et al. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. Clin Infect Dis. 2012;55:1352–61.
5. Klis FM, Mol P, Hellingwerf K, Brul S. Dynamics of cell wall structure in Saccharomyces cerevisiae. FEMS Microbiol Rev. 2002;26:239–56.
6. Klis FM, de Groot P, Hellingwerf K. Molecular organization of the cell wall of Candida albicans. Med Mycol. 2001;39 Suppl 1:1–8.
7. Bernard M, Latge JP. Aspergillus fumigatus cell wall: composition and biosynthesis. Med Mycol. 2001;39:9–17.
8. Reese AJ, Doering TL. Cell wall alpha-1,3-glucan is required to anchor the Cryptococcus neoformans capsule. Mol Microbiol. 2003;50:1401–9.
9. Fukazawa Y, Kagaya K, Shinoda T. Cell wall polysaccharides of pathogenic yeasts. Curr Top Med Mycol. 1995;6:189–219.
10. Douglas CM. Fungal beta(1,3)-D-glucan synthesis. Med Mycol. 2001;39:55–66.
11. Douglas CM, D'Ippolito JA, Shei GJ, Meinz M, Onishi J, et al. Identification of the FKS1 gene of Candida albicans as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother. 1997;41:2471–9.
12. Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, et al. Cloning of the Candida albicans homolog of Saccharomyces cerevisiae GSC1/FKS1 and its involvement in beta-1,3-glucan synthesis. J Bacteriol. 1997;179:4096–105.
13. Thompson JR, Douglas CM, Li W, Jue CK, Pramanik B, et al. A glucan synthase FKS1 homolog in cryptococcus neoformans is single copy and encodes an essential function. J Bacteriol. 1999;181:444–53.
14. Beauvais A, Bruneau JM, Mol PC, Buitrago MJ, Legrand R, et al. Glucan synthase complex of Aspergillus fumigatus. J Bacteriol. 2001;183:2273–9.
15. Schimoler-O'Rourke R, Renault S, Mo W, Selitrennikoff CP. Neurospora crassa FKS protein binds to the (1,3)beta-glucan synthase substrate, UDP-glucose. Curr Microbiol. 2003;46:408–12.
16. Kottom TJ, Limper AH. Cell wall assembly by Pneumocystis carinii. Evidence for a unique gsc-1 subunit mediating beta-1,3-glucan deposition. J Biol Chem. 2000;275:40628–34.
17. Li J, Burton RA, Harvey AJ, Hrmova M, Wardak AZ, et al. Biochemical evidence linking a putative callose synthase gene with (1→3)-beta-D-glucan biosynthesis in barley. Plant Mol Biol. 2003;53:213–25.
18. Cui X, Shin H, Song C, Laosinchai W, Amano Y, et al. A putative plant homolog of the yeast beta-1,3-glucan synthase subunit FKS1 from cotton (Gossypium hirsutum L.) fibers. Planta. 2001;213:223–30.
19. Kondoh O, Tachibana Y, Ohya Y, Arisawa M, Watanabe T. Cloning of the *RHO1* gene from *Candida albicans* and its regulation of beta-1,3-glucan synthesis. J Bacteriol. 1997;179:7734–41.
20. Tanaka K, Nambu H, Katoh Y, Kai M, Hidaka Y. Molecular cloning of homologs of RAS and RHO1 genes from Cryptococcus neoformans. Yeast. 1999;15:1133–9.
21. Katiyar SK, Alastruey-Izquierdo A, Healey KR, Johnson ME, Perlin DS, et al. Fks1 and Fks2 are functionally redundant but differentially regulated in Candida glabrata: implications for echinocandin resistance. Antimicrob Agents Chemother. 2012;56:6304–9.
22. Garcia-Effron G, Lee S, Park S, Cleary JD, Perlin DS. Effect of Candida glabrata FKS1 and FKS2 mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. Antimicrob Agents Chemother. 2009;53:3690–9.
23. Kang MS, Elango N, Mattia E, Au-Young J, Robbins PW, et al. Isolation of chitin synthetase from *Saccharomyces cerevisiae*. Purification of an enzyme by entrapment in the reaction product. J Biol Chem. 1984;259:14966–72.
24. Awald P, Zugel M, Monks C, Frost D, Selitrennikoff CP. Purification of 1,3-b-glucan synthase from *Neurospora crassa* by product entrapment. Exper Mycol. 1993;17:130–41.
25. Garcia-Effron G, Park S, Perlin DS. Correlating echinocandin MIC and kinetic inhibition of fks1 mutant glucan synthases for Candida albicans: implications for interpretive breakpoints. Antimicrob Agents Chemother. 2009;53:112–22.
26. Mizoguchi J, Saito T, Mizuno K, Hayano K. On the mode of action of a new antifungal antibiotic, aculeacin A: inhibition of cell wall synthesis in Saccharomyces cerevisiae. J Antibiot (Tokyo). 1977;30:308–13.
27. Baguley BC, Rommele G, Gruner J, Wehrli W. Papulacandin B: an inhibitor of glucan synthesis in yeast spheroplasts. Eur J Biochem. 1979;97:345–51.
28. Onishi J, Meinz M, Thompson J, Curotto J, Dreikorn S, et al. Discovery of novel antifungal (1,3)-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother. 2000;44:368–77.
29. Hammond M. Chemical and structure activity studies on the echinocandin lipopeptides. In: Rippon J, Fromtling R, editors. Cutaneous antifungal agents. New York: Marcel Dekker; 1993. p. 395–420.
30. Bartizal K, Abruzzo G, Trainor C, Krupa D, Nollstadt K, et al. In vitro antifungal activities and in vivo efficacies of 1,3-b-D-glucan synthesis inhibitors L-671,329, L-646,991, tetrahydroechinocandin B, and L-687,781, a papulacandin. Antimicrob Agents Chemother. 1992;36:1648–57.
31. Schmatz DM, Powles M, McFadden DC, Pittarelli LA, Liberator PA, et al. Treatment and prevention of *Pneumocystis carinii* pneumonia and further elucidation of the *P. carinii* life cycle with 1,3-b-glucan synthesis inhibitor L-671,329. J Protozool. 1991;38:151S–3.
32. Wiederhold NP, Lewis RE. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. Expert Opin Investig Drugs. 2003;12:1313–33.
33. Turner MS, Drew RH, Perfect JR. Emerging echinocandins for treatment of invasive fungal infections. Expert Opin Emerg Drugs. 2006;11:231–50.
34. Perlin DS. Current perspectives on echinocandin class drugs. Future Microbiol. 2011;6:441–57.
35. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, et al. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis. 2008;46:327–60.
36. Barchiesi F, Spreghini E, Tomassetti S, Arzeni D, Giannini D, et al. Comparison of the fungicidal activities of caspofungin and amphotericin B against Candida glabrata. Antimicrob Agents Chemother. 2005;49:4989–92.
37. Ernst EJ, Klepser ME, Ernst ME, Messer SA, Pfaller MA. In vitro pharmacodynamic properties of MK-0991 determined by time-kill methods. Diagn Microbiol Infect Dis. 1999;33:75–80.
38. Bowman JC, Abruzzo GK, Flattery AM, Gill CJ, Hickey EJ, et al. Efficacy of caspofungin against Aspergillus flavus, Aspergillus terreus, and Aspergillus nidulans. Antimicrob Agents Chemother. 2006;50:4202–5.
39. Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, et al. The antifungal echinocandin caspofungin acetate kills growing cells of Aspergillus fumigatus in vitro. Antimicrob Agents Chemother. 2002;46:3001–12.
40. Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, et al. Caspofungin activity against clinical isolates of fluconazole-resistant Candida. J Clin Microbiol. 2003;41:5729–31.
41. Bachmann SP, Patterson TF, Lopez-Ribot JL. In vitro activity of caspofungin (MK-0991) against Candida albicans clinical isolates

displaying different mechanisms of azole resistance. J Clin Microbiol. 2002;40:2228–30.
42. Niimi K, Maki K, Ikeda F, Holmes AR, Lamping E, et al. Overexpression of Candida albicans CDR1, CDR2, or MDR1 does not produce significant changes in echinocandin susceptibility. Antimicrob Agents Chemother. 2006;50:1148–55.
43. Bachmann SP, Ramage G, VandeWalle K, Patterson TF, Wickes BL, et al. Antifungal combinations against Candida albicans biofilms in vitro. Antimicrob Agents Chemother. 2003;47:3657–9.
44. Ferreira JA, Carr JH, Starling CE, de Resende MA, Donlan RM. Biofilm formation and effect of caspofungin on biofilm structure of Candida species bloodstream isolates. Antimicrob Agents Chemother. 2009;53:4377–84.
45. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of Candida biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother. 2002;46:1773–80.
46. Simitsopoulou M, Peshkova P, Tasina E, Katragkou A, Kyrpitzi D, et al. Species-specific and drug-specific differences in susceptibility of Candida biofilms to echinocandins: characterization of less common bloodstream isolates. Antimicrob Agents Chemother. 2013;57:2562–70.
47. Chen SC, Slavin MA, Sorrell TC. Echinocandin antifungal drugs in fungal infections: a comparison. Drugs. 2011;71:11–41.
48. Kofla G, Ruhnke M. Pharmacology and metabolism of anidulafungin, caspofungin and micafungin in the treatment of invasive candidosis: review of the literature. Eur J Med Res. 2011;16:159–66.
49. Pfaller MA, Espinel-Ingroff A, Bustamante B, Canton E, Diekema DJ, et al. Multicenter study of anidulafungin and micafungin MIC distributions and epidemiological cutoff values for eight Candida species and the CLSI M27-A3 broth microdilution method. Antimicrob Agents Chemother. 2014;58:916–22.
50. Pfaller MA, Messer SA, Woosley LN, Jones RN, Castanheira M. Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. J Clin Microbiol. 2013;51:2571–81.
51. Garcia-Effron G, Canton E, Peman J, Dilger A, Roma E, et al. Epidemiology and echinocandin susceptibility of Candida parapsilosis sensu lato species isolated from bloodstream infections at a Spanish university hospital. J Antimicrob Chemother. 2012;67:2739–48.
52. Spreghini E, Orlando F, Tavanti A, Senesi S, Giannini D, et al. In vitro and in vivo effects of echinocandins against Candida parapsilosis sensu stricto, Candida orthopsilosis and Candida metapsilosis. J Antimicrob Chemother. 2012;67:2195–202.
53. Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Moet GJ, et al. Comparison of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Etest methods with the CLSI broth microdilution method for echinocandin susceptibility testing of Candida species. J Clin Microbiol. 2010;48:1592–9.
54. Mora-Duarte J, Betts R, Rotstein C, Colombo AL, Thompson-Moya L, et al. Comparison of caspofungin and amphotericin B for invasive candidiasis. N Engl J Med. 2002;347:2020–9.
55. Kale-Pradhan PB, Morgan G, Wilhelm SM, Johnson LB. Comparative efficacy of echinocandins and nonechinocandins for the treatment of Candida parapsilosis infections: a meta-analysis. Pharmacotherapy. 2010;30:1207–13.
56. Colombo AL, Perfect J, DiNubile M, Bartizal K, Motyl M, et al. Global distribution and outcomes for Candida species causing invasive candidiasis: results from an international randomized double-blind study of caspofungin versus amphotericin B for the treatment of invasive candidiasis. Eur J Clin Microbiol Infect Dis. 2003;22:470–4.
57. Fernandez-Ruiz M, Aguado JM, Almirante B, Lora-Pablos D, Padilla B, et al. Initial use of echinocandins does not negatively influence outcome in Candida parapsilosis bloodstream infection: a propensity score analysis. Clin Infect Dis. 2014;58:1413–21.
58. Ghannoum MA, Chen A, Buhari M, Chandra J, Mukherjee PK, et al. Differential in vitro activity of anidulafungin, caspofungin and micafungin against Candida parapsilosis isolates recovered from a burn unit. Clin Microbiol Infect. 2009;15:274–9.
59. Kabbara N, Lacroix C, Peffault de Latour R, Socie G, Ghannoum M, et al. Breakthrough C. parapsilosis and C. guilliermondii blood stream infections in allogeneic hematopoietic stem cell transplant recipients receiving long-term caspofungin therapy. Haematologica. 2008;93:639–40.
60. Forrest GN, Weekes E, Johnson JK. Increasing incidence of Candida parapsilosis candidemia with caspofungin usage. J Infect. 2008;56:126–9.
61. Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. Aspergillus lentulus sp. nov., a new sibling species of A. fumigatus. Eukaryot Cell. 2005;4:625–32.
62. Hohl TM, Feldmesser M, Perlin DS, Pamer EG. Caspofungin modulates inflammatory responses to Aspergillus fumigatus through stage-specific effects on fungal beta-glucan exposure. J Infect Dis. 2008;198:176–85.
63. Pfaller MA, Marco F, Messer SA, Jones RN. In vitro activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus*, *Fusarium*, *Rhizopus*, and other filamentous fungi. Diagn Microbiol Infect Dis. 1998;30:251–5.
64. Espinel-Ingroff A. Comparison of In vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J Clin Microbiol. 1998;36:2950–6.
65. Nakai T, Uno J, Ikeda F, Tawara S, Nishimura K, et al. In vitro antifungal activity of Micafungin (FK463) against dimorphic fungi: comparison of yeast-like and mycelial forms. Antimicrob Agents Chemother. 2003;47:1376–81.
66. Motyl M, Nielsen Kahn J, Giacobbe R. In vitro susceptibiity of dermatophytes to CANCIDAS (caspofungin acetate); Chicago: ASM; 2003. p. Abstract M-1210.
67. Abruzzo GK, Flattery AM, Gill CJ, Kong L, Smith JG, et al. Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. Antimicrob Agents Chemother. 1997;41:2333–8.
68. Bartizal K, Gill CJ, Abruzzo GK, Flattery AM, Kong L, et al. In vitro preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). Antimicrob Agents Chemother. 1997;41:2326–32.
69. Del Poeta M, Cruz MC, Cardenas ME, Perfect JR, Heitman J. Synergistic antifungal activities of bafilomycin A(1), fluconazole, and the pneumocandin MK-0991/caspofungin acetate (L-743,873) with calcineurin inhibitors FK506 and L-685,818 against Cryptococcus neoformans. Antimicrob Agents Chemother. 2000;44:739–46.
70. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, et al. Wild-type MIC distributions and epidemiological cutoff values for the echinocandins and Candida spp. J Clin Microbiol. 2010;48:52–6.
71. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, et al. Wild-type minimum effective concentration distributions and epidemiologic cutoff values for caspofungin and Aspergillus spp. as determined by Clinical and Laboratory Standards Institute broth microdilution methods. Diagn Microbiol Infect Dis. 2010;67:56–60.
72. Arendrup MC, Cuenca-Estrella M, Lass-Florl C, Hope WW. Breakpoints for antifungal agents: an update from EUCAST

focussing on echinocandins against Candida spp. and triazoles against Aspergillus spp. Drug Resist Updat. 2013;16:81–95.
73. Pfaller MA, Diekema DJ, Andes D, Arendrup MC, Brown SD, et al. Clinical breakpoints for the echinocandins and Candida revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. Drug Resist Updat. 2011;14:164–76.
74. Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, et al. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. Antimicrob Agents Chemother. 2005;49:3264–73.
75. Pfaller MA, Messer SA, Moet GJ, Jones RN, Castanheira M. Candida bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008–2009). Int J Antimicrob Agents. 2011;38:65–9.
76. Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M. Geographic variations in species distribution and echinocandin and azole antifungal resistance rates among Candida bloodstream infection isolates: report from the SENTRY Antimicrobial Surveillance Program (2008 to 2009). J Clin Microbiol. 2011;49:396–9.
77. Castanheira M, Woosley LN, Diekema DJ, Messer SA, Jones RN, et al. Low prevalence of fks1 hot spot 1 mutations in a worldwide collection of Candida strains. Antimicrob Agents Chemother. 2010;54:2655–9.
78. Castanheira M, Messer SA, Jones RN, Farrell DJ, Pfaller MA. Activity of echinocandins and triazoles against a contemporary (2012) worldwide collection of yeast and moulds collected from invasive infections. Int J Antimicrob Agents. 2014;44:320–6.
79. Cleary JD, Garcia-Effron G, Chapman SW, Perlin DS. Reduced Candida glabrata susceptibility secondary to an FKS1 mutation developed during candidemia treatment. Antimicrob Agents Chemother. 2008;52:2263–5.
80. Garcia-Effron G, Chua DJ, Tomada JR, DiPersio J, Perlin DS, et al. Novel FKS mutations associated with echinocandin resistance in Candida species. Antimicrob Agents Chemother. 2010;54:2225–7.
81. Kahn JN, Garcia-Effron G, Hsu MJ, Park S, Marr KA, et al. Acquired echinocandin resistance in a Candida krusei isolate due to modification of glucan synthase. Antimicrob Agents Chemother. 2007;51:1876–8.
82. Laverdiere M, Lalonde RG, Baril JG, Sheppard DC, Park S, et al. Progressive loss of echinocandin activity following prolonged use for treatment of Candida albicans oesophagitis. J Antimicrob Chemother. 2006;57:705–8.
83. Miller CD, Lomaestro BW, Park S, Perlin DS. Progressive esophagitis caused by Candida albicans with reduced susceptibility to caspofungin. Pharmacotherapy. 2006;26:877–80.
84. Garcia-Effron G, Kontoyiannis DP, Lewis RE, Perlin DS. Caspofungin-resistant Candida tropicalis strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. Antimicrob Agents Chemother. 2008;52:4181–3.
85. Wiederhold NP, Grabinski JL, Garcia-Effron G, Perlin DS, Lee SA. Pyrosequencing to detect mutations in FKS1 that confer reduced echinocandin susceptibility in Candida albicans. Antimicrob Agents Chemother. 2008;52:4145–8.
86. Pfeiffer CD, Garcia-Effron G, Zaas AK, Perfect JR, Perlin DS, et al. Breakthrough invasive candidiasis in patients on micafungin. J Clin Microbiol. 2010;48:2373–80.
87. Thompson III GR, Wiederhold NP, Vallor AC, Villareal NC, Lewis II JS, et al. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to Candida glabrata infection. Antimicrob Agents Chemother. 2008;52:3783–5.
88. Lewis II JS, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. Rapid emergence of echinocandin resistance in Candida glabrata resulting in clinical and microbiologic failure. Antimicrob Agents Chemother. 2013;57(9):4559–61.
89. Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, et al. Candida spp. with acquired echinocandin resistance, France, 2004–2010. Emerg Infect Dis. 2012;18:86–90.
90. Pfaller MA, Jones RN, Castanheira M. Regional data analysis of Candida non-albicans strains collected in United States medical sites over a 6-year period, 2006–2011. Mycoses. 2014;57:602–11.
91. Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, et al. Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis. 2013;56:1724–32.
92. Pfaller MA, Castanheira M, Lockhart SR, Ahlquist AM, Messer SA, et al. Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of Candida glabrata. J Clin Microbiol. 2012;50:1199–203.
93. Pham CD, Iqbal N, Bolden CB, Kuykendall RJ, Harrison LH, et al. Role of FKS Mutations in Candida glabrata: MIC values, echinocandin resistance, and multidrug resistance. Antimicrob Agents Chemother. 2014;58:4690–6.
94. Lortholary O, Desnos-Ollivier M, Sitbon K, Fontanet A, Bretagne S, et al. Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 patients. Antimicrob Agents Chemother. 2011;55:532–8.
95. Tortorano AM, Prigitano A, Lazzarini C, Passera M, Deiana ML, et al. A 1-year prospective survey of candidemia in Italy and changing epidemiology over one decade. Infection. 2013;41:655–62.
96. Fekkar A, Meyer I, Brossas JY, Dannaoui E, Palous M, et al. Rapid emergence of echinocandin resistance during Candida kefyr fungemia treatment with caspofungin. Antimicrob Agents Chemother. 2013;57:2380–2.
97. Arendrup MC, Perkhofer S, Howard SJ, Garcia-Effron G, Vishukumar A, et al. Establishing in vitro-in vivo correlations for Aspergillus fumigatus: the challenge of azoles versus echinocandins. Antimicrob Agents Chemother. 2008;52:3504–11.
98. Arendrup MC, Perlin DS. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis. 2014;27:484–92.
99. Katiyar S, Pfaller M, Edlind T. Candida albicans and Candida glabrata clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother. 2006;50:2892–4.
100. Arendrup MC, Perlin DS, Jensen RH, Howard SJ, Goodwin J, et al. Differential in vivo activity of Anidulafungin, Caspofungin and Micafungin against C. glabrata with and without FKS resistance mutations. Antimicrob Agents Chemother. 2012;56(5):2435–42.
101. Howard SJ, Lestner JM, Sharp A, Gregson L, Goodwin J, et al. Pharmacokinetics and pharmacodynamics of posaconazole for invasive pulmonary aspergillosis: clinical implications for antifungal therapy. J Infect Dis. 2011;203:1324–32.
102. Slater JL, Howard SJ, Sharp A, Goodwin J, Gregson LM, et al. Disseminated Candidiasis caused by Candida albicans with amino acid substitutions in Fks1 at position Ser645 cannot be successfully treated with micafungin. Antimicrob Agents Chemother. 2011;55:3075–83.
103. Wiederhold NP, Najvar LK, Bocanegra RA, Kirkpatrick WR, Patterson TF. Caspofungin dose escalation for invasive candidiasis due to resistant Candida albicans. Antimicrob Agents Chemother. 2011;55:3254–60.
104. Lackner M, Tscherner M, Schaller M, Kuchler K, Mair C, et al. Position and numbers of FKS mutations in C. albicans selectively

influence in vitro and in vivo susceptibility to echinocandin treatment. Antimicrob Agents Chemother. 2014;58(7):3626–35.
105. Shields RK, Nguyen MH, Press EG, Kwa AL, Cheng S, et al. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. Antimicrob Agents Chemother. 2012;56:4862–9.
106. Beyda ND, John J, Kilic A, Alam MJ, Lasco TM, et al. FKS mutant Candida glabrata: risk factors and outcomes in patients with candidemia. Clin Infect Dis. 2014;59:819–25.
107. Jensen RH, Johansen HK, Arendrup MC. Stepwise development of a homozygous S80P substitution in Fks1p, conferring echinocandin resistance in Candida tropicalis. Antimicrob Agents Chemother. 2013;57:614–7.
108. Pasquale T, Tomada JR, Ghannoun M, Dipersio J, Bonilla H. Emergence of Candida tropicalis resistant to caspofungin. J Antimicrob Chemother. 2008;61:219.
109. Perlin DS. Echinocandin-resistant Candida: molecular methods and phenotypes. Curr Fungal Infect Rep. 2011;5(3):113–9.
110. Castanheira M, Woosley LN, Messer SA, Diekema DJ, Jones RN, et al. Frequency of fks mutations among Candida glabrata isolates from a 10-year global collection of bloodstream infection isolates. Antimicrob Agents Chemother. 2014;58:577–80.
111. Eng WK, Faucette L, McLaughlin MM, Cafferkey R, Koltin Y, et al. The yeast FKS1 gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin-dependent growth. Gene. 1994;151:61–71.
112. Johnson ME, Katiyar SK, Edlind TD. A new Fks hotspot for acquired echinocandin resistance in yeast, and its contribution to intrinsic resistance of Scedosporium species. Antimicrob Agents Chemother. 2011;55(8):3774–81.
113. d'Enfert C. Biofilms and their role in the resistance of pathogenic Candida to antifungal agents. Curr Drug Targets. 2006;7:465–70.
114. Mitchell KF, Taff HT, Cuevas MA, Reinicke EL, Sanchez H, et al. Role of matrix beta-1,3 glucan in antifungal resistance of non-albicans Candida biofilms. Antimicrob Agents Chemother. 2013;57:1918–20.
115. Desai JV, Bruno VM, Ganguly S, Stamper RJ, Mitchell KF, et al. Regulatory role of glycerol in Candida albicans biofilm formation. MBio. 2013;4:e00637-12.
116. Ben-Ami R, Garcia-Effron G, Lewis RE, Gamarra S, Leventakos K, et al. The fitness and virulence cost of fks1 mutations causing echinocandin-resistance in Candida albicans. J Infect Dis. 2011;204(4):626–35.
117. Perlin DS. Resistance to echinocandin-class antifungal drugs. Drug Resist Updat. 2007;10:121–30.
118. Walker LA, Gow NA, Munro CA. Fungal echinocandin resistance. Fungal Genet Biol. 2010;47:117–26.
119. Kartsonis N, Killar J, Mixson L, Hoe CM, Sable C, et al. Caspofungin susceptibility testing of isolates from patients with esophageal candidiasis or invasive candidiasis: relationship of MIC to treatment outcome. Antimicrob Agents Chemother. 2005;49:3616–23.
120. Pfaller MA, Diekema DJ, Ostrosky-Zeichner L, Rex JH, Alexander BD, et al. Correlation of MIC with outcome for Candida species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. J Clin Microbiol. 2008;46:2620–9.
121. Pfaller MA, Diekema DJ, Boyken L, Messer SA, Tendolkar S, et al. Effectiveness of anidulafungin in eradicating Candida species in invasive candidiasis. Antimicrob Agents Chemother. 2005;49:4795–7.
122. Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, et al. The PKC, HOG and Ca2+ signalling pathways coordinately regulate chitin synthesis in Candida albicans. Mol Microbiol. 2007;63:1399–413.
123. Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, et al. Stimulation of chitin synthesis rescues Candida albicans from echinocandins. PLoS Pathog. 2008;4, e1000040.
124. Cowen LE. Hsp90 orchestrates stress response signaling governing fungal drug resistance. PLoS Pathog. 2009;5, e1000471.
125. LaFayette SL, Collins C, Zaas AK, Schell WA, Betancourt-Quiroz M, et al. PKC signaling regulates drug resistance of the fungal pathogen Candida albicans via circuitry comprised of Mkc1, calcineurin, and Hsp90. PLoS Pathog. 2010;6, e1001069.
126. Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, et al. Hsp90 governs echinocandin resistance in the pathogenic yeast Candida albicans via calcineurin. PLoS Pathog. 2009;5, e1000532.
127. Singh-Babak SD, Babak T, Diezmann S, Hill JA, Xie JL, et al. Global analysis of the evolution and mechanism of echinocandin resistance in Candida glabrata. PLoS Pathog. 2012;8, e1002718.
128. Firon A, Lesage G, Bussey H. Integrative studies put cell wall synthesis on the yeast functional map. Curr Opin Microbiol. 2004;7:617–23.
129. Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, et al. Immune recognition of Candida albicans beta-glucan by dectin-1. J Infect Dis. 2007;196:1565–71.
130. Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, et al. Functional analysis of Candida albicans GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. Fungal Genet Biol. 2008;45:1404–14.
131. Lee KK, Maccallum DM, Jacobsen MD, Walker LA, Odds FC, et al. Elevated cell wall chitin in Candida albicans confers echinocandin resistance in vivo. Antimicrob Agents Chemother. 2012;56:208–17.
132. Stevens DA, Ichinomiya M, Koshi Y, Horiuchi H. Escape of Candida from caspofungin inhibition at concentrations above the MIC (paradoxical effect) accomplished by increased cell wall chitin; evidence for beta-1,6-glucan synthesis inhibition by caspofungin. Antimicrob Agents Chemother. 2006;50:3160–1.
133. Clemons KV, Espiritu M, Parmar R, Stevens DA. Assessment of the paradoxical effect of caspofungin in therapy of candidiasis. Antimicrob Agents Chemother. 2006;50:1293–7.
134. Healey KR, Katiyar SK, Raj S, Edlind TD. CRS-MIS in Candida glabrata: sphingolipids modulate echinocandin-Fks interaction. Mol Microbiol. 2012;86:303–13.
135. Healey KR, Katiyar SK, Castanheira M, Pfaller MA, Edlind TD. Candida glabrata mutants demonstrating paradoxical reduced caspofungin susceptibility but increased micafungin susceptibility. Antimicrob Agents Chemother. 2011;55:3947–9.
136. Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, et al. Genotypic evolution of azole resistance mechanisms in sequential Candida albicans isolates. Eukaryot Cell. 2007;6:1889–904.
137. Selmecki A, Forche A, Berman J. Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science. 2006;313:367–70.
138. Shin JH, Chae MJ, Song JW, Jung SI, Cho D, et al. Changes in karyotype and azole susceptibility of sequential bloodstream isolates from patients with Candida glabrata candidemia. J Clin Microbiol. 2007;45:2385–91.
139. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, et al. In vitro susceptibility of invasive isolates of Candida spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance. J Clin Microbiol. 2008;46:150–6.
140. Garcia-Effron G, Katiyar SK, Park S, Edlind TD, Perlin DS. A naturally occurring proline-to-alanine amino acid change in Fks1p in Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis accounts for reduced echinocandin susceptibility. Antimicrob Agents Chemother. 2008;52:2305–12.
141. Maligie MA, Selitrennikoff CP. Cryptococcus neoformans resistance to echinocandins: (1,3)beta-glucan synthase activity is sensitive to echinocandins. Antimicrob Agents Chemother. 2005;49:2851–6.

142. van Duin D, Casadevall A, Nosanchuk JD. Melanization of Cryptococcus neoformans and Histoplasma capsulatum reduces their susceptibilities to amphotericin B and caspofungin. Antimicrob Agents Chemother. 2002;46:3394–400.
143. Staab JF, Kahn JN, Marr KA. Differential Aspergillus lentulus echinocandin susceptibilities are Fksp independent. Antimicrob Agents Chemother. 2010;54:4992–8.
144. Odabasi Z, Paetznick V, Rex JH, Ostrosky-Zeichner L. Effects of serum on in vitro susceptibility testing of echinocandins. Antimicrob Agents Chemother. 2007;51:4214–6.
145. Paderu P, Garcia-Effron G, Balashov S, Delmas G, Park S, et al. Serum differentially alters the antifungal properties of echinocandin drugs. Antimicrob Agents Chemother. 2007;51:2253–6.
146. Wiederhold NP, Najvar LK, Bocanegra R, Molina D, Olivo M, et al. In vivo efficacy of anidulafungin and caspofungin against Candida glabrata and association with in vitro potency in the presence of sera. Antimicrob Agents Chemother. 2007;51:1616–20.
147. Foldi R, Szilagyi J, Kardos G, Berenyi R, Kovacs R, et al. Effect of 50% human serum on the killing activity of micafungin against eight Candida species using time-kill methodology. Diagn Microbiol Infect Dis. 2012;73:338–42.
148. Kovacs R, Gesztelyi R, Berenyi R, Doman M, Kardos G, et al. Killing rates exerted by caspofungin in 50 % serum and its correlation with in vivo efficacy in a neutropenic murine model against Candida krusei and Candida inconspicua. J Med Microbiol. 2014;63:186–94.
149. Arendrup MC, Rodriguez-Tudela JL, Park S, Garcia-Effron G, Delmas G, et al. Echinocandin susceptibility testing of Candida spp. Using EUCAST EDef 7.1 and CLSI M27-A3 standard procedures: analysis of the influence of bovine serum albumin supplementation, storage time, and drug lots. Antimicrob Agents Chemother. 2011;55:1580–7.
150. Arendrup MC, Garcia-Effron G, Lass-Florl C, Lopez AG, Rodriguez-Tudela JL, et al. Echinocandin susceptibility testing of Candida species: comparison of EUCAST EDef 7.1, CLSI M27-A3, Etest, disk diffusion, and agar dilution methods with RPMI and isosensitest media. Antimicrob Agents Chemother. 2010;54:426–39.
151. Pfaller MA, Castanheira M, Messer SA, Rhomberg PR, Jones RN. Comparison of EUCAST and CLSI broth microdilution methods for the susceptibility testing of 10 systemically active antifungal agents when tested against Candida spp. Diagn Microbiol Infect Dis. 2014;79:198–204.
152. Andes D, Diekema DJ, Pfaller MA, Bohrmuller J, Marchillo K, et al. In vivo comparison of the pharmacodynamic targets for echinocandin drugs against Candida species. Antimicrob Agents Chemother. 2010;54:2497–506.
153. Eschenauer GA, Nguyen MH, Shoham S, Vazquez JA, Morris AJ, et al. Real-world experience with echinocandin MICs against Candida species in a multicenter study of hospitals that routinely perform susceptibility testing of bloodstream isolates. Antimicrob Agents Chemother. 2014;58:1897–906.
154. Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, et al. Interlaboratory variability of Caspofungin MICs for Candida spp. Using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother. 2013;57:5836–42.
155. Ben-Ami R, Hilerowicz Y, Novikov A, Giladi M. The impact of new epidemiological cutoff values on Candida glabrata resistance rates and concordance between testing methods. Diagn Microbiol Infect Dis. 2014;79:209–13.
156. Pfaller MA, Messer SA, Diekema DJ, Jones RN, Castanheira M. Use of micafungin as a surrogate marker to predict susceptibility and resistance to caspofungin among 3,764 clinical isolates of Candida by use of CLSI methods and interpretive criteria. J Clin Microbiol. 2014;52:108–14.
157. Pfaller MA, Diekema DJ, Jones RN, Castanheira M. Use of anidulafungin as a surrogate marker to predict susceptibility and resistance to caspofungin among 4,290 clinical isolates of Candida by using CLSI methods and interpretive criteria. J Clin Microbiol. 2014;52:3223–9.
158. Arendrup MC, Rodriguez-Tudela JL, Lass-Florl C, Cuenca-Estrella M, Donnelly JP, et al. EUCAST technical note on anidulafungin. Clin Microbiol Infect. 2011;17:E18–20.
159. Espinel-Ingroff A, Pfaller MA, Bustamante B, Canton E, Fothergill A, et al. Multilaboratory study of epidemiological cutoff values for detection of resistance in eight Candida species to fluconazole, posaconazole, and voriconazole. Antimicrob Agents Chemother. 2014;58:2006–12.
160. Perlin DS. Antifungal drug resistance: do molecular methods provide a way forward? Curr Opin Infect Dis. 2009;22:568–73.
161. Pfaller MA, Andes D, Arendrup MC, Diekema DJ, Espinel-Ingroff A, et al. Clinical breakpoints for voriconazole and Candida spp. revisited: review of microbiologic, molecular, pharmacodynamic, and clinical data as they pertain to the development of species-specific interpretive criteria. Diagn Microbiol Infect Dis. 2011;70:330–43.
162. Perlin DS. Echinocandin resistance, susceptibility testing and prophylaxis: implications for patient management. Drugs. 2014;74:1573–85.
163. Stevens DA, Espiritu M, Parmar R. Paradoxical effect of caspofungin: reduced activity against Candida albicans at high drug concentrations. Antimicrob Agents Chemother. 2004;48:3407–11.
164. Stevens DA, White TC, Perlin DS, Selitrennikoff CP. Studies of the paradoxical effect of caspofungin at high drug concentrations. Diagn Microbiol Infect Dis. 2005;51:173–8.
165. Rueda C, Cuenca-Estrella M, Zaragoza O. Paradoxical growth of Candida albicans in the presence of caspofungin is associated with multiple cell wall rearrangements and decreased virulence. Antimicrob Agents Chemother. 2014;58:1071–83.
166. Bizerra FC, Melo AS, Katchburian E, Freymuller E, Straus AH, et al. Changes in cell wall synthesis and ultrastructure during paradoxical growth effect of caspofungin on four different Candida species. Antimicrob Agents Chemother. 2011;55:302–10.
167. Shields RK, Nguyen MH, Du C, Press E, Cheng S, et al. Paradoxical effect of caspofungin against Candida bloodstream isolates is mediated by multiple pathways but eliminated in human serum. Antimicrob Agents Chemother. 2011;55:2641–7.
168. Wiederhold NP, Kontoyiannis DP, Chi J, Prince RA, Tam VH, et al. Pharmacodynamics of caspofungin in a murine model of invasive pulmonary aspergillosis: evidence of concentration-dependent activity. J Infect Dis. 2004;190:1464–71.
169. Klont RR, Mennink-Kersten MA, Ruegebrink D, Rijs AJ, Blijlevens NM, et al. Paradoxical increase in circulating Aspergillus antigen during treatment with caspofungin in a patient with pulmonary aspergillosis. Clin Infect Dis. 2006;43:e23–5.
170. Zanette RA, Kontoyiannis DP. Paradoxical effect to caspofungin in Candida species does not confer survival advantage in a Drosophila model of candidiasis. Virulence. 2013;4:497–8.
171. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007;20:133–63.
172. Koh AY, Kohler JR, Coggshall KT, Van Rooijen N, Pier GB. Mucosal damage and neutropenia are required for Candida albicans dissemination. PLoS Pathog. 2008;4, e35.
173. Kennedy MJ, Volz PA, Edwards CA, Yancey RJ. Mechanisms of association of Candida albicans with intestinal mucosa. J Med Microbiol. 1987;24:333–41.
174. Magill SS, Swoboda SM, Johnson EA, Merz WG, Pelz RK, et al. The association between anatomic site of Candida colonization, invasive candidiasis, and mortality in critically ill surgical patients. Diagn Microbiol Infect Dis. 2006;55:293–301.

175. Magill SS, Swoboda SM, Shields CE, Colantuoni EA, Fothergill AW, et al. The epidemiology of Candida colonization and invasive candidiasis in a surgical intensive care unit where fluconazole prophylaxis is utilized: follow-up to a randomized clinical trial. Ann Surg. 2009;249:657–65.
176. Miranda LN, van der Heijden IM, Costa SF, Sousa AP, Sienra RA, et al. Candida colonisation as a source for candidaemia. J Hosp Infect. 2009;72:9–16.
177. Voss A, Hollis RJ, Pfaller MA, Wenzel RP, Doebbeling BN. Investigation of the sequence of colonization and candidemia in nonneutropenic patients. J Clin Microbiol. 1994;32:975–80.
178. Richet HM, Andremont A, Tancrede C, Pico JL, Jarvis WR. Risk factors for candidemia in patients with acute lymphocytic leukemia. Rev Infect Dis. 1991;13:211–5.
179. Harriott MM, Noverr MC. Importance of Candida-bacterial polymicrobial biofilms in disease. Trends Microbiol. 2011;19:557–63.
180. Cheng S, Clancy C, Hartman D, Hao B, Nguyen M. Candida glabrata intra-abdominal candidiasis is characterized by persistence within the peritoneal cavity and abscesses. Infect Immun. 2014;82(7):3015–22.
181. Fekkar A, Dannaoui E, Meyer I, Imbert S, Brossas JY, et al. Emergence of echinocandin-resistant Candida spp. in a hospital setting: a consequence of 10 years of increasing use of antifungal therapy? Eur J Clin Microbiol Infect Dis. 2014;33(9):1489–96.
182. Blanchard E, Lortholary O, Boukris-Sitbon K, Desnos-Ollivier M, Dromer F, et al. Prior caspofungin exposure in patients with hematological malignancies is a risk factor for subsequent fungemia due to decreased susceptibility in Candida spp.: a case-control study in Paris, France. Antimicrob Agents Chemother. 2011;55:5358–61.
183. de la Torre P, Reboli AC. Micafungin: an evidence-based review of its place in therapy. Core Evid. 2014;9:27–39.
184. van Burik JA, Ratanatharathorn V, Stepan DE, Miller CB, Lipton JH, et al. Micafungin versus fluconazole for prophylaxis against invasive fungal infections during neutropenia in patients undergoing hematopoietic stem cell transplantation. Clin Infect Dis. 2004;39:1407–16.
185. Chou LS, Lewis RE, Ippoliti C, Champlin RE, Kontoyiannis DP. Caspofungin as primary antifungal prophylaxis in stem cell transplant recipients. Pharmacotherapy. 2007;27:1644–50.
186. Mattiuzzi GN, Alvarado G, Giles FJ, Ostrosky-Zeichner L, Cortes J, et al. Open-label, randomized comparison of itraconazole versus caspofungin for prophylaxis in patients with hematologic malignancies. Antimicrob Agents Chemother. 2006;50:143–7.
187. Doring M, Hartmann U, Erbacher A, Lang P, Handgretinger R, et al. Caspofungin as antifungal prophylaxis in pediatric patients undergoing allogeneic hematopoietic stem cell transplantation: a retrospective analysis. BMC Infect Dis. 2012;12:151.
188. Xu SX, Shen JL, Tang XF, Feng B. Newer antifungal agents for fungal infection prevention during hematopoietic cell transplantation: a meta-analysis. Transplant Proc. 2013;45:407–14.
189. Ziakas PD, Kourbeti IS, Mylonakis E. Systemic antifungal prophylaxis after hematopoietic stem cell transplantation: a meta-analysis. Clin Ther. 2014;36(2):292–306.e1.
190. Scott LJ. Micafungin: a review of its use in the prophylaxis and treatment of invasive Candida infections. Drugs. 2012;72:2141–65.
191. Hope WW, Castagnola E, Groll AH, Roilides E, Akova M, et al. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: prevention and management of invasive infections in neonates and children caused by Candida spp. Clin Microbiol Infect. 2012;18 Suppl 7:38–52.
192. Ostrosky-Zeichner L. Candida glabrata and FKS mutations: witnessing the emergence of the true multidrug-resistant Candida. Clin Infect Dis. 2013;56:1733–4.
193. Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, et al. FKS mutations and elevated echinocandin MIC values among Candida glabrata isolates from U.S. population-based surveillance. Antimicrob Agents Chemother. 2010;54:5042–7.
194. Pfaller MA, Castanheira M, Messer SA, Moet GJ, Jones RN. Echinocandin and triazole antifungal susceptibility profiles for Candida spp., Cryptococcus neoformans, and Aspergillus fumigatus: application of new CLSI clinical breakpoints and epidemiologic cutoff values to characterize resistance in the SENTRY Antimicrobial Surveillance Program (2009). Diagn Microbiol Infect Dis. 2011;69:45–50.
195. Chapeland-Leclerc F, Hennequin C, Papon N, Noel T, Girard A, et al. Acquisition of flucytosine, azole, and caspofungin resistance in Candida glabrata bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. Antimicrob Agents Chemother. 2010;54:1360–2.
196. Bizerra FC, Jimenez-Ortigosa C, Souza AC, Breda GL, Queiroz-Telles F, et al. Breakthrough candidemia due to multidrug-resistant Candida glabrata during prophylaxis with a low dose of micafungin. Antimicrob Agents Chemother. 2014;58:2438–40.
197. Ruggero MA, Topal JE. Development of echinocandin-resistant Candida albicans candidemia following brief prophylactic exposure to micafungin therapy. Transpl Infect Dis. 2014;16(3):469–72.

Antifungal Targets, Mechanisms of Action, and Resistance in *Candida albicans*

30

Robert A. Akins and Jack D. Sobel

1 Introduction

Antifungal resistance at the gene level has been studied in *C. albicans* for about a decade now. Cloning of *C. albicans* genes by homology to resistance genes in *S. cerevisiae*, and heterologous expression of *C. albicans* genes in *S. cerevisiae* has allowed rapid progress in identifying and studying the five major *C. albicans* genes involved in resistance to clinically used antifungals: ABC transporter genes CDR1 and CDR2, major facilitator efflux gene MDR1, and ergosterol biosynthesis genes ERG11 and ERG3. Analysis of these genes indicates that resistance involves alterations to the enzyme targeted by FLZ, encoded by ERG11, and upregulation of P-glycoprotein-type ABC transporters and major facilitators (MFS) that probably efflux azoles, terbinafine, and perhaps caspofungin. Potential alterations to ERG3 or its regulation have been understudied in *C. albicans*. Resistant isolates from clinical samples, especially in oropharyngeal candidiasis (OPC), typically display stepwise mutations in more than one of these genes. However, it is clear from in vivo and in vitro studies that mutations of these major genes do not completely account for the evolution of high-level azole resistance in some clinical isolates. More work is needed that is independent of heterologous studies in *S. cerevisiae*, to identify other genes that contribute to resistance in *C. albicans*. Very little is understood about reversible, adaptive resistance of *C. albicans*, despite its potential clinical significance. Most clinical failures to control non-OPC infections occur with in vitro-susceptible strains. There has been important discovery of tolerance mechanisms to azoles. Heterologous studies in *S. cerevisiae* on regulation of target genes have been less useful, due to differences in regulation in *C. albicans*. Nevertheless, recent has progress has been made in identifying genes that regulate CDR1 or ERG genes.

R.A. Akins, Ph.D. (✉) • J.D. Sobel, M.D.
Wayne State University School of Medicine, Detroit, MI, USA
e-mail: rakins@med.wayne.edu

There is no shortage of reviews on the subject of antifungal resistance in *Candida* [1–12]. To the extent that this review is useful, it stands on the shoulders of these reviewers. I particularly recommend Sanglard and Bille's book chapter [10] for its broad scope and meticulousness, Lamb and Kelly's clear description of the toxic sterol model for azole inhibition [5], an excellent compendium and analysis of ERG11 mutations from the folks and affiliates at the Janssen Research Foundation [13], and a focused and up-to-date review and modeling of efflux mechanisms of resistance [14]. The focus of this review is *C. albicans*, but in areas in which *Candida* research is underdeveloped, we resort to available information on *S. cerevisiae* or *C. glabrata* as a portent of things to come.

2 Ergosterol Biosynthesis Genes and Antifungal Resistance

The ergosterol biosynthetic pathway is a target for many antifungals (Table 30.1). This pathway converts acetic acid to ergosterol, using largely the same enzymes as in the mammalian biosynthesis of cholesterol. The ergosterol pathway is really two pathways. Syntheses up to farnesyl pyrophosphate (ERG20) constitute the isoprenoid pathway, generating isoprenoids, carotenoids, prenyl groups for membrane attachments, tRNA modifications, etc. The bulk of the intermediates flow on via Erg9p, to ergosterol. In *S. cerevisiae*, genes at or above ERG20 are essential for growth, and cannot be rescued by supplementing with ergosterol. In contrast, most of the downstream genes are not essential for viability; those that are, e.g., ERG11, can be grown in the presence of ergosterol.

The identity of the *C. albicans* genes based on homology to the *S. cerevisiae* genes is unambiguous and likely orthologous, ranging from 39 to 73 % identical and 53–84 % similar over the length of the entire open reading frame, with the exception of ERG8. Fungal ERG8, encoding phosphomevalonate kinase, has no ortholog in man, or in multicellular

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_30

Table 30.1 Ergosterol biosynthetic pathway: enzymes, gene homologies, inhibitors, and resistance mechanisms within the pathway

Product	Gene	Activity	Ca/Sc ID (sim)	Ca/Hs ID (sim)	Inhibitor	Resistance	Hypersusceptible
Acetyl-CoA							
Acetoacetyl-CoA	ERG10	Acetyl-CoA C-acetyltransferase	63(74)	42(56)			
HMG-CoA	ERG13(HCS1)	3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase	67(80)	49(66)			
Mevalonate	HMG1, HMG2	3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase	46(59)	38(52)	Statins		
Mevalonate phosphate	ERG12	Mevalonate kinase	39(53)	31(49)			
Mevalonate pyrophosphate	ERG8	Phosphomevalonate kinase	40(56)	No ortholog			
Isopentyl pyrophosphate	ERG19	Mevalonate pyrophosphate decarboxylase	56(65)	43(55)			
Dimethylallyl phosphate	IDI1	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase	52(67)	50(68)			
Geranyl phosphate	ERG20	Dimethylallyltranstransferase	73(84)	45(63)			
Farnesyl pyrophosphate		Geranyltranstransferase					
Squalene	ERG9	Squalene synthase	56(70)	46(62)	Zaragozic acid (Z)	↑ScERG9 (Z,A,T)	↑ScERG9 (AmB)
2,3-Oxidosqualene	ERG1	Squalene epoxidase	52(68)	37(53)	Terbinafine (T)	↑ERG1	
Lanosterol	ERG7	Lanosterol synthase	62(74)	39(57)			
4,4,-Dimethyl-8,14,24-trienol	ERG11	C14-Lanosterol demethylase	64(78)	39(53)	Azoles (A)	Erg11p, ↑ERG11, ΔErg11(?)	
4,4-Dimethyl zymosterol	ERG24	C14-Sterol reductase	57(70)	32(48)	Morpholines (M)	Scerg24p, ΔErg24(A), ↑ERG24	
	ERG25	C4-Sterol methyloxidase	58(69)	39(53)	PF1163A		
4-Methyl zymosterol	ERG26	C4-Sterol decarboxylase	69(80)	32(52)			
Zymosterol	ERG27	C3-Sterol ketoreductase	60(74)	23(42)			
Fecosterol	ERG6	C-24 Sterol methyl-transferase	65(77)	none		ΔErg6(AmB); ↑ERG6(A)	ΔErg6(T,M)
	ERG28	Erg scaffold	57(74)	38(65)			
Episterol	ERG2	C8-Sterol isomerase	58(69)	58(69)	Morpholines		ΔErg2(T,A)
Ergosta-5,7,24(28) trienol	ERG3	C5-Sterol desaturase	51(61)	45(60)		ΔErg3(A, AmB)	↑ERG3
Ergosta-5,7,22,24(28) tetraenol	ERG5	C22-Sterol desaturase	71(82)	none	Azoles?		
Ergosterol	ERG4	C24-Sterol reductase	58(71)	25(40)			

Sequential products are listed vertically, next to the enzyme responsible for that product. ERG28 may act as a scaffold for genes acting upstream. %identity (%similarity) of the *C. albicans* gene (Ca) to the *S. cerevisiae* (Sc) or *Homo sapiens* (Hs) homolog were derived from Blast analysis on the NCBI website. Mutation symbolism: ergXp: point mutations in the ErgX protein; Δergx: disruption of ERGX (both alleles); ↑ERGX: overexpression of ERGX. Sc prefix indicates that data is only available for *S. cerevisiae*. Drugs to which mutations confer resistance or hypersusceptibility are in () if different than the inhibitor targeting that enzyme. Evidence and references for these are in the text

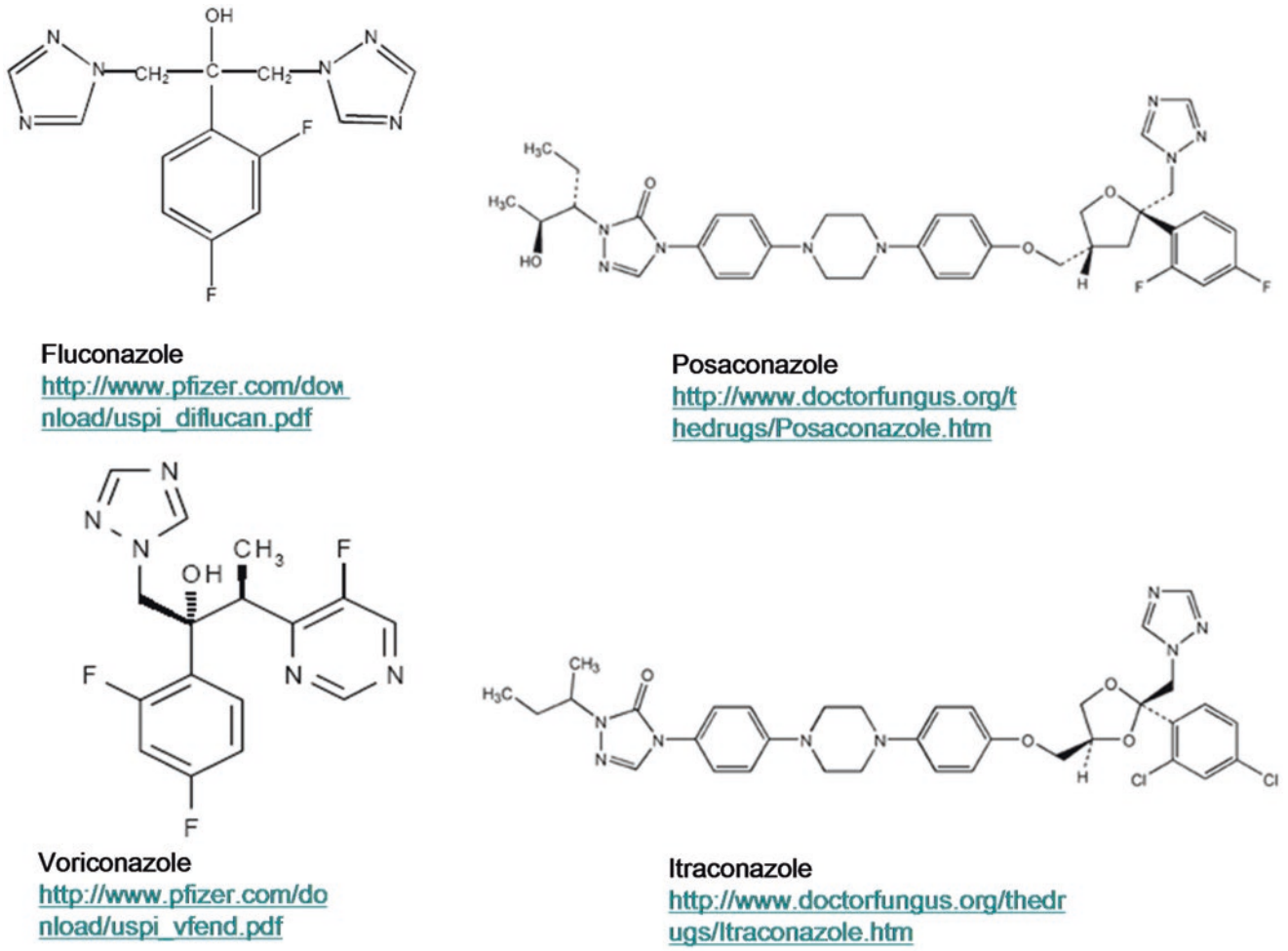


Fig. 30.1 Structures of azoles in clinical use or trials

animals [15]. CaERG8 does have orthologs in bacteria, and perhaps in plants. Human phosphomevalonate kinase has orthologs down to *C. elegans*. This lack of a human ortholog and the fact that ERG8 in *S. cerevisiae* is essential [16] make pErg8 an ideal target for antifungals. However, in one report, CaERG8 is not essential [17]; it is not yet known if this is a fundamental difference between species or if another kinase in *C. albicans* suffices in the absence of CaErg8p. CaERG5 and CaERG6 have no homologs in man, since they catalyze reactions found in ergosterol, not in cholesterol.

There are a variety of azoles (Fig. 30.1) which all act by binding and inhibiting Erg11p, a cytochrome P450 enzyme lanosterol 14α-demethylase (Fig. 30.2). This inhibition does not block the pathway at lanosterol, although lanosterol concentrations do increase. Instead, lanosterol with its 14-methyl group intact is acted on by downstream enzymes to generate 14-methylated intermediates (Fig. 30.3). The model, based on observations in *S. cerevisiae*, is that one of

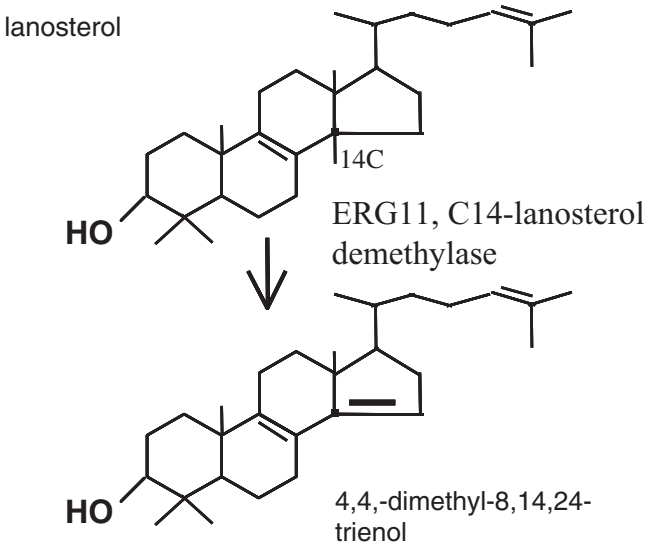


Fig. 30.2 C-14 lanosterol demethylase encoded by ERG11

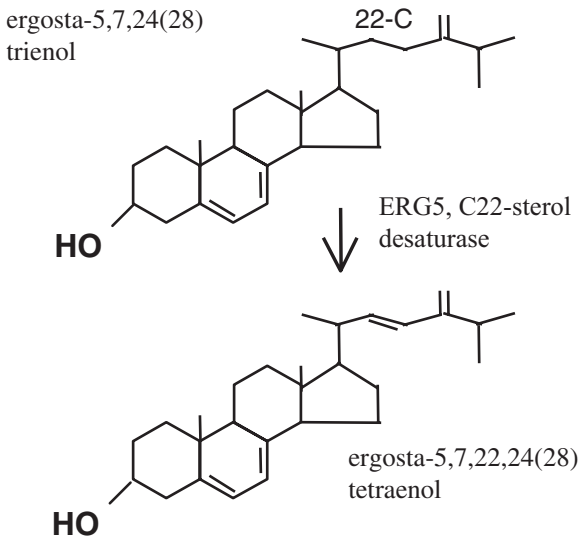


Fig. 30.3 22-C sterol desaturase encoded by ERG5

these intermediates, 14-methylergosta-8,24(28)-dien-3,6-diol, is toxic and is responsible for growth inhibition. The main evidence for this is that mutation or deletion of the gene encoding Erg3p, which forms this toxic sterol from 14-methyl fecosterol, confers resistance to azoles [18, 19]. In this model, reviewed in detail by its authors [5], it is not the lack of ergosterol that is inhibitory, but the accumulation of the toxic intermediate. They propose that the hydroxyl group on the sixth carbon disrupts hydrophobic interactions of the intermediate with plasma membrane phospholipids, permeabilizing the membrane.

The toxic diol model is not universally accepted for *C. albicans*. Early observations showed that viable ERG11 mutants accumulated significant amounts of the diol [20, 21]. However, this may be a quantitative difference, since the ERG11 mutants show reduced growth. Furthermore, the identity of the diol in these papers is inconsistent with earlier analysis [22]. The ERG11 mutant is not characterized; in other work, CaERG11 mutants have had other defects, for example in respiration [23]. Because of these problems, and since CaERG3 mutations behave like ScERG3 mutations, discussed below, the toxic sterol model probably applies to *C. albicans*, although it may require more for complete inhibition than in *S. cerevisiae*.

2.1 ERG11

At least 12 point mutations among more than 80 polymorphisms in ERG11 confer or are associated with azole resistance (Table 30.2). Sequential publications report new mutations, suggesting that the inventory is not yet saturated. Compelling evidence that the mutation caused resistance in four of these was determined by expressing the wild-type *C. albicans* ERG11 gene in *S. cerevisiae*, after using site-directed mutagenesis to alter a single base to the mutant sequence, followed by measurement of demethylase activity in vitro or determining resistance of the *S. cerevisiae* transformant. Nearly as compelling were the four additional mutant genes which were cloned into a high copy plasmid and expressed in *S. cerevisiae* to determine resistance of the transformants relative to control clones by Chau et al. [24]. Strong evidence for another four mutants was provided by showing reduced susceptibilities of demethylase activity from clinical resistant strains. Associative evidence was found for an additional mutation's link to resistance, meaning that it was repeatedly found only in resistant or quasi-resistant (SDD, susceptible, dose dependent = MIC between 8 and 32 μg/mL) isolates, never in susceptible isolates. Some mutations alter heme binding and reduce demethylase activity, while others alter azole binding without affecting heme binding or demethylase. Recent reviews discuss how these mutations fit into a tentative structural model of the enzyme [5, 13].

Although this integral membrane protein has not been crystallized, homology models based on the crystal structure of CYP51 of *Mycobacterium tuberculosis* have been constructed [25–29]. Despite differences among these papers, it is clear that azoles bind near the heme group in the enzyme, and mutations that interfere with this interaction confer resistance to azoles. These mutations preferentially affect the short-chained azoles, FLZ and voriconazole, because of additional stabilizing interactions of the long chains of posaconazole and itraconazole with residues along the inner channel 2. Conversely, mutations along channel 2 may confer resistance only to the latter azoles by precluding their binding.

Table 30.2 does not include mutations which have been seen in susceptible isolates, listed in [13] and [24], since these are less likely to be functionally related to resistance. We refer to these as polymorphisms. Despite this, some of these may play a role in resistance. Some polymorphisms, when combined with resistance mutations, further increase the level of resistance of their host [30]. Conversely, mutant S405K alone confers moderate resistance, but is susceptible in combination with some other polymorphisms [13]. These observations suggest that more independent isolates need to be screened for polymorphisms and mutations, and more of these need to be functionally tested, ideally by site-directed mutagenesis and expression in *S. cerevisiae* or in *C. albicans*.

Does an increased level of Erg11p confer azole resistance? Overexpression of ScERG11 in *S. cerevisiae* on a centromeric plasmid, driven by the GAL1 promoter, conferred galactose-dependent high-level azole resistance [31]. Overexpression of Erg11p in *C. glabrata* is associated with chromosomal duplication [32, 33]. Interestingly, the duplication results in an eightfold, not a twofold, increase in

Table 30.2 Mutations in ERG11 that confer FLZ resistance, and their methods of determination

Mutation	Effect	Method(s)	References
Y132H(F)	Altered FLZ binding, no reduction in activity; resistant transformants	SDM, confers SDD, confers R in combination with S405F; SDM-EA;↑Ca(Sc) confers R growth in combination with G450E; combinations may confer R to posaconazole	[24, 30, 419]
T315A	Altered FLZ binding, altered heme binding, reduced activity; resistant transformants	SDM confers R; SDM-EA	[420]
R467K	Altered heme, reduced FLZ binding; resistant transformants	SDM confers SDD; confers R in combination with G464S; SDM-EA	[30, 421, 422]
G464S	Altered heme, reduced FLZ binding, reduced activity; resistant transformants	SDM-EA; ↑Ca(Sc) confers SDD, confers increased SDD in combination with G129A (a susceptible polymorphism), confers R in combination with R467K, may confer R to posaconazole in combination with G307S	[24, 30, 423, 424]
A61V	Resistant clinical strain with other mutations, resistant transformants	↑Ca(Sc) confers SDD growth	[24]
K143E+ T229A	Found together in resistant isolates, resistant enzyme, resistant transformants	↑Ca(Sc) confers SDD growth; EA	[13, 24, 425]
S405F	Found in resistant isolates, unique mutation in some, also found in sensitive isolates in combination with other mutations, resistant transformants	↑Ca(Sc) confers SDD; confers R in combination with Y132H	[13, 30]
G450E	Found with other mutations in many resistant strains, resistant enzyme; resistant transformants	↑Ca(Sc) confers R growth in combination with Y132H; EA	[13, 24, 425]
F72L			
	Found in one resistant strain with other mutations, resistant enzyme	EA	[425]
F126L	Found in one strain with other mutations, resistant enzyme	EA	[425]
E266D	Found with other mutations in many resistant strains, resistant enzyme	EA	[13, 425]
F449L	Found in strains with other mutations, resistant enzyme	EA	[13, 425]
F105L	Found in resistant isolates, unique ERG11 mutation in some	Correlative, located in substrate access channel in model	[13, 424]

Abbreviations: SDD = susceptible, dose-dependent (resistant in vitro to 8–32 μg/mL FLZ); R: resistant, (in vitro to 64+ μg/mL FLZ). Methods: SDM = Heterologous expression in *S. cerevisiae* of wild-type *C. albicans* gene after site-directed mutagenesis; SDM-EA = in vitro enzyme assay extracts from *S. cerevisiae* transformed with *C. albicans* gene which was altered by SDM; ↑Ca(Sc) = overexpression in *S. cerevisiae* of cloned genes from resistant isolates of *C. albicans*; EA = in vitro assays of enzymatic activity of extracts of resistant clinical isolates. Several known and new mutations were reported by Chau et al. [24], but their link to resistance is uncertain since they only appeared in resistant isolates that had other known resistance mutations and/or upregulation of CDR2. New mutations include A107T, G448V, V452A, V509M, Y257H, and G307S

mRNA, suggesting that ERG11 expression is normally limited by a repressor which is titrated out by the duplication. Resistance via chromosome duplication has not been demonstrated in *C. albicans*, although it does result from chromosome loss [34, 35]. Tandem gene duplications have not been demonstrated to be a resistance mechanism in *C. albicans*. Overexpression of ERG11 is seen in some clinical resistant *C. albicans* isolates; however, the level of expression is poorly correlated with resistance (Sect. 4 and [24]).

Our recent development of a reliable multicopy shuttle plasmid for *C. albicans* has allowed us to address this issue without resorting to heterologous expression. The selective marker in this plasmid is the wild-type gene encoding IMP dehydrogenase; only when overexpressed can this gene confer resistance to the IMP analog mycophenolic acid (MPA), forcing amplification of the plasmid to about 40 copies per cell. Wild-type genes cloned into the plasmid, behind vector-derived or native promoters, overexpress their product [36]. Overexpression of a fusion product consisting of mostly CaErg11p in *C. albicans* confers moderate FLZ resistance [36], and overexpression of the native protein driven by its own promoter confers significant resistance to azoles (unpublished observations). This is expected, since overproduction of target should allow continued demethylation by enzyme that is not bound by azole. In these transformants, normal to near-normal levels of ergosterol accumulate even in 64 μg/mL FLZ.

What is the effect of disruption of ERG11? Recently, disruption of both alleles of ERG11 in *C. albicans* was reported. The disruptant is aerobically viable, and is resistant to high concentrations of FLZ [37]. In contrast, in *S. cerevisiae*,

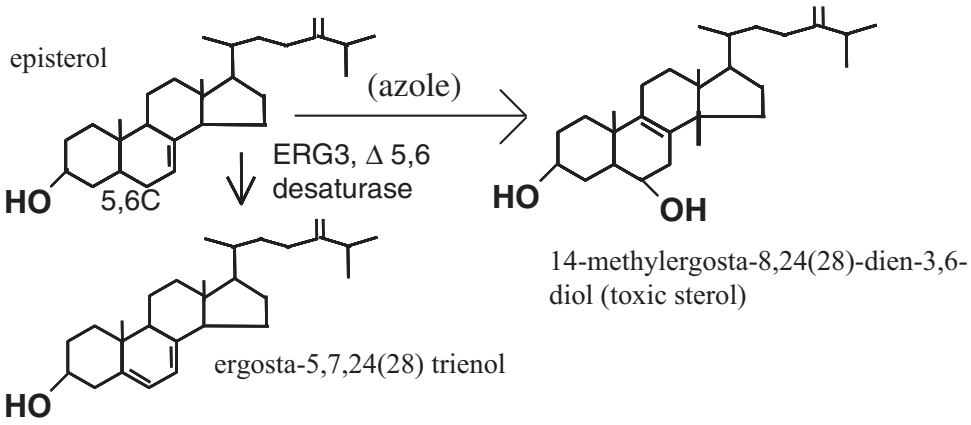


Fig. 30.4 5,6-C sterol desaturase encoded by ERG3

this deletion, or inactivating point mutations, is lethal unless supplemented with ergosterol and fatty acid under anaerobic conditions, or unless there is a second mutation in ERG3. A number of ScERG11 mutants identified as viable and azole resistance turned out to have secondary mutations in ERG3 [18–20, 38, 39]. On one level, the disruption in *C. albicans* is gratifying, since it removes the target of inhibition, so the cell is resistant. However, this poses a problem for the model for azole mechanism of action, and/or calls into question the genotype of the ERG11 disruptant strain [37]. Deletion of the gene should introduce the same growth-inhibited phenotype as wild-type cells inhibited by FLZ, because it should generate an inhibitory concentration of 14-methylergosta-8,24(28)-dien-3,6-diol. That is, the disruptant should be viable but capable of very slow growth at best even in the absence of azole.

One interpretation of the effects of disrupting CaERG11 [37] assumes that the toxic sterol model is basically correct but that the disruptant is complex. It proposes that the viability/growth of the disruptant results either from a regulatory change or from a secondary mutation that reduces this toxic intermediate. The likely target for either is ERG3. This interpretation is favored by the means by which the authors isolated the disruption in the second ERG11 allele. This derived from the single allele disruptant by selection on amphotericin B, which selects for strains devoid of ergosterol, rather than by a second round of URA3 blasting, which was attempted but not successful. While it is clear that the second ERG11 allele had been deleted, other mutations may have been selected for, and again the prime suspect is ERG3. Consistently, 14-methyl fecosterol is detected in the disruptant, but possibly not the toxic sterol. The authors are keenly aware of this possibility and do mention that they sequenced the ERG3 genes in the disruptants and found no changes. However, it is not clear whether they looked at its expression or function. More analysis of ERG3 in the ERG11 disruptant is warranted.

A tenuous alternative interpretation of the CaERG11 disruption emphasizes that deleting the target is different than inhibiting it by azoles, proposing that azoles inhibit more than one target. In this view, the original model is an oversimplification. This is not likely a quantitative issue, in which the azole only partially inhibits Erg11p, since that should be less inhibitory than the deletion in the context of the model. A candidate for a second site of inhibition is *Erg5p*, another P450 enzyme acting downstream of Erg3p (Fig. 30.4). Indeed, azoles inhibit in vitro activity of ScErg5p, a $^{\Delta 22}$ desaturase P450 enzyme, with almost the same efficacy as Erg11p [40]. In this modified model, azoles contribute to accumulation of toxic sterol in two ways: by blocking demethylation of lanosterol and by constricting the pathway below Erg3p, at Erg5p. Deletion of ERG11 does not dramatically reduce growth, in part because active Erg5p hypothetically reduces the pool of 14-methylergosta-8,24(28)-dien-3,6-diol. This explains the growth of the disruptants in the absence of azole. ERG11 disruptants are azole resistant because, independent of and prior to exposure to azole, they downregulated downstream genes or deactivated downstream enzymes, so that azole exposure does not generate needed levels of 14-m ethylergosta-8,24(28)-dien-3,6-diol. Consistently, Sanglard's group showed that the ERG11 disruptant accumulates reduced amounts of intermediates at and downstream of 14-methyl fecosterol [37]. In contrast, wild-type cells inhibited by azoles accumulate significant amounts of these downstream derivatives [18] and presumably have induced levels of Erg3p [41].

This alternative rationalization is complex, and requires assumptions that lack an experimental basis. The more likely explanation is the first, that the disruption of the second allele of ERG11 forced simultaneous selection for a mutation that affects ERG3. In a separate study, ERG11 was underexpressed by disrupting one allele and placing the second under control of the tetracycline repressor. Under these conditions, growth was reduced by 90 % [17]. While the authors are not clear whether complete inactivation of expression was cidal, these observations suggest that expression is essential for

growth. Until these issues are resolved, it is premature to conclude that ERG11 is not essential for growth in wild-type *C. albicans*, or that its deletion confers resistance to azoles.

ERG3 encodes the Δ5,6 desaturase acting late in the ergosterol biosynthesis pathway. Erg3p is responsible for converting tolerated 14-methyl intermediates, which accumulate because of azole inhibition of 14C-lanosterol demethylase, into the toxic sterol 14-methylergosta-8,24(28)-dien-3,6-diol [18, 19]. Therefore, ERG3 inactivation should and does confer azole resistance. Wild-type strains exposed to azoles typically accumulate euburicol, obtusifoliol, and the toxic sterol, whereas ERG3 mutants accumulate mostly ergosta-7, 22-dienol in the absence of azole, instead of ergosterol, and mostly 14a-methyl fecosterol after azole exposure, both in *S. cerevisiae* and *C. albicans* [2, 5, 6, 8]. Deletions of both alleles of CaERG3 conferred high-level azole resistance [37], suggesting that diol formation by Erg3p is inhibitory in *C. albicans* as in *S. cerevisiae*. However, there are differences among yeast species. For example, ERG3 deletion mutants are azole resistant in *S. cerevisiae* [19] and *C. albicans* [37], but not in *C. glabrata* [39]. Most spontaneous azole-resistant (recessive) mutations in *S. cerevisiae* occur in the ERG3 gene, which would not be expected in the diploid *C. albicans*. However, if lineages exist or arise that are heterozygous for inactivating mutations in ERG3, then ERG3 mutations could be or become a common mechanism of resistance, restricted to these lineages. It is also reasonable to expect that mutations that repress transcription of ERG3 could confer resistance in clinical isolates, independent of lineage. Sequencing and expression analysis of this gene in clinical isolates therefore need more attention.

ERG1 encodes squalene epoxidase, which when inhibited by terbinafine results in ergosterol depletion and accumulation of squalene (Fig. 30.5). Terbinafine is fungistatic for most *Candida* species, but fungicidal for filamentous fungi [42–44]. Terbinafine becomes fungicidal for *C. albicans* in combination with calcineurin inhibitors, an effect also seen with azoles [45, 46]. Strains that are resistant to azoles are normally not cross-resistant to terbinafine [47], unless resistance is based on overexpression of CDR1.

Analysis of terbinafine-resistant genes in *C. albicans* isolates is not reported, but studies in *S. cerevisiae* are instructive. Single-point mutations in ERG1 in *S. cerevisiae* result in terbinafine resistance. Mutants maintain ergosterol in their membranes despite squalene accumulation, suggesting that normal cells are inhibited by the depletion of ergosterol rather than by accumulation of squalene [48, 49]. Unpublished information on the *S. cerevisiae* database (http://db.yeastgenome.org/cgi-bin/locus.pl?locus=erg1) indicates that deletion of ERG1 is lethal unless maintained under anaerobic conditions to allow uptake of exogenous ergosterol. More analysis of CaERG1 is warranted, to determine whether *C. albicans* follows the example of *S. cerevisiae*.

Expression of ERG1 and other ERG genes is increased about fivefold upon exposure to terbinafine in susceptible strains of *C. albicans* or *S. cerevisiae* [50, 51]. Can overexpression of ERG1 confer terbinafine resistance? Screening of our *C. albicans* overexpression library resulted in the isolation of ERG1-overexpressant that was highly resistant to terbinafine, without cross-resistance to azoles (ICAAC or unpublished data). This is consistent with results in *A. fumigatus*, in which a plasmid overexpressing ERG1 conferred resistance to terbinafine [52]. Parenthetically, overexpression of a naphthalene-degrading enzyme, salicylate 1-monooxygenase, conferred terbinafine resistance in *A. nidulans*, presumably by enhancing its degradation [53].

Genes other than the target ERG1 can affect susceptibility to terbinafine. Overexpression of *C. albicans* efflux genes CDR1, CDR2, or MDR1 in *S. cerevisiae* results in resistance to terbinafine and azoles [54–56]. Consistently, clinical strains that overexpress CDR1 are more resistant to terbinafine [42].

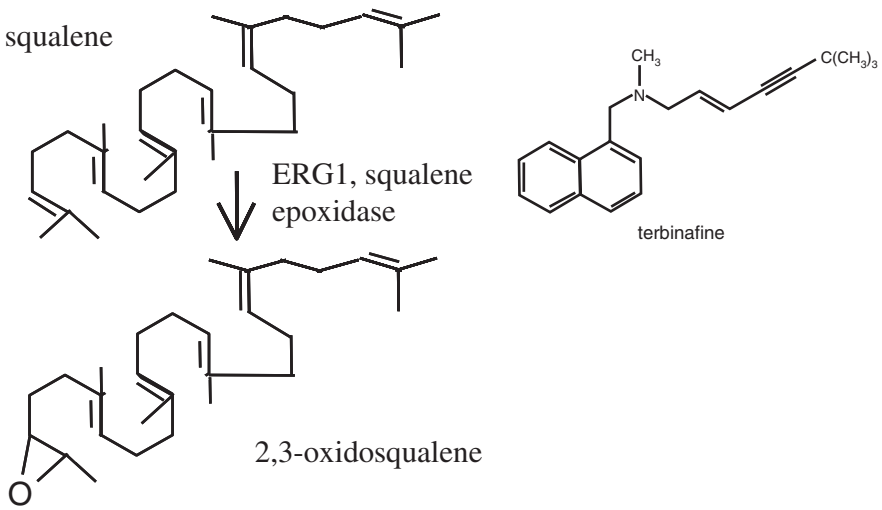


Fig. 30.5 Squalene epoxidase, encoded by ERG1, inhibited by terbinafine, http://www.doctorfungus.org/thedrugs/Terbinafine.htm

Disruption of CDR1 in *C. albicans* confers hypersusceptibility to terbinafine and azoles [57]. Disruption of MDR1 in C. *dubliniensis* does not alter susceptibility to terbinafine, even though its overexpression in *S. cerevisiae* results in resistance [58]. Mutations in ERG2 or ERG11 result in hypersusceptibility to terbinafine, azoles, inhibitors of sphingolipid synthesis, and other agents [59]. Disruption of ERG1 is likely to be lethal, as it is in *S. cerevisiae* except under conditions that allow uptake of exogenous ergosterol. However, transposon-mediated disruption of ERG1 was achieved in *C. glabrata*, resulting in increased resistance to both terbinafine and azoles [60]. Disruption of ERG3 does not alter terbinafine susceptibility, even though it confers azole resistance [37]. Disruption of CYB5, encoding a cytochrome component of ERG3, results in hypersusceptibility to terbinafine and azoles [61]. Disruption of CDC35, encoding adenylate cyclase, or CAP1, encoding cyclase-associated protein, confers hypersusceptibility to terbinafine and azoles, suggesting a role for cAMP signaling involving regulation of CDR1 [62].

ERG6 encodes C-24 sterol methyltransferase (Fig. 30.6), which catalyzes a reaction not shared in cholesterol biosynthesis. Hence it is an attractive target for antifungals. Its disruption in *S. cerevisiae* confers pleiotropic defects that include slow growth, poor mating, poor uptake of tryptophan, increased permeability, and increased cation and antifungal susceptibilities, suggesting increased membrane permeability [63–66]. Its disruption in *C. albicans* conferred hypersusceptibility to terbinafine, cycloheximide, fenpropiomorph, and tridemorph, but not to azoles, and resistance to AMB [67]. The authors surmise that these hypersusceptibilities were due to increased permeability. However, since no direct assays for this were reported, and since deletion of ScERG6 reduced activity of efflux pump Pdr5p [68], these conclusions are premature. Antifungal inhibitors that target ERG6 should be potent synergens with existing antifungals, if it is shown that antifungal susceptibilities remain high in an ERG6 disruptant that is overexpressing CDR1 or MDR1, to determine whether the increased efflux negates benefits of ERG6 disruption.

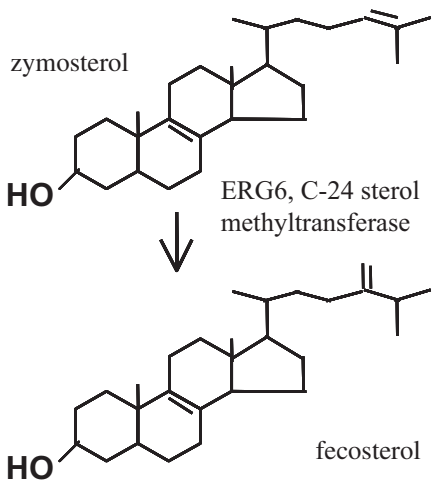


Fig. 30.6 C-24 sterol methyltransferase, encoded by ERG6

ERG24 encodes C14-sterol reductase, which finalizes the C-14 modifications initiated by Erg11p. It is the target of the fungicide fenpropimorph. In *S. cerevisiae*, this inhibition results in the accumulation of ignosterol (ergosta-8,14 dien 3β-ol) (Fig. 30.7). This indicates that downstream enzymes delta 8-7 isomerase, delta 5-desaturase, and delta 22-desaturase are inactive on sterols that retain the C14 = 15 double bond, and that ignosterol is not tolerated. It perturbs the membrane and inhibits uptake of glucose and pyrimidines. Mutations in ScERG24 confer resistance, and ScERG24 disruptants are aerobically viable, but only on defined media, a reflection of their dependency on their increased Ca^{++} in the media. Suppressor mutants of ScERG24 resistance mutants, *fen1* and *fen2*, have been identified (reviewed in [69, 70]). FEN1 encodes a fatty acid chain elongase [71], and FEN2 encodes a plasma membrane H^{+}-pantothenate symporter [72]. The common effect of mutations in these two genes is a reduction in membrane very-long-chain fatty acids and sphingolipids, so perhaps the fungicidal effect of accumulating sterol intermediates at the level of Erg24p depends on their interactions with these lipids.

CaERG24 was cloned by its ability to complement an *S. cerevisiae* erg24 mutant. Its disruption in *C. albicans* is viable, slow growing, slightly resistant to azoles and nystatin, and hypersusceptible to cycloheximide, cerulenin, fluphenazine, and brefeldin A, suggesting increased permeability. Disruptants were unable to germinate and showed reduced virulence in a mouse disseminated candidiasis model [73]. These results indicate that CaErg24p is like ScErg24p and is a potential target for next-generation antifungals.

Mutations in or overexpressions of CaERG24 have not been published. However, we find that its overexpression on our multicopy plasmid confers resistance to azoles; these transformants have not been tested yet for resistance to fenpropimorph (unpublished results). It is not obvious to us why overexpression of the C-14 reductase should confer resistance to inhibitors of C-14 demethylase; sterol analysis and permeability studies of these transformants are warranted.

ERG25 encodes C4-sterol methyloxidase, which, acting in concert with Erg26p, sequentially removes the two C4-methyl groups. Its disruption in *S. cerevisiae* results in sterol auxotrophy, indicating that the accumulated 4,4 dimethylzymosterol is not a tolerated membrane sterol. The auxotrophy can be suppressed, either by a second mutation in ERG11 or by azoles. In this situation, azoles actually promote growth of the *erg25* mutant. The intermediate that accumulates is 24-methylenelanosterol, which apparently cannot be partially acted on by Erg3p to create a toxic sterol [74]. This suggests that another route to azole resistance is

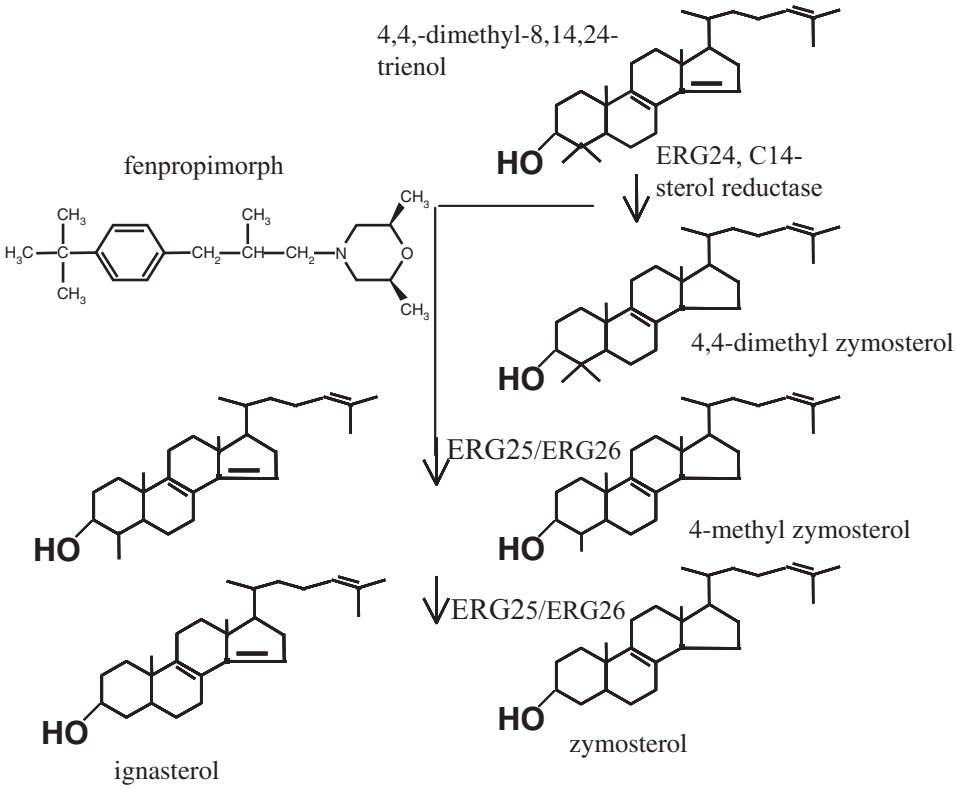


Fig. 30.7 Formation of zymosterol from sequential actions of C14-sterol reductase, C4-sterol methyloxidase, and C4-decarboxylase, encoded by ERG24,25,26. Formation of ignosterol by inhibition of C14-sterol reductase with fenpropimorph (left branch). Structure of fenpropimorph from http://www.hclrss.demon.co.uk/fenpropimorph.html

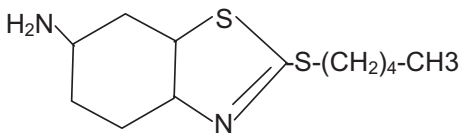


Fig. 30.8 Structure of APB

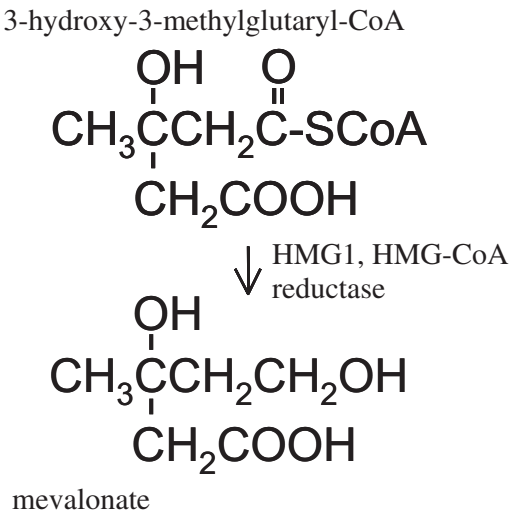


Fig. 30.9 HMG-CoA reductase, encoded by HMG1

by inactivating mutations in ERG25. More intriguing is the notion that downregulation of ERG25 should confer phenotypic resistance during azole exposure. The cloned CaERG25 gene is able to rescue *erg25* mutants in *S. cerevisiae* [75], but whether disruptants and azole suppression will be the same in *C. albicans* has not yet been determined.

Inhibitor studies suggest that blocking C4 demethylation will have similar effects in *C. albicans*. A natural antifungal agent, PF1163A derived from Penicillium, reportedly inhibits ScErg25p, since wild type cells are inhibited, but cells overexpressing Erg25p are not [76]. The agent also inhibits *C. albicans*, but resistance has not been reported [77]. Another agent, 6-amino-2-*n*-pentylthiobenzothiazole (APB) (Fig. 30.8), inhibits *C. albicans* in vitro and is effective in treating systemic candidiasis in mice [78, 79]. It blocks C4 demethylation, preferentially the second demethylation, in *C. albicans* and *S. cerevisiae*, as determined by an accumulation of C4-methylated intermediates [80, 81], but whether the specific target is Erg25p or Erg26p is not known.

HMG1 encodes the single *C. albicans* homolog encoding β-hydroxymethylglutarate reductase (Fig. 30.9), the rate-limiting and committed step in cholesterol biosynthesis in humans and the target of the statins such as lovastatin and zocor [82]. In *S. cerevisiae*, paralogous genes HMG1 and HMG2 each encode this activity and together are essential. The human enzyme can complement the double disruptant. The two enzymes are regulated differently. Hmg1p is limited by palmitoleic acid availability, unaffected by oleic acid, and

strongly inhibited by ergosterol. In contrast, Hmg2p is unaffected by palmitoleic acid, inhibited by oleic acid, and only slightly inhibited by ergosterol [83, 84]. Overexpression of a truncated but active form of HMG1 resulted in accumulation of normal levels of squalene, but near-normal levels of downstream intermediates, suggesting that Hmg1p is rate limiting for the early portion of the pathway, but that there are other rate-limiting steps after squalene [85].

Few studies have reported mutations in ScHMG1/2 that confer resistance to statins [86]; none for CaHMG1 have been reported. However, lovastatin inhibits growth of *C. albicans* and acts synergistically with FLZ to reduce the MIC (FIC 0.08), although this requires high concentrations of the statin and the synergy is media dependent. What effects inhibitory concentrations of lovastatin had on sterol composition, and whether the lovastatin alone or in combination was fungicidal was not reported. Lovastatin alone did not appear to cause changes in expression levels of HMG1 or selected genes in the ergosterol pathway, and changes in these genes after combined treatment approximated changes after FLZ alone [87]. Consistently, in *S. cerevisiae*, lovastatin is highly synergistic with azoles and results in inhibition of sterol esterification [88]. Perhaps the synergy results in part from a modest induction by lovastatin of ERG3 [89], which would potentiate azole effects.

Statins may be clinically useful in combination antifungal therapy, but a more potent, fungal specific statin derivative would likely be more useful and potentially fungicidal, than lovastatin.

Parenthetically, prenylation appears to be essential for *C. albicans*, since disruption of RAM2, encoding a subunit of farnesyltransferase, is lethal. However, mammalian inhibitors of this enzyme are not effective on *C. albicans* [90].

2.2 Amphotericin B (AMB)

This polyene antifungal forms a complex with a higher affinity for membrane ergosterol than for cholesterol, accounting for its specificity. Binding by this complex is fungicidal, resulting in cell permeabilization [91, 92]. Detailed aspects of AMB mechanism and resistance can be found in Chap. xx.

AMB-resistant isolates from clinical samples of *C. albicans* are rare, and it is difficult to obtain resistant mutants in vitro with single-step selection. While that is great news clinically, it has hampered understanding of the cellular response to AMB. Inhibition of ergosterol biosynthesis with azoles results in subsequent phenotypic resistance to AMB, consistent with the model that ergosterol is its primary binding site [93–95]. Consistently, mutations (ERG3, ERG11) that deplete *C. albicans* or *C. glabrata* of ergosterol result in AMB resistance in laboratory [39, 96] or patient isolates [97–99]. Induction of *C. albicans* CDR1 by adriamycin resulted in tolerance to AMB [100], but clinical strains that overexpress CDR1 are generally not resistant to polyenes.

Some studies implicate the fungal cell wall in AMB resistance. *C. albicans* shows increasing transient resistance, "phenotypic resistance," or PR, to AMB as it moves into stationary phase. This change is not seen if cell walls are removed with zymolyase or other wall-degrading enzymes, or weakened with mercaptoethanol [101–103]. Ultrastructural changes in the periplasm and wall correlate with increasing PR [104]. We showed that intrinsic AMB resistance in *C. lusitaniae* is regulated by a high-frequency switching mechanism. Cells of most strains switched from resistant to susceptible, concomitantly from round to elongate in cell shape, at a frequency of about 1 per 100 to 1000 cells. Susceptible, elongate cells were hypersensitive to zymolyase, and zymolyase rendered resistant cells susceptible to AMB [105]. Consistently, in *A. flavus*, AMB-resistant mutants were isolated by stepwise selection. Mutant spheroplasts were as susceptible as wild type, but intact cells were resistant, suggesting that cell wall alterations conferred resistance [106].

These data gave rise to the prediction that mutations that alter cell wall structure, or perhaps alter key targets within the cell wall, and will alter susceptibility to AMB. However, appropriate mutants or candidate genes for this have not yet been identified or analyzed. Consistent with this expectation, microarray analysis of an AMB- and FLZ-resistant derivative of *C. albicans* implicated some cell wall maintenance genes. The mutant was isolated by stepwise selection on increasing concentrations of AMB [107]. The resistance was not stable past 28 generations of growth in the absence of selective pressure, so it may have been "phenotypic." Nonetheless, the derivative strain was depleted of ergosterol and instead had predominantly lanosterol and 24-methylene lanosterol. Microarray analysis of the mutant, in the absence of AMB, showed some increases in ERG6, ERG25, and ERG5, and an increase in cell wall maintenance gene PHR2. However, these experiments need confirming, since they were just based on duplicate microarrays and validated only by nonquantitative PCR, with often marginal fold changes in expression.

Much of the AMB-resistance pathway is still unknown. Consistently, we recovered five different genes from our *C. albicans* overexpression library, whose overexpression confers resistance to AMB. Only one of these is wall associated, one may be a stress-response sensor, and the rest have no associated functions to date.

2.3 Regulation of Ergosterol Biosynthesis Genes

Studies of the regulation of ERG genes in *C. albicans* are just beginning. Extrapolating from studies of *S. cerevisiae*, we expect that the pathway is feedback inhibited by ergosterol or

other late sterol derivatives at the enzymatic and transcriptional levels. Upregulation of ERG genes results from downstream inhibition by antifungals or disruptions. ScERG10 is negatively regulated by sterols [108]. ScERG2 is negatively regulated by an intermediate between zymosterol and ergosterol [109].

Strong regulation of ScHmg1p occurs at translational and degradational levels in addition to transcriptional [108]. Promoter/reporter fusion constructs in *S. cerevisiae* were used to ask which of the ERG genes were transcriptionally regulated in response to inhibitors of HMG1, ERG9, or ERG11 [89]. Several genes stood out as being strongly upregulated compared to other genes in the pathway. All inhibitors upregulated ERG12, ERG8, and ERG19 in the isoprenoid subpathway, and ERG9 and ERG2 in the sterol-specific latter pathway. ERG 10, HMG1, and ERG20 were upregulated by statins, and ERG11 and ERG6 were modestly upregulated by azoles. ERG3, ERG4, and ERG5 were downregulated by FLZ. In contrast, an earlier study showed dramatic induction of ScERG3 by statins or ketoconazole [41].

ScErg9p, encoding squalene synthase, is the first enzyme in the pathway dedicated just to sterols as opposed to earlier points in the isoprenoid pathway. Therefore it should be and is a focal point of regulation, since sterols are needed in vast excess of other isoprenoid derivatives. ScERG9 is upregulated by defects in downstream genes ERG3, ERG7, and ERG24 or by inhibitors (azole, zaragozic acid) and by heme-activated protein transcription factors HAP1 and HAP2/3/4 [110]. ScERG9 is indirectly but specifically upregulated by Slk19p and downregulated by MFS protein Tpo1p. Upregulation increases flow through the pathway and increases ergosterol content, generating azole/terbinafine/zaragozic acid resistance, but nystatin hypersusceptibility [111]. Studies of the regulation of CaERG9 are not yet reported. More complex levels of regulation are probably also operative. For example, ScERG3 is upregulated by mutations in ScHMG1, ScERG2, ScERG4, ScERG5, and ScERG6 [112]. This result indicates that more expression analysis is needed, and the complexity offers the opportunity for significant differences in regulation between species.

One of the pathways that branch from pre-CaERG9 ergosterol biosynthesis results in synthesis and secretion of farnesol, a derivative of farnesyl pyrophosphate. In vitro, farnesol acts as a quorum sensor, in that its gradual accumulation by growing cells inhibits the yeast-to-hyphal transition without itself being inhibitory [113]. Farnesol accumulation increases eightfold upon inhibition of CaErg9p by zaragozic acid, even more rapidly after azole inhibition [114, 115]. The authors suggested that this accumulation is partly responsible for the fungicidal or fungistatic effects of the antifungals, and for their ability to inhibit biofilm formation, but this remains to be demonstrated.

Expression studies in *C. albicans* have generally confirmed expectations that negative regulation based on sterol levels is operative, since inhibitors or downstream mutations result in upregulation of several ERG genes. Which genes are upregulated depends on how the individual study was conducted. *C. albicans* strains with mutations in post-ERG11 genes, notably ERG6 or ERG24, show increases in expression of ERG11, ERG7, and ERG25 [116]. In an RT-PCR study, azoles and terbinafine induced expression of ERG9, ERG11, ERG25, and ERG3 from 1 to 5 h after exposure, and prevented downregulation of ERG1 and ERG7 [51]. Some of these changes were shown to require a histone deacetylase activity, since its inhibition by trichostatin A prevented the ERG gene inductions [117]. In a promoter-fusion study, ERG11 was induced by azoles and terbinafine after a lag period, suggesting that the induction resulted from the depletion of ergosterol occurring in the first 4–5 h after exposure [118]. In an older microarray study, 24-h exposure to itraconazole resulted in upregulation of most of the ERG genes [119]. However, this study may be flawed in that it compared expression levels to parallel untreated 24-h cultures which were in post-log phase by that time. Baseline levels of the ERG genes would be reduced under those conditions, inflating the levels of the itraconazole-treated culture.

More recent microarray studies have focused on comparing resistant to susceptible cultures, or comparing effects of very-short-term exposures to azole. In the Karababa et al. microarray study, ERG3, ERG6, and ERG25 were upregulated four- to sixfold in strains overexpressing CDR1, but were not upregulated by short-term exposure to fluphenazine [120]. In contrast, the ERG genes did not show major changes in expression among lineages that evolved resistance to azoles after in vitro selection, other than a threefold decrease in ERG1 in one of the adapted strains [121]. In stepwise selected azole-resistant strains examined by Roger et al., only ERG2 showed an increase [122]. Since these microarray-based analyses of different azole-resistant strains do not show common alterations in specific ERG genes, and also differ from similar studies in *S. cerevisiae*, where, for example, ERG8 and ERG13 are downregulated [123], the implication is that they do not play pivotal roles in the resistant phenotypes. Alternatively, in individual strains, individual changes in ERG gene expressions may be important in the context of other mutations that are lineage specific.

Despite the systematic analysis of ERG gene expression following exposure to antifungals, recent work has identified one of the regulators. CaUPC2, homologous to sterol uptake genes ScUPC2 and ScECM22, is important for ERG gene inductions and antifungal resistance [124]. Disruption of this gene, encoding a zinc finger transcription factor, confers hypersusceptibility to azoles, terbinafine, and lovastatin, as well as cell wall-acting agents Nikkomycin Z and calcofluor white. Disruptants are unable to upregulate ERG2 or ERG11 in response to fluconazole, and show depleted levels of ergosterol, and reduced ability to import labeled cholesterol.

Thus, CaUPC2 may regulate expression of ergosterol biosynthesis and sterol uptake genes. Potentially, hyperactive point mutations in CaUPC2 could confer azole/terbinafine resistance. This study suggests that regulation of ERG genes is important in maintaining normal levels of susceptibility to antifungals, so it is not inconsistent with the mixed messages from microarray studies which suggest that there is no dominant pathway to antifungal resistance by overexpression or upregulation of specific ERG genes.

Identifying putative regulatory genes by homology to *S. cerevisiae* is not always so successful. ROX1 has been identified as a transcriptional repressor of ERG genes in *S. cerevisiae*, but its closest *C. albicans* homolog RFG1 is involved instead in filamentation [125]. However, these genes are not orthologous; their proteins share only a short region of homology, an HMG domain indicative only of a DNA-binding protein, not of a specific function. A corresponding *C. albicans* gene, if it exists, will have to be discovered with a functional assay.

A short publication recently documented a 2.5-fold upregulation of ERG3 by disrupting EFG1, and makes the claim that resistance is the result of this upregulation [126]. EFG1 encodes a helix-loop-helix transcriptional activator which is known as a regulator of morphogenesis and virulence [127–129]. This recent claim that overexpression of ERG3 mediates resistance is at odds with our results (Table 30.3) and with the expectation that overexpression of ERG3 should increase susceptibility, not resistance, to azoles, by increasing production of the toxic sterol intermediate (Fig. 30.4). While the publication does support that ERG3 is upregulated in EFG1 disruptants, it does not support the conclusion that the resulting resistance is mediated by ERG3, since expressions of hundreds of other genes were also affected by the disruption, and since expression of ERG3 was only monitored at a single time point in the absence of FLZ.

Table 30.3 Overexpression of ERG and PDR genes alters susceptibility to antifungals

Gene	FLZ	TER
ERG1	C	R
ERG5	C	C
ERG9	C	C
ERG20	C	C
ERG3	HS	C
PDR16	HS	C
ERG6-7	R	C
ERG11	R	C
ERG24	R	C

Genes were amplified with elongase and cloned as *SphI* fragments into pMPA9MAL1
Clones were verified by sequencing
C: transformants show the same susceptibility as controls; HS: hypersusceptible; R: resistant

2.4 ERG Gene Overexpression Study

Our laboratory has initiated a study on the effects of overexpression of CaERG genes on antifungal resistance. Each gene (Table 30.3) was amplified from a susceptible *C. albicans* genome by PCR and cloned into our high copy plasmid [36]. Recombinant plasmids were transformed into *C. albicans*, and tested for susceptibility in an agar-based assay. Overexpression of ERG1 resulted in terbinafine resistance without cross-resistance to FLZ, as expected. Likewise, ERG11 or ERG 6 overexpression conferred azole resistance, and ERG3 overexpression conferred hypersusceptibility.

There were some surprises in this preliminary study. We did not expect from existing work that overexpression of ERG24 would confer resistance, since its disruption confers slight azole resistance [73]. Overexpression of CaPDR16 conferred hypersusceptibility, but was expected to confer resistance, since disruption of ScPDR16 confers hypersusceptibility [130].

One might have predicted that overexpression of genes upstream of ERG11 would confer azole hypersusceptibility if they resulted in increased production of the toxic sterol. Alternatively, the overexpression might confer resistance if they resulted in increased levels of Erg11p. Neither was observed, since overexpression of Erg1p, Erg9p, and Erg20p had no effect on azole susceptibility. This suggests that they are not rate-limiting steps in production of lanosterol, so that their overproduction does not increase lanosterol pools. Since upregulation of ScERG9 confers azole resistance [110, 111], we may be witnessing another pathway difference between *C. albicans* and *S. cerevisiae*.

3 Efflux of Antifungals as a Resistance Mechanism

3.1 ABC Transporters

CDR1 and CDR2 are ABC transporters, transmembrane efflux pumps that use ATP to move a variety of small hydrophobic compounds out of the cell. These are the major efflux mechanisms of clinical significance in *C. albicans*. They are homologous to *S. cerevisiae* efflux pump PDR5 and were identified by their ability to complement *pdr5* mutants [54, 131]. They are related to the transmembrane human P-glycoprotein encoded by MDR1. By extrapolation from studies on human MDR1, one expects that the *Candida* pumps will work by binding of small hydrophobic substrate molecules to high-affinity sites, probably within the plasma membrane in the cytoplasmic leaflet, which then pass substrate to a secondary site on the extracellular leaflet, from which it is released. Binding and movement of substrate to each of the two sites will require binding and hydrolysis of 1

ATP, each by a different nucleotide-binding domain (NBD). A recent review models these expectations [14]. How such a broad group of structurally unrelated compounds can be effluxed by a single pump is still an open question.

Overexpression of CDR1 or CDR2 confers resistance to azoles including fluconazole, ketoconazole, miconazole, voriconazole, and itraconazole, and to terbinafine and cycloheximide. Overexpressing strains have increased ability to efflux nystatin [132], but this did not confer resistance to nystatin [133]. Based on clinical strains that overexpress CDR1 or CDR2 but are susceptible to posaconazole, one assumes that the latter is not an effective substrate [24]. Many other agents are apparently effluxed by CDR1 or CDR2, and it is clear that the two pumps have only partly overlapping specificities (reviewed in [14]).

In *S. cerevisiae*, there are at least 31 genes encoding ABC proteins, 11 in the PDR family [134]. In the *C. albicans* genome, there are only six members of this family that have both dual nucleotide-binding domains (NBD), each followed by six-transmembrane domains (Table 30.4). CDR1 and

Table 30.4 ABC transporter genes in *C. albicans* and their homologs and functions in *S. cerevisiae*

	CA gene	CA gene ID	SC homolog	%ID (similar)	Function of SC gene	Function of CA gene
PDR family						
1	CDR1	CaO19.6000	PDR5	54(71)	Pleiomorphic drug resistance	Pleiomorphic drug resistance
2	CDR2	CaO19.5958	PDR5	53(69)	Pleiomorphic drug resistance	Pleiomorphic drug resistance
3	CDR3	CaO19.1312	PDR5	48(65)	Pleiomorphic drug resistance	Unknown, not resistance[a], opaque-specific
4	CDR4	CaO19.5079	PDR5	51(68)	Pleiomorphic drug resistance	Unknown, not resistance[a]
			PDR11	33(49)	Sterol uptake	
			PDR12	44(62)	Weak acid efflux	
			YNR070W	55(71)	Unknown	
5	CDR99	19.8533,4	PDR5	56(73)	Pleiomorphic drug resistance	Unknown
6	SNQ2	CaO19.5759	SNQ2	54(73)	Drug resistance, not azoles, partially overlaps PDR5	Unknown
			AUS1	34(52)	Sterol uptake	
7	ADP1	CaO19.8090	ADP1	50(65)	Unknown	Unknown
8	Unnamed	CaO19.4531	YOL075c	34(53)	Unknown	Unknown
MRP family						
9	YCF1	CaO19.13832	YCF1	55(71)	Vacuolar glutathione-conjugate-bilirubin, cadmium transporter activity	Unknown
			VMR1	35(51)	Vacuolar metal resistance	
			NFT1	25(46)	Unknown	
10	MLT1	CaO19.5100	BPT1	38(57)	Like YCF1	Unknown
11	Unnamed	CaO19.6382		43(62)		Unknown
12	YOR1	CaO19.1783	YOR1	47(64)	Efflux organic anions, oligomycin resistance	Unknown
			YBT1(BAT1)	32(52)	Bile acid transporter	
MDR family						
13	ATM1	CaO19.1077	ATM1	64(75)	Mito Fe/S transporter	Unknown
14	HST6		STE6	30(51)	Secretes α-factor	Probably secretes mating peptide
15	MDL1	CaO19.10146	MDL1	46(64)	Mito peptide transporter	Unknown
16	MDL2	CaO19.5600,5599	MDL2	38(54)	Mito peptide transporter	Unknown
ALD family						
17	PXA1	CaO19.7500	PXA1	42(57)	Peroxisomal transport LCFA	Unknown
18	PXA2	CaO19.12720	PXA2	40(67)	Heterodimer with PXA1	Unknown
Unclassified						
19	Unnamed	CaO19.10632	None		Unknown half transporter, conserved in many fungi	Unknown

Information regarding *S. cerevisiae* genes and family groupings is based on [134]. *C. albicans* genes were listed based on Blast searches with each *S. cerevisiae* gene. Genes in the PDR family were also searched by blasting the conserved NBD domains in the CDR family [426] against the *C. albicans* genome database [135]. % identities and similarities of the homologs from *C. albicans* versus *S. cerevisiae* were from Blast alignment results. Functions associated with the *C. albicans* genes are discussed in the text; functions associated with *S. cerevisiae* genes are readily accessed at http://www.yeastgenome.org/

[a]Incomplete analysis does not implicate resistance

CDR2 are about ~1500 amino acids long, 100 kb apart on chromosome 3, and are 83 % identical and 91 % similar [135]. Four other genes in *C. albicans* share extensive homology. CDR99 on Ctg19-10079 is the closest in sequence, 69 % identical, 81 % similar to CDR1, followed by CDR4 on chromosome 1 at 59 % identical, 73 % similar, CDR3 on chromosome 4 (53 % identical, 68 % similar), and SNQ1 on chromosome 6 (39 % identical, 57 % similar). CaO19.4531 has an additional seventh TM domain in the *N*-terminal half, and is only 24 % identical, 42 % similar to CDR1. ADP1 has a half-transporter structure with one NBD and a six-transmembrane domain, which are 22 % identical and 44 % similar in this region to CDR1. Each member of this family has nearly identical patterns of TM domains (Fig. 30.10). Each member also has highly conserved NBD domains (Fig. 30.11). No other proteins in the *C. albicans* database show the conserved NBD characteristic of this family.

Among these, only CDR1–4 have been functionally analyzed, and only CDR1 and CDR2 are so far associated with azole resistance. Many lines of evidence argue that these two genes are major determinants of resistance in isolates from clinical samples. For example, expression of these genes on a high copy plasmid in *S. cerevisiae*, in which endogenous ABC transporters were deleted, conferred high-level resistance to azoles [54, 55, 136]. Deletion of CDR1 confers hypersusceptibility to azoles in *C. albicans* [57]. Overexpression of CDR1 and CDR2 is common among resistant clinical isolate [137–140] and in laboratory isolates selected for azole resistance [121, 141, 142], although some susceptible clinical isolates also overexpress CDR1 [140]. Deletion of CDR2 in *C. albicans* confers hypersusceptibility, in strains already deleted for CDR1 [54]. Expression of CDR2 is elevated in revertants of hypersusceptible mutants in which CDR1 had been disrupted [54].

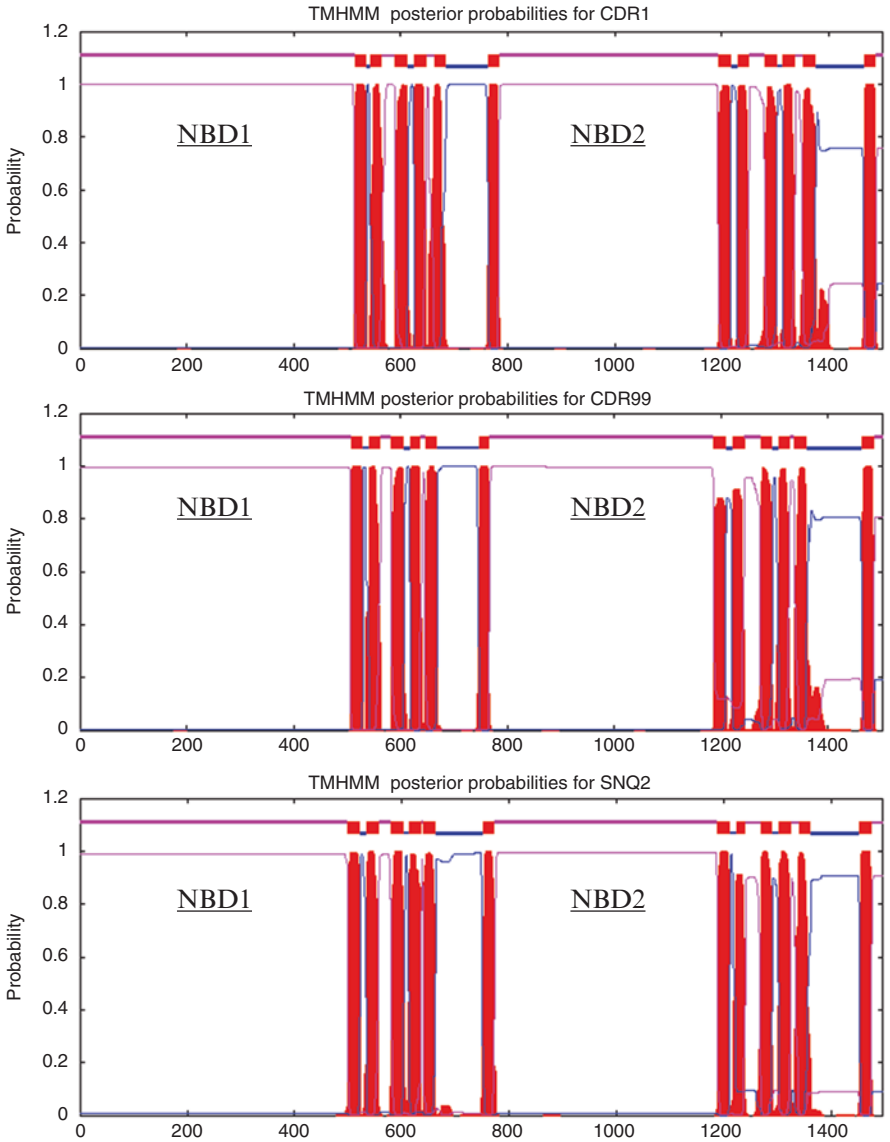
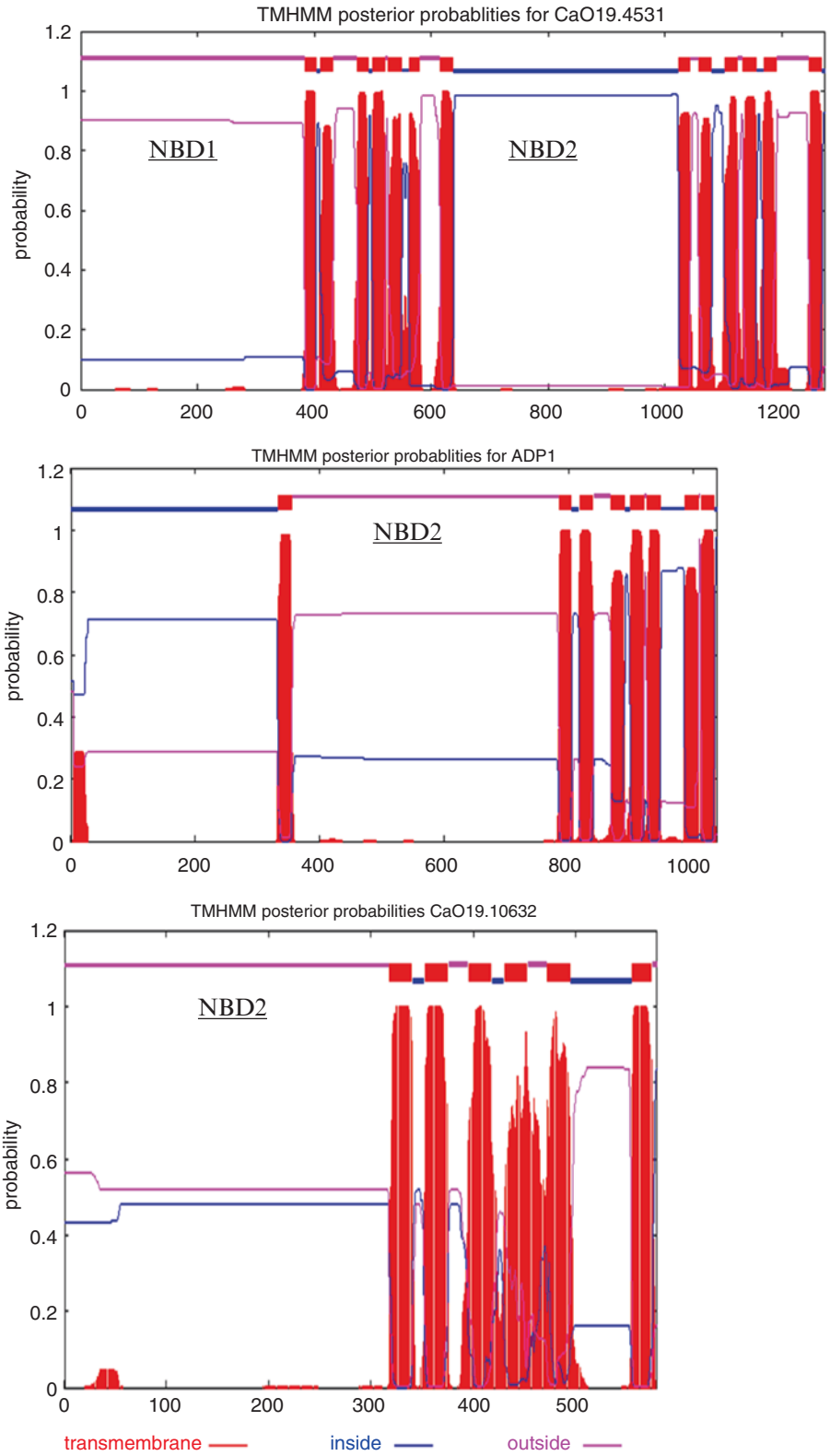


Fig. 30.10 Relative positions of transmembrane helices and nucleotide-binding domains in the *C. albicans* CDR gene family. Determined using TMHMM server 2.0 software, http://www.cbs.dtu.dk/services/TMHMM-2.0/

Fig. 30.10 (continued)



Heterologous overexpression in *S. cerevisiae* of CaCDR3 did not confer a resistant phenotype. Neither CDR3 or CDR4 is induced by FLZ. Disruption of CDR3 or CDR4 did not confer hypersusceptibility [143, 144]. Therefore it appears that these genes are not involved with resistance. However, these disruptions are inconclusive, and the implication that neither gene functions in multidrug resistance is premature, pending analysis of disruptants created in a strain disrupted

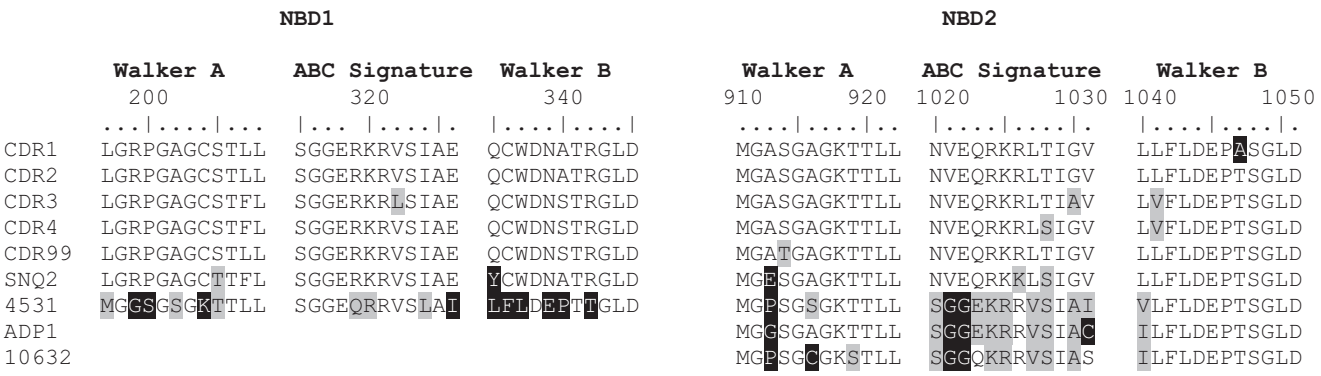


Fig. 30.11 Conserved nucleotide-binding domains in the *C. albicans* CDR family. Most sequences in this domain are identical. Conserved changes are highlighted in *gray*, unconserved changes relative to CDR1 are white letters on black background. Modified from [144]

for CDR1 and CDR2, and pending overexpression analysis in *C. albicans*.

Structurally, CDR1 is a typical ABC transporter in many respects, composed of a pair of tandemly duplicated six-pass transmembrane domains, each with conserved, nonidentical nucleotide-binding domains (NBDs) with ATPase activity (Fig. 30.10). The NBDs are upstream of the TM domains, and each has a highly conserved ABC signature motif with flanking Walker A and B motifs [144]. CDR1 has the fungus-specific cysteine in the conserved Walker sequence of NBD1, instead of the invariant lysine found in non-fungal ABC transporters (Fig. 30.11). Replacement of this cysteine with a lysine in CaCDR1, placed in a *S. cerevisiae* hyperexpression system, diminishes ATPase activity and confers hypersusceptibility to antifungals, without altering protein localization or stability. Replacement of the conserved lysine in the Walker box of NBD2 has similar but not identical effects, from which the authors conclude that the two NDBs have different functions [145]. The authors, however, have not dismissed an alternative explanation that the two might have identical functions that are not evident since the mutations introduced into each are not equivalent. Site-directed mutagenesis of C193 or K901 in Walker box 1 and 2 (outside the most conserved sequences shown in Fig. 30.2) diminishes ATPase activity, whereas changes at other conserved positions in the boxes do not [14]. Another structure-function study shows that a mutation in transmembrane domain 11, converting threonine at position 1351 to phenylalanine, blocks resistance to antifungals and FLZ efflux, without altering ATPase activity, nucleotide/substrate binding, or protein localization and stability [146].

Six-transmembrane domains are on the carboxy side of each NBD in the CDR family of transporters. Based on studies with human Mdr1p, one expects that some drug-binding sites in Cdr1p would occur in the carboxy-terminal transmembrane domain 12. Consistently, deletion of this domain from CDR1 and expression in a multicopy plasmid in *S. cerevisiae pdr5* mutant resulted in loss of resistance relative to intact cloned CDR1. However, the loss was selective and drug dependent. For example, CDR1-mediated resistance to azoles, oligomycin, chloramphenicol, and benomyl was retained in the ΔTM12 strain, but resistance to cycloheximide, anisomycin, and nystatin was lost. The ΔTM12 strain retained the CDR1-dependent ability to efflux estradiol and to hydrolyze ATP [132]. Point mutations in TM10 of ScPDR5 alter substrate (azole) and inhibitor (FK506) specificity [147]; effects of analogous mutations in CDR1/2 have not yet been reported. However, site-directed mutagenesis of CaCDR1 overexpressed in *S. cerevisiae* showed that some mutations in TMS11 or in the sequence between Walker box A and signature C in NBD1 conferred hypersusceptibility to anisomycin, cycloheximide, fluconazole, miconazole, and nystatin. Other mutations in TMS6 or in the sequence between Walker A box and signature C sequences in NBD2 conferred hypersusceptibility to a subgroup of these substrates. One mutation in TMS6 causes mislocalization of the protein unless the cell was grown in cycloheximide [14]. Clearly, many more site-directed mutations are needed to tell this story.

The fluorescent dye rhodamine 6G has been used to monitor efflux via CDR1, since the dye has been shown to accumulate in FLZ-susceptible cells not overexpressing CDR1, and conversely to move into the supernatant in FLZ-resistant cells overexpressing CDR1 or CDR2, but not MDR1. This efflux is energy and temperature dependent, as expected of active transport mechanism [148]. It had been assumed that the dye was directly effluxed by Cdr1p, but could have been an indirect mechanism activated by Cdr1p activity. Direct involvement with substrates has now been demonstrated. Shukla et al. showed that a heterologously overexpressed CaCDR1-GFP pump binds to photoaffinity reagents that bind to human P-glycoprotein. Binding of these reagents is competed out by putative Cdr1p substrates nystatin and miconazole, in a manner suggested by at least two substrate-binding sites [149]. Similarly, Gauthier et al.

showed that Cdr1p and Cdr2p cross-link to a photoaffinity reagent derived from rhodamine 6G, showing their direct involvement in efflux and enabling the localization of binding sites. They cloned the genes into a high copy vector behind the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, and expressed the proteins in *S. cerevisiae* transformants; CDR2 had been modified to alter CTG codons to TCT to maintain serines in those positions. The transformants were resistant to fluconazole, ketoconazole, and itraconazole, and showed increased efflux of rhodamine 6G [150], as predicted in earlier work [54, 55]. They went on to show that the photoaffinity reagent IAARh123, known to cross-link to human MDR1, specifically cross-linked to both CDR1 and CDR1. They then cloned and expressed *N*- and *C*-terminal "halves" of CDR2 in their *S. cerevisiae* system, and showed that each half could bind rhodamine 6G independently, although both halves were required for resistance. Cells expressing CDR1 were hypersusceptible to FK520, an immunosuppressant shown to compete for binding sites on human MDR1, whereas cells overexpressing CDR2 were resistant to FK520. Furthermore, cells expressing CDR1, but not CDR2, showed strong synergy between fluconazole and FK520 at subinhibitory doses [150]. Similar results were reported by [55]. Cells expressing CDR2, but not CDR1, were hypersusceptible to hydrogen peroxide and resistant to diamide [150]. Thus, there are clear differences between these two highly homologous pumps.

More recently, specific overexpression of CDR1 in *C. albicans* has confirmed its importance in conferring multidrug resistance in its normal host [133]. Its overexpression was achieved by creating a fusion of the *N*-acetylglucosamine-inducible, glucose-repressible HEX1 promoter to the coding region of CDR1. This construct was cloned into a shuttle plasmid and transformed into *C. albicans* strain CAI4. Transformants showed *N*-acetylglucosamine-dependent increases in CDR1 mRNA and CDR1p antigen, and rhodamine 6G efflux, during 3 h of induction, with no corresponding increases in glucose-grown cells. Cells grown in *N*-acetylglucosamine showed increased resistance to typical CDR1 "substrates," including azoles and terbinafine, confirming that the elevated level of CaCdr1p had the same effect on *C. albicans* as it did in *S. cerevisiae.* Surprisingly, transformants were also resistant when grown in glucose, when the cloned gene should have been repressed. The differences in induction experiments and in vivo resistance were not adequately addressed by the authors, but the lack of glucose repression of resistance may have been due to derepression by depletion of glucose or other effects occurring at the extended growth time points. The study, therefore, would have been tighter had they included those time points in their mRNA and antigen assays, to show corresponding increases in CDR1 in glucose cultures. Without this data, and to some extent even with it, the devil's advocate is left with the argument that increased CDR1 results in an unknown secondary effect which in turn is responsible for the phenotype of the transformants. This is an argument worth minimizing, especially since the study used YNB broth as its test media, in which most strains of *C. albicans* adapt and grow in YNB plus FLZ at ≤64 μg/mL [151].

Immunosuppressant FK520 is known to be synergistic with azoles to produce a fungicidal effect. The following observations led the authors to conclude that this synergy is due to direct competition of FK506 with FLZ for CDR1-mediated efflux: (1) cells overexpressing wild-type CDR1 accumulate FLZ in the presence of FK506, but not so for cells overexpressing mutant CDR1 (T1351F), which does not bind FK506; (2) cells overexpressing CDR1 are hypersusceptible to the combination, but those overexpressing the T1351F mutant gene are not [146]. In other words, residual FK506-resistant efflux of FLZ by the mutant is sufficient for low-level resistance. This argument is somewhat weakened by the observation that the level of FLZ accumulation in the mutant is only marginally lower than in controls not expressing CDR1 at all. Furthermore, it is controversial, since other studies indicate that the immunosuppressants act by inhibiting the calcineurin-mediated stress response pathway. Another explanation to the Shukla et al. study might be that a high level of CDR1-mediated efflux is detrimental to cells with an activated calcineurin pathway.

Cdr1p and the related Pdh1p in *C. glabrata* are homologous to and presumably efflux antifungals like *C. albicans* CDR1. There is evidence that the *C. glabrata* pumps may be regulated by phosphorylation. Their overexpression in the *S. cerevisiae* hyperexpression system shows that they confer resistance to azoles, terbinafine, and rhodamine 6G, but not polyenes, and allow efflux of rhodamine 6G. FK506 and oligomycin reversed the increased resistance conferred by Cdr1p/Pdh1p, whereas verapamil and cyclosporine A, inhibitors of human MDR1, did not. In this system, Cdr1p/Pdh1p show glucose-dependent phosphorylation. For Pdh1p, this is presumably by protein kinase A, since (a) the phosphoprotein is recognized by antibodies that react with PKA substrates (those having phosphoserine or phosphothreonine followed by arginine), but not with standard phosphoserine or phosphotyrosine antibodies; (b) the phosphorylation is prevented by the PKA inhibitor H-89, which increases FLZ susceptibility; and (c) phosphorylation requires glucose, which activates the PKA pathway. This phosphorylation might be required for Pdh1p activity, since H-89 and other PKA inhibitors reverse FLZ resistance. Phosphorylation of Cdr1p is not detected with antibodies to PKA substrates, but it is detected with anti-phosphothreonine and anti-phospho-Akt substrate antibodies, as is Pdh1p [152]. Phosphorylation of Cdr1p may also be required for its function, since dephosphorylated membrane fractions containing Cdr1p show reduced ATPase activity [152], and since serine-to-alanine

mutations at major sites of phosphorylation, S307 and S484, diminish rhodamine 6G efflux. Double mutants are completely defective in efflux. Mutation at S307, near NBD1, or the double mutant, but not at S484 alone, showed increased susceptibility to a host of xenobiotics. Surprisingly, the double mutant is still much more resistant to FLZ than controls not expressing any Cdr1p, despite its completely inactive rhodamine 6G efflux activity. Phosphorylation at S307 and S484 is presumably affecting the NBD1/NBD2 interactions in the homodimer, and intragenic revertants of S307A/S484A double mutants presumably mimic the configuration normally induced by phosphorylation [153]. All of these modifications, as it should be remembered, are occurring in the heterologous hyperexpression system in *S. cerevisiae*. It remains to be determined whether phosphorylation of either pump occurs in *C. albicans*, how that affects their function, and how it is regulated.

Studies cited above suggest that resistance in isolates from clinical samples to azoles, largely mediated by overexpression of CDR1/2, might be overcome by simultaneous treatment of ABC inhibitors such as FK506, FK520, or a propafenone GP382 [55, 146, 150]. From a clinical perspective, this approach may be limited, since the inhibitors appear to target only Cdr1p, not Cdr2p, and since resistance mutations arise at high frequency. Disulfiram is another candidate for a CDR antagonist, since it appears to inhibit human MDR1 [154, 155]. This oxidant, known for its use as a deterrent-based treatment for alcoholism, inhibits human acetaldehyde dehydrogenase, resulting in the accumulation of acetaldehyde and associated nausea following ethanol consumption. Shukla et al. [156] showed that disulfiram treatment of plasma membranes isolated from *S. cerevisiae* that overexpress CaCDR1 results in inhibition of its ATPase and nucleotide-binding activities. They further show that disulfiram acts synergistically with antifungals that are substrates of CDR1 to inhibit the CDR1 overexpressing strain. The authors, however, imply that disulfiram reverses CDR1-mediated resistance by specific reactions with CDR1. The agent likely inhibits activities of many enzymes, not just CDR1, the only activity looked at in the study; hence it is fungicidal alone at slightly higher concentrations than when used in the synergy study. Would, for example, a strain that is FLZ resistant due to overexpressed ERG11 also be rendered susceptible by subinhibitory concentrations of disulfiram? In addition, if the synergy is specific for CDR1, can a concentration of disulfiram be found that is less effective on the overexpressing strain than on the parent at the same FLZ concentration? This potential lack of specificity may be a deterrent to its clinical use, as is its lack of fungicidal or fungistatic effects on *C. albicans* in ex vivo blood cultures, even at much higher doses (unpublished observations).

3.1.1 Regulation of CDR1/CDR2 Genes

Regulatory Sequences

Understanding how CDR1 and CDR2 are regulated is clinically important, since in resistant isolates, these genes and others are often upregulated together, suggesting that resistance is due to mutations in regulatory genes.

Studies in *S. cerevisiae* highlight the complexity of regulation of multidrug resistance genes. ScPDR1 and ScPDR3 encode the master regulators in the PDR network. The proteins are Gal4p-like Zn(II)2Cys6 transcription factors which form homo- and hetero-dimers that are constitutively nuclear and bind to their target response element (PDRE, consensus 5′-TCCGCGGA-3′). Differing numbers and combinations of binding sites upstream of individual PDR target genes determine whether PDR1, PDR3, or both are required for activation or repression of each gene. Point mutations in the regulators can cause hyperactivation as well as inactivation. Some target PDR genes are also controlled by stress-induced regulators encoded by YAP1, YAP2, and MSN2/MSN4 [134].

How are the *C. albicans* ABC transporter genes regulated? CDR1 is expressed at all phases of growth, but declines about twofold during midlog phase. It is dramatically induced, in a 60-min time frame, by progesterone, β-estradiol, miconazole, nystatin, vinblastine, and heat shock, but less dramatically by cycloheximide, vinblastine, and FLZ [157]. Using an integrated CDR1/GFP fusion strain, others showed that FLZ does induce CDR1 expression after sustained exposure (e.g., overnight), and the induction is concentration dependent and reverses upon withdrawal of FLZ. In this system, azoles in general induce CDR1 from six- to eightfold, cycloheximide sixfold, and calcofluor and 5-fluorocytosine about twofold, while amphotericin B and peroxide at subinhibitory concentrations did not induce [158].

Two laboratories have scrutinized the promoter and upstream regulatory sequences of CDR1. Using a CDR1/luciferase fusion on a multicopy plasmid, Puri et al. identified the transcript start site and defined the promoter region within -345, with a distal miconazole response element at -847 to -1147, which contained an AP-1 sequence that showed DNA-protein-binding activity. This 1 kb region contained several upstream activating and silencing domains [159]. In a follow-up study, the group identified sites protected from DNaseI within the -289 region. Mutation of one of these resulted in enhanced transcription, thus identifying it as a negative regulatory element (NRE). The NRE was used to affinity purify a DNA-binding protein, not yet analyzed. The sequence of the NRE was CCAACTGATTGAAAC. A different protected sequence at -243 to -234, TCTTTTCCACT, was a basal regulatory element BRE, needed for low-level transcription, since mutating it decreased transcription [160].

Independently, de Michel et al. used a CDR1/luciferase fusion in an integrative plasmid to analyze the upstream regulatory region. They defined a 21 bp drug-response element (DRE) at -480 to -420, with a homologous DRE in CDR2 at -240 to -180 (CGGA(A/T)ATCGGATATTTTTTTT). These sequences were necessary and sufficient for inductions mediated by steroid hormones, amorolfine, terbinafine, and fluphenazine, but again, not by FLZ. Both DREs showed specific DNA-protein-binding activity. In addition, CDR1 but not CDR2 contained a BRE (-860 to -810) needed for uninduced low-level transcription [161]. More recently, this group has showed that 40 of 42 genes that are upregulated in response to fluphenazine have the upstream DRE element [120].

Karnani et al. [162] have also identified a steroid response region in the -696 to -521 region of CDR1. Two elements within this region, SRR1 and SRR2, confer steroid inducibility on CDR1 or heterologous recombinant promoters, and show specific DNA binding from nuclear extracts of steroid-induced *C. albicans*. SRE1 responds to progesterone, whereas SRE2 responds to progesterone and β-estradiol. Disruption of genes encoding regulatory proteins involved in morphogenesis (SSK1, EFG1, TUP1, CPH1, NRG1, TPK1, or RAS1) did not result in a clear definition of which activators are interacting with the SRR elements. Comparing promoters of other genes regulated by steroids, a consensus sequence was found that may represent binding sites for the activator: -661 to -165-AAGAA-13 to 152 bp-CGCAA-21 to 68 bp-ATTGG-603 to -84.

Until recently, little was known about the regulators of the efflux genes in *C. albicans*, largely owing to significant differences in their regulation relative to *S. cerevisiae*, discussed below. The studies cited above will clearly fill this gap in the immediate future. In addition, new findings which use approaches that rely less on similar regulatory mechanisms in the two yeasts have recently opened up this field of study.

Regulatory Proteins

A definitive study reporting TAC1 as an important transcriptional activator of CDR1 was recently reported [163]. This gene was identified by its Zn(2)-Cys(6) finger domain and its locus, linked to mating type. Its nuclearly localized product interacts with the drug-response element (CGG triplets) in the promoters of CDR1, CDR2, and other genes. Its disruption results in hypersusceptibility to azoles and inability to upregulate CDR1 or CDR2 by fluphenazine. Tac1p binds to DRE elements upstream of CDR1 and CDR2. A codominant point mutation in TAC1 confers constitutive upregulation of CDR1 [163] and is consistent with the observation that homozygosity at the linked mating-type locus is strongly correlated with azole resistance [35].

Potential activators of CDR1 were also screened by integrating a hybrid CDR1 promoter/lacZ fusion into *S. cerevisiae*, and then transforming with a *C. albicans* library on a multicopy plasmid. This identified CaNDT80, homologous to a meiosis-specific transcription factor in *S. cerevisiae* with a novel DNA-binding motif. Disruption of CaNDT80 confers hypersusceptibility to azoles and decreases the azole-induced expression of CDR1 [164].

It is possible, since Tac1p binds to DRE elements, that Ndt80p binds to the basal regulatory element (BRE) in the CDR1 promoter, identified by Sanglard's group [161]. However, the putative binding site for Ndt80p, based on its *S. cerevisiae* homolog (GNCRCAAA(A/T)), does not correspond to the region defined as the BRE by Sanglard's or Prasad's group. Therefore, the data from the three laboratories suggests that three activators may be required for CDR1 expression, with Tac1p being the limiting factor. Verification of this will require identification and mutation of the binding site(s) for Ndt80p and disruption of the gene encoding the DNA-binding protein that binds to the BRE detected by Gaur et al. [160].

Older approaches to analyzing the candidate regulators of resistance genes in *C. albicans* have been to identify and clone homologs of regulatory genes in *S. cerevisiae*, and then transform *S. cerevisiae* strains, deleted for those genes, with the cloned *C. albicans* homolog. This is a powerful method, but has its limitations, as the following examples show. In *S. cerevisiae*, yAP1 regulates expression of a large number of genes in response to oxidative stresses and to azoles. Notably, it upregulates ScFLR1, an MFS protein, to confer resistance to FLZ. The *C. albicans* homolog CAP1 complements yAP1 function in deletion strains [165]. However, overexpression of CAP1 in *C. albicans* does not confer FLZ resistance; in contrast, it downregulates MDR1 [166]. ScPDR1 and ScPDR3 regulate expression of many genes including ABC transporter PDR5 [167–170], the homolog of CaCDR1, which confers FLZ resistance when overexpressed [171]. *C. albicans* genes have been identified that complement PDR1/3 deletion strains and are PDR5 dependent for FLZ resistance. One of these, FCR1, was identified as a Zn_2C_6-type zinc finger regulatory protein like PDR1/3. However, deletion of this gene in *C. albicans* confers hyperresistance to FLZ, the opposite of expected from *S. cerevisiae* [172]. A second complementing gene, FCR3, encodes a leucine zipper regulatory protein that upregulates PDR5. Its role in *C. albicans* is not yet reported [173].

Regulation of expression by PDR1 in *S. cerevisiae* requires other proteins that regulate the activity of the transcription factors. ScPDR13 in *S. cerevisiae* was discovered by screening a multicopy plasmid library for transformants that were cross-resistant to oligomycin and cycloheximide, normally mediated by two different ABC transporters YOR1 and PDR5, respectively. Both transporters were upregulated and required in library transformants that overexpressed PDR13. Resistance also required a functional PDR1. A point mutation in a PDR13 gene was identified that had the same effect as overexpression of the wild-type gene. Overexpression

of PDR13 did not elevate the level of Pdr1p, nor did it require the native PDR1 promoter for its effect, suggesting that its interaction with Pdr1p was posttranslational. PDR13 encodes a heat-shock protein in the Hsp70 family, so initial model was that this protein is needed to refold Pdr1p, and likely other targets, to an active conformation. Consistently, PDR13 disruptants are cold sensitive and slow growing [174], and fail to induce transcription of other genes not regulated by PDR1, for example, CUP1 and CRS5, normally induced by copper stress [175]. Subsequent studies showed that select, hyperactive mutants of PDR1 are resistant without the need for Pdr13p, and that Pdr13p is cytoplasmic, and ribosome associated, and interacts with a DnaJ-related Hsp40 chaperone subunit encoded by ZUO1 [176, 177]. Resistance is afforded by either subunit if they are expressed in a manner that allows them to be free of association with the ribosome, where the complex has its chaperone activity. Moreover, the peptide-binding domain of Pdr13 is not needed for its activation of Pdr1, both suggesting that the activation mechanism is not by folding [178]. Considered together, it appears that activation of many stress-related transcription factors may be dependent on interaction with individual chaperone subunits, which in turn require some form of modification, presumably stress related, that frees them from their normal location and function. It has not yet been determined whether an identified CaPDR13 homolog plays a similar role in resistance in *C. albicans*.

3.2 Drug Efflux and Membrane Composition

Human P-glycoprotein is enriched in cholesterol and sphingolipid-rich microdomains, rafts and caveolae, which are more rigid than the rest of the plasma membrane, and which increase dramatically in MDR human cells. There is controversial evidence that its efflux activity is responsible for translocation of phospholipids and cholesterol from the inner to the outer leaflet of the plasma membrane [179]. Experimentally induced movement of P-glycoprotein from rafts to non-raft membrane, e.g., by cholesterol depletion, results in a reduction in efflux capacity [180–182].

There is evidence that yeast membrane proteins cluster into distinct raft microdomains in the ER for transport to the plasma membrane, an area that needs much more study [183–189]. Applying this to *Candida*, Prasad's group has shown that the plasma membrane of *C. albicans* is asymmetric with respect to phospholipids, with phosphatidylethanolamine predominantly (96%) in the cytoplasmic leaflet. Disruption of CDR1 and CDR2 results in a further decrease in the amount of this phospholipid in the external leaflet, suggesting that the pumps, in an additive fashion and like human MDR1, may be responsible for the energy-dependent externalization of this aminophospholipid, and hence act as a floppase [190].

Since the fluidity of the membrane in the vicinity of human MDR1 has been shown to affect its activity, Prasad's group therefore expressed CDR1 in isogenic strains of *S. cerevisiae* with various erg mutants that generally have increased membrane fluidity. Mutants *erg2* and *erg3*, with more fluid membranes, showed increased floppase activity and decreased accumulation of labeled FLZ, whereas erg4 mutants, with less fluid membranes, showed decreased floppase activity and near-normal FLZ accumulation. Differences in susceptibility to FLZ were not impressive, but in this study, the host strain still had endogenous ABC transporter genes that increased baseline susceptibility [191].

Prasad's group later showed that *erg6* and *erg16* mutants were hypersusceptible to azoles except FLZ, to terbinafine, and other agents, and that these mutants had more fluid membranes [59]. These cells, when de-energized, accumulated more rhodamine 6G or labeled FLZ than controls, suggesting increased passive transport due to increased permeability. Increases in membrane fluidity alone are not likely explanations for resistance, since cells in which fluidity had been increased by benzyl alcohol were not resistant. However, membrane sphingolipid from the *erg* mutants, with depleted sterols, were more readily extracted, indicating a disruption in the interactions in the membrane that normally hold sphingolipid in place. This effect was eliminated by growing the mutants in ergosterol. Consistently, inhibition of sphingolipid biosynthesis with fumonisin B1, wild-type cells became hypersusceptible to multiple antifungals and showed poor efflux of rhodamine 6G, without increases in membrane fluidity. Disruptions to ergosterol or sphingolipids resulted in poor surface localization of Cdr1p-GFP, hyper-expressed in *S. cerevisiae* missing its major ABC pumps [59]. Similar results were obtained when expressing CDR1, but not MDR1, in *S. cerevisiae* [192]. These data suggest that it is not the altered fluidity of the plasma membrane per se that increases susceptibility, nor the associated increased permeability, but rather the disruptions to ergosterol-sphingolipid-rich raft domains, which appear to be essential for CDR1 localization and hence function.

In *S. cerevisiae*, deletions of various nonessential ERG genes result in altered membrane sterols and increased drug susceptibility. Although it is generally assumed that this results from increased permeability, more recent study shows that it can be accounted for by decreased activity of Pdr5p. This decrease is largely due to partial mislocalization of the pump and is not directly correlated with differing fluidities among individual *erg* mutants. Efflux is reduced most by ERG4, almost as defective as PDR5 disruptants, with ERG4>ERG6>ERG2>ERG3 [68].

In a *C. albicans* strain selected for resistance to gradually increasing concentrations of FLZ, and overexpressing CDR1, CDR2, and ERG11, the membranes were increasingly fluid, and had less ergosterol and reduced externalization of phos-

phatidylethanolamine [193]. The authors suggest that these changes may contribute to resistance, perhaps by activating efflux. Alternatively, they may be a consequence of increased efflux due to the elevated levels of the pumps.

3.3 Efflux by Major Facilitators

CaMDR1, formerly BEN1, is a major facilitator (MFS) protein that is specific for FLZ among the azoles, not to be confused as a homolog of human MDR1, which is an ABC transporter. Major facilitators are proton antiporters whose energy derives from proton gradients established by independent proton-translocating ATPases. These transmembrane proteins typically confer resistance to inhibitory substances, and are found in bacteria, yeasts, and man. In *S. cerevisiae*, there are at least 23 such genes, some having 12, and some having 14 transmembrane domains [194]. In *C. albicans*, there are no genes with strong similarity to MDR1, but there are seven related MFS proteins, all about 30 % identical, and 50 % similar (Table 30.5). Extrapolating from *S. cerevisiae* homologs, these proteins may be located in the plasma membrane and efflux polyamines and in some cases antifungals. However, only one of these, FLU1, has been analyzed in relation to resistance (discussed below). CaMDR1 apparently is induced by and effluxes a partially overlapping set of hydrophobic compounds compared to the CDR proteins: benomyl, methotrexate, cycloheximide, benztriazoles, 4-nitroquinoline-*N*-oxide, FLZ, and sulfometuron methyl [195–197]. Its disruption confers hypersusceptibility in clinical isolates whose resistance is associated with elevated expression of MDR1; therefore, MDR1 is a major factor in resistance in isolates from clinical samples to FLZ. However, disruption of MDR1 in FLZ-susceptible strains has no effect, undoubtedly because the gene is not expressed under normal circumstances in these strains [198] … a lesson to be remembered when analyzing gene disruptions in general. In one report, disruption of MDR1 did not increase susceptibility above the levels conferred by disruption of CDR1 [199].

Little is known about MDR1 regulation, except that it is not induced by exposure to FLZ, but is upregulated by benomyl and apparently by mutation in a transregulatory factor in some FLZ-resistant strains [138, 200, 201]. Thus, resistance in isolates from clinical samples via MDR1 likely results from mutation in genes encoding activators or repressors of MDR1 that result in its upregulation. Its closest homolog in *S. cerevisiae*, FLR1 (Table 30.5), shares many of the same substrates and expression patterns. FLR1 regulation is induced dramatically but only transiently after exposure to benomyl. Once adapted to growth, the gene is downregulated. In *S. cerevisiae*, yAP1 regulates expression of a large number of genes in response to oxidative stresses and to azoles [194]. Notably, it upregulates FLR1, to confer resistance to FLZ. The *C. albicans* homolog CAP1 complements yAP1 function in deletion strains [165]. However, overexpression of CAP1 in *C. albicans* does not confer FLZ resistance as expected; in contrast, it downregulates MDR1 [166].

C. albicans undergoes adaptive growth responses to FLZ as does *S. cerevisiae*, and these are favored by acidic media [151]. By analogy to FLR1, this suggests a role for MDR1, despite its apparent lack of induction by FLZ. The earlier studies discussed above may have missed the hypothetical, transient up- and downregulation. Assuming nonetheless that transient induction or activation of MDR1 is responsible for short-term adaptive growth in FLZ, the data invite the speculation that, once intracellular FLZ concentration is reduced by efflux, a second mechanism is responsible for the more enduring capacity for growth, despite FLZ at concentrations that maintain ergosterol depletion ([151] and unpublished observations).

Table 30.5 Major facilitator genes in *C. albicans*, their relationship to *S. cerevisiae* genes, and putative functions

CA gene	Name (CHR)	MDR1 %ID (similar)	SC homolog	CA-SC %ID (similar)	CA function	SC function
MDR1	CaO19.13047(6)	100	FLR1	44(63)	FLZ resistance	FLZ resistance
FLU1	CaO19.6577(7)	33(52)	TPO1	42(58)	Possible FLZ resistance[a]	Polyamine transport, resistance CHX, AMB
TPO1	CaO19.7148(7)	29(48)		43(59)	Unknown	
–	CaO19.7974(3)	27(49)		31(51)	Unknown	
TPO3	CaO19.4737(1)	33(52)	TPO3	61(74)	Unknown	Polyamine transport
NAG3	CaO19.9705(6)	32(52)		35(55)	Unknown	
TPO4	CaO19.8104(R)	30(50)	TPO4	49(67)	Unknown	Polyamine transport
–	CaO19.3395(6)	31(50)	YHR048W	44(62)	Unknown	Unknown, reg by FLZ

Genes were identified by Blast comparison with the *C. albicans* genome database. Their % identities and % similarities to CaMDR1, determined by two gene Blast comparisons are indicated in column 3. The closest homolog in *S. cerevisiae* is indicated, with its % identity and similarity to its homolog. Functions for most of the *C. albicans* genes are inferred by homology.

[a]Moderate resistance of *C. albicans* gene in *S. cerevisiae* [199].

3.3.1 FLU1

Another multidrug transporter in the MFS class is encoded by FLU1 (Table 30.5) and was identified by its ability to restore resistance to fluconazole and cycloheximide to *S. cerevisiae pdr5* mutants. Like MDR1, this resistance did not extend to the other azoles. Its disruption in *C. albicans* only resulted in slight hypersusceptibility to FLZ, and a more pronounced hypersusceptibility to mycophenolic acid. The expression levels of this gene did not vary between susceptible and resistant matched pairs of a small number of clinical isolates. However, these isolates were not analyzed before and after FLZ exposure; thus it remains possible that resistant isolates had mutations that allowed more rapid or higher levels of upregulation only following exposure. No changes in labeled FLZ accumulation could be shown in FLU1 disruptants, whereas increased accumulation was seen in CDR1 disruptants [199]. The effects of FLU1 disruption in a CDR1/CDR2 disruptant, or of FLU1 overexpression on accumulation in the *S. cerevisiae* FLU1/*pdr5* strain or in *C. albicans*, were not reported, so the jury is still out on whether FLU1 can contribute to resistance. Considering the similarities among all the MFS transporters in Table 30.5, it may be that yet other genes contribute to resistance.

It should be emphasized that there are clinical isolates whose resistance seems to result from efflux mechanisms that are not attributable to CDR1, CDR2, or MDR1 (next section).

4 Evidence that Resistance in Clinical Isolates of *C. albicans* Is Complex

4.1 In Vitro Evolution of FLZ Resistance

In vitro "evolution" studies looking at the rise of FLZ resistance among populations of yeasts have been done for *S. cerevisiae* and *C. albicans*. This approach asks what types of mutation give rise to FLZ resistance when selecting among a population of cells in culture, not individual cells on agar. This imposes an extra criterion, since the resistant mutants must also retain some degree of fitness or be overgrown by more fit-resistant mutants.

In *S. cerevisiae*, the outcome depended on the type of selective pressure. When using a single-step selection at a high FLZ concentration (128 μg/mL), mutants were repeatedly isolated that had recessive mutations in ERG3, which also resulted in the overexpression of ERG11. Such an outcome would not be expected in diploid strains of *S. cerevisiae*, or in the diploid *C. albicans*. A global screening of all individual gene deletion strains showed that defects in many genes other than ERG3 result in FLZ resistance (sterol metabolism genes ERG6, ERG28, OSH1, SCS2, and eight other genes). These mutant genes did not arise in the mutant selection experiment because they are unfit. In contrast, in a second stepwise selection for resistant mutants, initially at 16 μg/mL, then 32 μg/mL, and finally 128 μg/mL, a semi-dominant mutation in PDR1 was repeatedly isolated. This mutation arose early on in the selection process, and was followed by a single, second mutation in an unknown gene, which increased the MIC. The PDR1 mutation resulted in overexpression of ABC transporter genes PDR5 and SNQ2, but not in the major facilitator gene FLR1 nor consistently in ERG11 [141].

There are two lessons to be learned from this study; both underscore the complex nature of azole resistance. First, many genes can potentially mutate to confer azole resistance. Second, among individual populations, the mutation responsible for the resistance can vary, and depends on the type of selective pressure. Extrapolating, in vivo selection, which potentially introduces more uncharacterized selective pressures than in vitro, could further impact the types of resistance genes that could predominate in individual patients.

In a second population-based selection for FLZ resistance, this time in *C. albicans*, the outcome was more complex. Again, individual cultures evolved differing patterns of resistance as FLZ concentrations were increased incrementally. Overexpressions of CDR1, CDR2, MDR1, and ERG11 were episodic, as were other mutational changes [142]. Resistance, once established, was stable in the absence of selection for many generations, as were the levels of overexpression. Microarray analysis of these in vitro-evolving populations showed two patterns. In one, overexpression of CDR2 was predominant. In the second, overexpression of MDR1 was the major change, occurring either early or late in the evolution of stable resistance, along with changes in many other genes associated with oxidative stress or lipid metabolism. Clinical isolates showed genomic patterns in line with those that evolved in vitro [121].

In an independent study, Kohli et al. selected for resistance to gradually increasing concentrations of FLZ, and showed that incremental increases in expression of CDR1, CDR2, and ERG11 had occurred [193].

The take-home lesson from this approach is that genetic alterations that result in FLZ resistance occur incrementally and involve potentially many genes.

4.2 In Vivo Evolution of FLZ Resistance

Comparing resistance genes among sequential isolates, recovered from the same patient, that have decreasing susceptibility to FLZ, is an in vivo version of the in vitro evolution studies discussed above. Their story is much the same. Development of resistance occurs incrementally, with contributions from mutations in several known genes and most likely in genes yet to be discovered.

In a study of sequential, matched isolates from OPC patients, development of FLZ resistance was complex [137]. For example, overexpression of only CDR1 conferred only moderate FLZ resistance in isolates from one patient, whereas similar level expressions of CDR1 in isolates from a different patient conferred moderate resistance in some isolates, or very high resistance in other isolates, without any other apparent changes in CDR2, MDR1, or ERG11. Progression to higher resistance in a third patient did not involve increased expression levels of CDR1, CDR2, MDR1, or ERG11; therefore either resistant point mutations ocurred in one or more of these genes, or a different mechanism not involving these genes was involved. The efflux genes are clearly important in resistance but do not tell the whole story. Moderate increases in some genetic backgrounds are apparently sufficient for resistance, but not in others. The following question remains: What genetic differences contribute to this effect?

In a second study, serial isolates from an HIV patient had sequential alterations that were correlated with gradual increases in MICs to FLZ. Initial increases in MDR1 apparently resulted in moderate MICs, followed by several alterations in ERG11, and finally by increases in one of the CDR genes, to generate fully resistant derivatives [138].

A third study of four FLZ-resistant *C. albicans* strains isolated from HIV patients again emphasizes that there are still unknown mechanisms involved in resistance in isolates from clinical samples. All four isolates showed reduced accumulation of labeled FLZ, but only two of these showed increased levels of CDR1 and CDR2 expression. The other two isolates presumably use an unknown efflux mechanism, or suppress uptake [139].

A fourth study, which compared large numbers of unmatched clinical isolates, supports the argument that azole resistance is complex and not yet defined. The study compared expression levels and sequences of a large collection of clinical isolates, half FLZ resistant and half susceptible. Overexpression of CDR1 and CDR1 was common among the resistant isolates, overexpression of MDR1 was less common, and overexpression or mutation of ERG11 was not correlated with resistance. However, a significant percentage of susceptible isolates also overexpressed these genes. The authors concluded that molecular based assays for resistance using these genes were not sufficiently predictive, and that other mechanisms and mitigating genetic factors need more study [140].

Perhaps the most rigorous study of azole resistance in clinical isolates used RT-PCR to establish a baseline of expression levels of ERG11, CDR1, CDR2, and MDR1, relative to control genes, among FLZ-susceptible clinical isolates [24]. They then compared these levels to those found in 38 resistant isolates, considering whether these were resistant to FLZ, voriconazole, or posaconazole. Only one isolate was resistant to posaconazole. Consistent with some of the earlier studies, overexpression of ERG11 and MDR1 was not common among resistant isolates. CDR1 was overexpressed in less than half, whereas CDR2 was overexpressed in all but three resistant clinical isolates. However, CDR2 was also overexpressed in some susceptible isolates. Resistant isolates were more polymorphic in ERG11 sequences than susceptible isolates, and seemingly fewer mutations were needed for FLZ or voriconazole resistance than for posaconazole resistance. However, the test for whether these mutations were responsible for resistance was to clone them into a multicopy plasmid and transform them into *S. cerevisiae* to confer reduced susceptibility. Despite controls, this assay is suspicious in that overexpression of even wild-type ERG11 sequences in *C. albicans* confers high-level resistance (unpublished observations). This study is also limited in that it does not consider point mutations in CDR1/CDR2 genes. Furthermore, it only looked at expression levels of strains in the absence of azoles; therefore potential differences in responses of resistant strains to azoles are overlooked. Despite these limitations, the study indicates that it is likely that multiple mutations, including those affecting CDR2 or CDR1 expression and ERG11p binding to azoles, are responsible for in vitro resistance among clinical isolates.

Karababa et al. performed a thoughtful and careful analysis of gene expression using microarrays, comparing genes that were induced or repressed in a susceptible lab strain incubated in fluphenazine to induce CDR1 or in benomyl to induce MDR1, to genes altered between matched pairs of clinical isolates overexpressing either CDR1 or MDR1 [120]. In each case, multiple genes in addition to CDR1 or MDR1 were altered. By comparing these sets of genes, they identified the subsets of changes common to drug-exposed susceptible strains and resistant versus susceptible clinical strains. Comparing fluphenazine-exposed susceptible to CDR1-expressing resistant strains, this includes CDR1 (eightfold overexpression) and CDR2 (25- to 40-fold overexpression), upregulation of heat-shock protein HSP12, glutathione peroxidase GPX1, and potential lipid transportase RTA3. Interestingly, overexpression of ERG3, ERG6, and ERG25 was unique to the clinically resistant strains. The group further shows that comparisons of microarray results from other laboratories are useful in defining common subsets of genes that respond similarly to different types of stress, allowing much-needed focus on a manageable number of genes to analyze in more detail. The main limitation of this study, imposed by the complexity of microarray analysis, and the amount of labor and expense involved, is that exposed susceptible strains are only examined at a single time after exposure to the antifungal agent. Thus, clinically resistant strains may be expressing a broader range of genes than exposed susceptible cells, but the two might have more in common given longer exposure times.

Many of the genes induced in MDR1-expressing clinical resistant isolates encoded genes in the aldo-keto reductase family (IFD and related genes); these were not seen among

induced genes in the CDR group [120]. Consistently, some of these genes were detected independently in two studies using a proteome approach, again, only in strains overexpressing MDR1 [202, 203]. One of these genes, YPR127, was altered both by overexpression and disruption, without effect on susceptibilities to azoles or oxidative stress. This serves as a reminder that many genes that are co-regulated with resistance genes may not be important or limiting for a normal response to drugs or for resistance in isolates from clinical samples. Some subset presumably will be, so the near future belongs to those who are clever at teasing only those that have an impact.

5 Phenotypic Resistance and Tolerance

The implication of PDR1/3 mutants in *S. cerevisiae*, and of clinical resistant isolates that overexpress CDR1 and CDR1, is that mutations occur in regulatory genes in *C. albicans* that give rise to stable resistance in isolates from clinical samples. Phenotypic or adaptive resistance is not mutation based, but is defined as a reversible, regulated alteration in phenotype which allows growth in concentrations of antifungal that were inhibitory prior to the alteration. It is likely due to upregulation or activation of activators, which are then downregulated once selective pressure is removed. Phenotypic resistance may involve other genes in addition to the CDR genes. Epigenetic changes could also contribute to phenotypic resistance. Tolerance is a term used somewhat loosely here to mean a static response in which the organism survives at inhibitory concentrations but cannot grow, and hence is not resistant or phenotypically resistant. *C. albicans* is tolerant of azoles, since they are not fungicidal under most conditions. Tolerance more traditionally is used to describe a decreasing susceptibility to an agent after repeated exposures, but in this context has come to mean the opposite of intolerance, i.e., the opposite of cidal, e.g., [46].

Several observations suggest that tolerance or phenotypic resistance is more important than stable azole resistance in the pathobiology of most *Candida* infections. Most clinical failures to FLZ occur with susceptible isolates. Stable azole resistance in non-OPC isolates is rare. For example, in one study, 40 of 40 breakthrough fungemias among neonates and infants were due to isolates that were susceptible to FLZ in vitro [204]. Among VVC patients, less than 4 % of isolates were FLZ resistant, and in only one patient was there a documented transition to resistance during treatment. Indeed, FLZ susceptibility seemed irrelevant to RVVC patient outcome under FLZ therapy [205]. In our hands, most or all of these FLZ-resistant isolates are unstable and revert to susceptibility (unpublished observations), suggesting that their apparent resistance was phenotypic. Numerous small- and large-scale surveys of bloodstream isolates of *C. albicans* indicate that 97–100 % are highly susceptible in vitro to FLZ and other azoles [206–211], despite breakthrough infections which occur at a rate of about 10 %, with high attributable mortality [212, 213]. Stable azole resistance is not a risk factor for patient outcome in non-OPC candidiasis patients [214]. The fungistatic response of *C. albicans* to FLZ, i.e., its tolerance of FLZ, is a critical factor here. Certainly, host factors such as prolonged neutropenia are crucial, but only because FLZ is only static, not cidal, in these patients. It needs underscoring that *C. albicans*' ability to tolerate exposure to FLZ, and probably to other forms of stress, is a prelude to phenotypic resistance, and is a precondition for mutation and resistance. On the latter point, FLZ exposure has been shown to increase mutagenic responses in *C. albicans*, such as chromosomal nondisjunctions, which in turn expose recessive alleles of as-yet unknown genes that cause azole resistance [34].

Another argument that tolerance to FLZ is important in clinical therapy is that, in the single infection site in which *C. albicans* is not tolerant, FLZ therapy is greatly enhanced. This optimal site for azole treatment is vaginal. Here, a single dose (150 mg) typically suffices to rapidly eradicate the infection [215]. In contrast, in oral mucosal infections, multiple doses are typically required, for example, 14 daily doses of 100–400 mg [216]. The key difference is in the vaginal microenvironment, which contains about 20 mM acetic acid [217]. The combination of FLZ and 20 mM acetic acid is fungicidal [151]. It remains to be seen whether cases of recurrent VVC [218] occur in patients in which vaginal acetic acid is depleted.

5.1 Phenotypic Resistance

In vitro, most isolates mount a reversible, adaptive response within a few hours in acidic media ([151] and unpublished results). On a longer timescale, and perhaps using different mechanism, *C. albicans* can adapt to increasing concentrations of FLZ. In one study, the resistance was reversed by subculturing in the absence of FLZ, and no increase in MDR1 or CDR1 was detected [219]. Similar results are seen in some clinical FLZ-resistant isolates [220]. Studies of phenotypic resistance are largely ignored, probably since molecular analysis of stable mutations is more tractable.

What mechanisms might be responsible for adaptive responses to FLZ in *C. albicans*? In one study, a series of *C. albicans* isolates from a BMT patient on FLZ showed the development of FLZ-"resistant" isolates. These were shown to have elevated expression of CDR genes, but not ERG genes. No mutations occurred in ERG11 genes, and sterol composition was not affected. In contrast to the stably resistant isolates recovered from OPC patients, these isolates gradually lost resistance upon subculture in nonselective

media, concomitant with a decrease in expression of CDR genes. Conversely, subpopulations of initially susceptible isolates showed transient resistance upon exposure to FLZ, correlated with upregulation of transporters [221, 222]. An earlier study also induced FLZ-resistant isolates by subculturing 12+ days in 8+ μg/mL FLZ, and the resistant phenotype was lost upon subculturing 14+ days. In contrast to the study above, these isolates did not show elevated expression of CDR1, MDR1, or ERG11. Although no mention was made of CDR2, the possibility is that the phenotypic resistance mechanism of this isolate was different [219]. These two studies support the premise that phenotypic resistance is a regulatory response. Potentially, this involves upregulation of some of the same genes which, when mutated, confer resistance.

Is "phenotypic switching" in *C. albicans* responsible for some forms of phenotypic resistance? Most strains of *C. albicans* undergo high-frequency switching that alters their cellular and colony morphology [223]. There are indications that switching promotes survival under selective in vivo conditions [224, 225]. Switching is a regulatory process that involves transcriptional reprogramming by histone acetylases and deacetylases [226–230]. Resistance to antifungals may also be affected by switching. Early studies linking resistance and switching were not impressive, and the demonstration that CDR3 is induced in opaque cells is uninterpretable in this context, since it has not yet been correlated with resistance [143]. Soll's group recently demonstrated that primary isolates from HIV patients undergo switching at a 100-fold elevated rate, and that, independent of antifungal therapy, specific morphologies have dramatically different susceptibilities to azoles and fluorocytosine. However, this was not true among the various subtypes deriving from laboratory strains that switch [231]. This is an exciting demonstration of the richer potential range of phenotypes of primary isolates and of in vivo pressures relative to laboratory strains and in vitro conditions. The authors cite as unpublished observations that some of the usual suspect genes are induced by switching; assuming that this data will be forthcoming, this suggests that resistance genes are just a subset of target genes whose regulation is altered by the switching regulator(s).

Phenotypic resistance is different than the "trailing effect" seen by select isolates in RPMI 1640 media under NCCLS conditions for MIC. The latter is seen only in a minority of isolates and occurs at pH >6 [232, 233]; adaptive growth is seen by most isolates, preferentially in acidic media (unpublished results and [151]). Trailing strains are those which are inhibited for 24 h under NCCLS conditions, but show slower growth at higher antifungal concentrations, blurring the assignment of a specific MIC, and often show fully confluent growth after another 24-h incubation, even at high concentrations. One study showed that trailing strains show a higher than average number of polymorphisms in ERG11, but not generally higher levels of expression [234]. CDR1 was lower in the absence of FLZ, and inducible by FLZ to a greater extent, in trailing strains compared to susceptible but dose-dependent (SDD) strains. This study was limited due to lack of comparison to susceptible controls, and does not establish a clear mechanism by which trailing strains differ from susceptibles. Another study also showed that a trailing strain of *C. albicans* was inducible for ERG1, ERG11, CDR1, and CDR2, and that an inhibitor that interfered with these inductions blocked its trailing response to FLZ [117]. Again, this study does not address what key differences exist between trailing and non-trailing strains. Lower priorities have seemingly been applied to studies on trailing mechanisms, since these strains do not seem to pose a greater threat of resistance in systemic infections than susceptible strains [235].

5.2 Tolerance Pathways

These proposed inducible pathways for tolerance are not hypothetical. Two pathways to date are implicated in tolerance: cAMP-protein kinase A, and Ca-calmodulin-calcineurin pathways. A third, the protein kinase C-cell integrity pathway, is likely involved, but no published studies are yet available. Since PKC is a regulator of cell wall integrity in *S. cerevisiae* [236], we expect that inhibition of genes in that pathway will act synergistically with azoles. Microarray studies already implicate their involvement in responses of *S. cerevisiae* to caspofungin (next section).

The calcineurin pathway in fungi is a stress response, signal transduction pathway recently recognized as essential for tolerance of *C. albicans* to FLZ (Fig. 30.12). Calcineurin is a heterodimeric phosphatase; calcium-dependent calmodulin binds to calcineurin to activate its phosphatase. In *S. cerevisiae*, Ca^{++}-bound calmodulin binds to calcineurin, which can then remove an inhibiting phosphate on ScCzr1p, allowing it to enter the nucleus and activate transcription of stress response genes. The pathway is conserved in many fungi, although the phenotypic consequences of its inactivation vary with the species [237]. In *C. albicans*, calcineurin subunits are encoded by CNA1 and CNB1. While disruption of both alleles of CNA1 is not lethal, disruptants are killed when exposed to FLZ and other azoles, terbinafine, amorolfine, calcofluor white, Congo red, caffeine, SDS, brefeldin A, and mycophenolic acid. Disruptants were more sensitive to Na^+, Li^+, and Ca^{++}; could not survive in serum; and were avirulent in a mouse infection model [46, 238]. Similar effects result from inhibition of calcineurin by cyclosporin A, which binds to cyclophilin and the complex binds and inhibits calcineurin, or by FK506, which binds to FKBP12, preventing it from tethering calcineurin to its target proteins [45, 239, 240]. The fungicidal synergy of cyclosporin A and

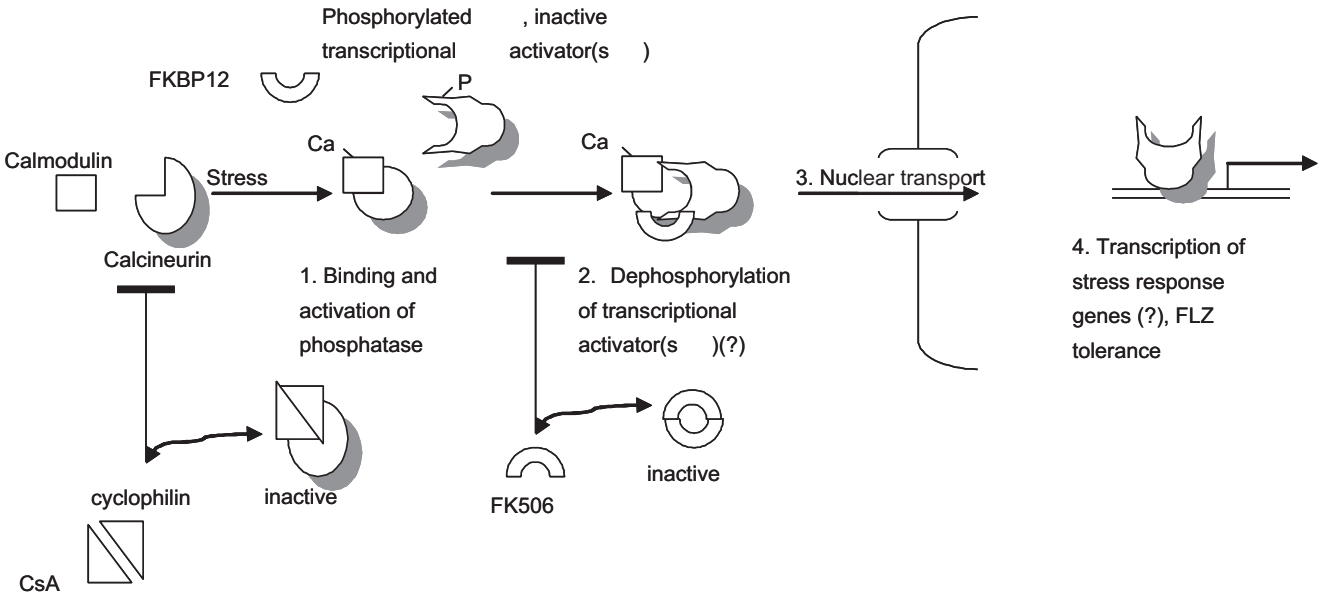


Fig. 30.12 Calcineurin pathway for inducing tolerance of FLZ by activation of stress response genes. FLZ exposure imposes a stress which mobilizes Ca^{++}, activating calmodulin, which binds and activates the phosphatase calcineurin (1). With the help of FKBP12, activated calcineurin binds and dephosphorylates transcriptional activators, not yet defined in *C. albicans* (2). This allows nuclear import of the activator (3), which upregulates expression of stress response genes (4). Which of these is critical for survival in FLZ is not yet known. Cyclosporin (CsA) blocks the pathway by binding to endogenous cyclophilin to inhibit calcineurin, and FK506 blocks by complexing with and inhibiting FKBP12

FLZ is not dependent on transporters CDR1, CDR2, MDR1, or FLU1, since the synergy is still evident in disruptants [241]. These are the only likely targets for these two agents, since the deletion of cyclophilin and FKBP12 results in lack of synergy of either inhibitor with FLZ. These observations indicate that the pathway is essential for responding to a variety of stress signals.

Consistent with findings in *S. cerevisiae*, disruption of CaCNA1 altered colony morphology. CaFKS1, a β-glucan synthase subunit, and CaPMC1, a calcium efflux protein, were upregulated by calcium or FLZ in a calcineurin-dependent manner, whereas CaCDR1, CaFKS3, CaPMR1, and CaPMR2 were not. Deletion of CaPMC1 conferred sensitivity to Ca^{++} but not to Li^{+} or FLZ [46, 238]. Which gene, regulated by CNA1/CNB1, is required for tolerance to FLZ is therefore not known.

Similar phenotypes were conferred by disruption of CNB1 [242]. These authors point out that inhibitors affecting wall structure, in contrast to those affecting membranes, are not synergistic with CsA. They show that an FLZ-resistant mutant with a mutation in ERG11 is susceptible to the FLZ-FK506 synergy, whereas FLZ-resistant mutants showing overexpression of CDR1 are not.

CaCZR1, based on its homology and ability to substitute for ScCZR1 in *S. cerevisiae*, was predicted to be the likely downstream target of calcineurin in *C. albicans*. However, its disruption did not reduce virulence in a disseminated mouse model, did not eliminate synergy between FK506 and fluconazole, did not confer hypersensitivity to salt stress, and changed susceptibility to fluconazole only modestly [243]. These observations show either that CaCRZ1 is not the primary target of calcineurin or that there is a redundant gene.

Together, these papers show the paramount importance of the calcineurin pathway in conferring FLZ tolerance, and underscore that we do not know the critical genes that regulate or mediate the pathway to affect FLZ tolerance.

5.3 cAMP-Protein Kinase A Pathway

This pathway in *S. cerevisiae* is required for growth, carbohydrate synthesis, and recovery (release from G_0) after stress, and is an antagonist of the calcineurin stress response pathway [244]. This antagonism is mediated by phosphorylation of the nuclear localization signal of ScCrz1p, preventing its activation of calcineurin response genes [245].

The cAMP-PKA pathway in *C. albicans* is likely required to facilitate the recovery process and resume growth after various stress conditions, perhaps an initial inhibition by FLZ. Crucial genes in this second pathway essential for FLZ tolerance are CDC35, encoding the adenyl cyclase enzyme, and CAP, the cAMP-associated protein. Disruption of either gene results in hypersusceptibility to azoles and terbinafine, as does incubation of wild-type strains with adenyl cyclase inhibitor MDL-12330A. These hypersusceptibilities were

overcome with exogenous cAMP. The FLZ-induced expression of CDR1 was blocked by the deletions or inhibitor [62].

On this basis, we speculate that phenotypic resistance to FLZ may be a two-step process: a tolerance response mediated by a calcineurin-induced mechanism, to allow survival, and then resumption of growth from the inhibited state, mediated by cAMP-PKA activation of targets that remain to be identified. In this model, the apparent antagonism between the two pathways is actually a timing mechanism. Activated PKA phosphorylates the *C. albicans* equivalent of ScCrz1p to shut down the calcineurin pathway once the stress response has achieved its goals.

6 Novel Mechanisms for Azole Resistance?

Sc*PDR16* was identified as one of the many target genes regulated by ScPDR1 [246]. Its disruption confers hypersusceptibility to azoles. Additional disruption of the paralogous ScPDR17 increases this hypersusceptibility and renders the double mutant hypersusceptible to a broader range of agents. PDR17 disruptants have inverted ratios of phosphatidylethanolamine to phosphatidylserine, whereas PDR16 disruptants have slightly reduced levels of ergosterol but dramatically increased levels of intermediates episterol and fecosterol. De-energized double disruptants showed enhanced uptake of rhodamine 6G, suggesting increased membrane permeability and possibly explaining the enhanced drug sensitivities. Since both proteins are related to Sec14p, which regulates synthesis or trafficking of phosphatidylcholine in the Golgi, the authors proposed that Pdr16/17p controls sterol and phospholipid synthesis [130]. The *Candida* homolog CaPDR16 is also upregulated in *C. albicans* clinical isolates that overexpress CDR1 and CDR2. A GFP reporter gene, fused to the CaPDR16 promoter, is upregulated in a clinical resistant isolate relative to a matched susceptible isolate, indicating that the resistant isolate has an upregulated regulatory gene, or an activated gene product [247], possibly TAC1. However, our *C. albicans* transformants overexpressing PDR16 on a high copy plasmid are hypersusceptible to FLZ and resistant to AMB (unpublished observation). The basis for this difference may be in the different levels of overexpression, or in more fundamental functional differences between the *C. albicans* versus *S. cerevisiae* genes.

ALK8 is a *C. albicans* homolog to alkane-inducible cytochrome P450 genes, and data show that its overexpression in ABC pump-disrupted strains of *C. albicans* or *S. cerevisiae* conferred multidrug resistance, including azoles. Alk8p was shown to hydroxylate lauric acid in vitro, a reaction that was competed out with fluconazole. Like FLU1, no correlation of overexpression of ALK8 with resistance among clinical isolates was established, but information on how this correlation was sought is lacking [248]. Nevertheless, these data suggest that another mechanism for resistance in *C. albicans* may be drug detoxification initiated by P450-mediated hydroxylations. However, no modifications to antifungals have yet been directly documented.

Circumstantial evidence has been presented for FLZ resistance by inhibiting *uptake*, but so far only in *C. lusitaniae* [249]. However, one study does show that uptake of FLZ in *C. albicans* is by facilitated diffusion (energy independent and saturable) [250], and therefore that a carrier protein is another potential target for resistance by mutation or tolerance by regulation. This mechanism may not apply to the more hydrophobic azoles, which may enter more readily by passive diffusion.

Conversely, one study provides strong evidence that *sequestration* of FLZ into vesicular vacuoles is at least part of a resistance mechanism [251]. Resistant isolates recovered from a cancer patient after prolonged FLZ treatment showed increased levels of FLZ accumulation, mostly into a high-speed pellet subfraction, correlated with dramatically increased density of vesicular vacuoles seen by electron microscopy. Sequestering of toxic agents into vacuoles is not a new mechanism of resistance. For example, the yeast ScYCF1 ABC transporter is a vacuolar pump that moves arsenite from the cytoplasm into vacuoles, acting in synergy with a plasma membrane transporter to reduce susceptibility [252].

6.1 Is FLZ Mutagenic?

In one study, *C. albicans* exposed in vitro to FLZ generated FLZ-resistant derivatives showing loss of one copy of chromosome 3 or 4 via nondisjunctions. The implication is that these chromosomes carry recessive resistant alleles. Since these mutants have normal expression levels of CDR1, CDR2, MDR1, and ERG11, alternative mechanisms of resistance are probably involved [34]. However, this implication rests on the unproved assumption that the chromosome losses are linked to FLZ resistance mutations. Another study reported a strong correlation among clinical strains between FLZ resistance and loss of chromosome 5, generating homozygosity at the mating-type locus [35]. This loss would also generate homozygosity at ERG11 and TAC1. However, when loss of chromosome 5 is selected for by sorbose selection [253], or screening for mating-type homozygotes, instead of FLZ selection, there is no correlation [254, 255]. Together, these results only suggest that chromosome loss is a common result of growth inhibition, but that this may facilitate the selection for recessive mutations that confer FLZ resistance.

Is there any evidence that FLZ is directly mutagenic at the DNA sequence level, by induction of some form of adaptive mutagenesis? Adaptive mutagenesis has been redefined from

its original heretical forms into one in which cells increase mutation rates in response to growth-inhibiting stress, so that mutant genes can allow growth to resume. Mutations are not targeted to those genes in any fundamental way [256]. Adaptive mutagenesis in *S. cerevisiae* has been shown to be dependent on mutagenic nonhomologous end joining of dsDNA breaks and on error-prone translesion DNA synthesis by polymerase ζ [257–259]. In *C. albicans*, there are no studies. From sequence analysis of ERG11, it seems that the genes from FLZ-resistant strains are a richer source of DNA polymorphisms than the genes from susceptible strains [13]. This suggests adaptive mutagenesis, but this disparity is minimal in other studies [260].

6.2 Mitochondrial Respiration and Antifungal Susceptibility

Susceptibility to FLZ in *C. glabrata* and in *S. cerevisiae* is dependent on mitochondrial function. Petite mutants arise at very high frequencies, and are induced by ethidium bromide [261, 262], in which some or all mitochondrial DNA is deleted. These respiratory defective strains are FLZ resistant [263–268]. At least part of the basis for this seems to be a retrograde downregulation of expression of PDR5, the *S. cerevisiae* homolog of CDR1, by a functional F_0 component of the mitochondrial ATPase. This downregulation is lost in petites or in strains with point mutations in the ATPase [269], derepressing PDR5. Apparently, the same link exists in *C. glabrata* [265, 270]. Furthermore, FLZ-resistant *C. glabrata* petites need not have irreversible mtDNA deletions. Petites that arose from insertion mutations in several mitochondrial biogenesis genes were reversibly FLZ resistant. Their respiratory deficiency was not due to alterations in the mitochondrial genome. The deficiency and the FLZ resistance reverted at a very high rate, suggesting that an epigenetic mechanism was determining respiratory competence [271].

It is possible that there is more to mitochondrial based resistance than induction of CDR1. It has been suggested that sterol-depleted mitochondria spew out ROS that inhibit or kill the cell; therefore dysfunctional mitochondria are benign. However, there is some evidence to the contrary [272]. Another perplexing link between mitochondrial function and sterol metabolism is seen in ERG3 mutants in *S. cerevisiae*. Respiratory competent cells with ERG3 deletion are resistant to fluconazole, whereas petites with ERG3 deletions are not; petites are resistant only with a functional ERG3 gene [263].

The above studies argue that there is a complex link between mitochondrial function and antifungal susceptibilities. The demonstration that a reversible mitochondrial dysfunction in *C. glabrata* is responsible for FLZ resistance raises the question of whether a similar effect may occur in *C. albicans*. However, the relationship in *C. albicans* is difficult to study, since forming petites in this species is difficult. There is a report of induction of mitochondrial dysfunction by ethidium bromide in *C. albicans*, accompanied by a slight increase in tolerance for AMB [273], but there was no demonstration of an altered mitochondrial genome and no testing for azole resistance. Inhibition of mitochondrial protein synthesis and cytochrome function with erythromycin also resulted in increased tolerance for AMB [274]. Similarly, respiratory-defective *C. albicans* were isolated after exposure to acriflavine, and were resistant to histatin [275], but again, defects in the mitochondrial DNA were not demonstrated. In our hands, it is possible to isolate ethidium bromide-resistant mutants, but these are not respiration defective (unpublished data). Other reports show reduced adhesion or virulence of putative *C. albicans* petites, but do not characterize changes in mitochondrial DNA nor alterations in antifungal susceptibility [276–279]. Growth of *C. albicans* in defined anaerobic media does not require ergosterol and results in almost complete conversion of yeast to hyphal forms which are highly resistant to azoles, AmB, terbinafine, and zaragozic acid [280]. This, and the efflux pump-independent resistance of *C. albicans* within anaerobic biofilms [281], suggests that there is a link between respiring mitochondria and antifungal susceptibility in *C. albicans* that warrants further study.

7 Hitting Targets Outside the Ergosterol Pathway

Because of the prospect of increasing azole resistance, it is important to better understand mechanisms of action of other antifungals, either so that they may be used instead of, or to synergize with, azoles or so that new, fungal specific genes involved in those mechanisms may be targeted by next-generation antifungals. Some of the antifungal agents in our proposal will never be used clinically, but identifying genes and pathways that respond to these may uncover new targets or help understand actions of clinical antifungals. Others, e.g., fluorocytosine, could be used more effectively if we could block potential resistance mechanisms or at least prescreen effectively for resistant isolates before treatment.

7.1 Echinocandins

Echinocandins are natural lipopeptides, which now include synthetic derivatives, notably caspofungin (Merck; derived from pneumocandin B0 produced by *Zalerion arboricola*), micafungin (Fujisawa; derived from echinocandin B produced by *Coleophoma empetri*), and anidulofungin (Vicuron;

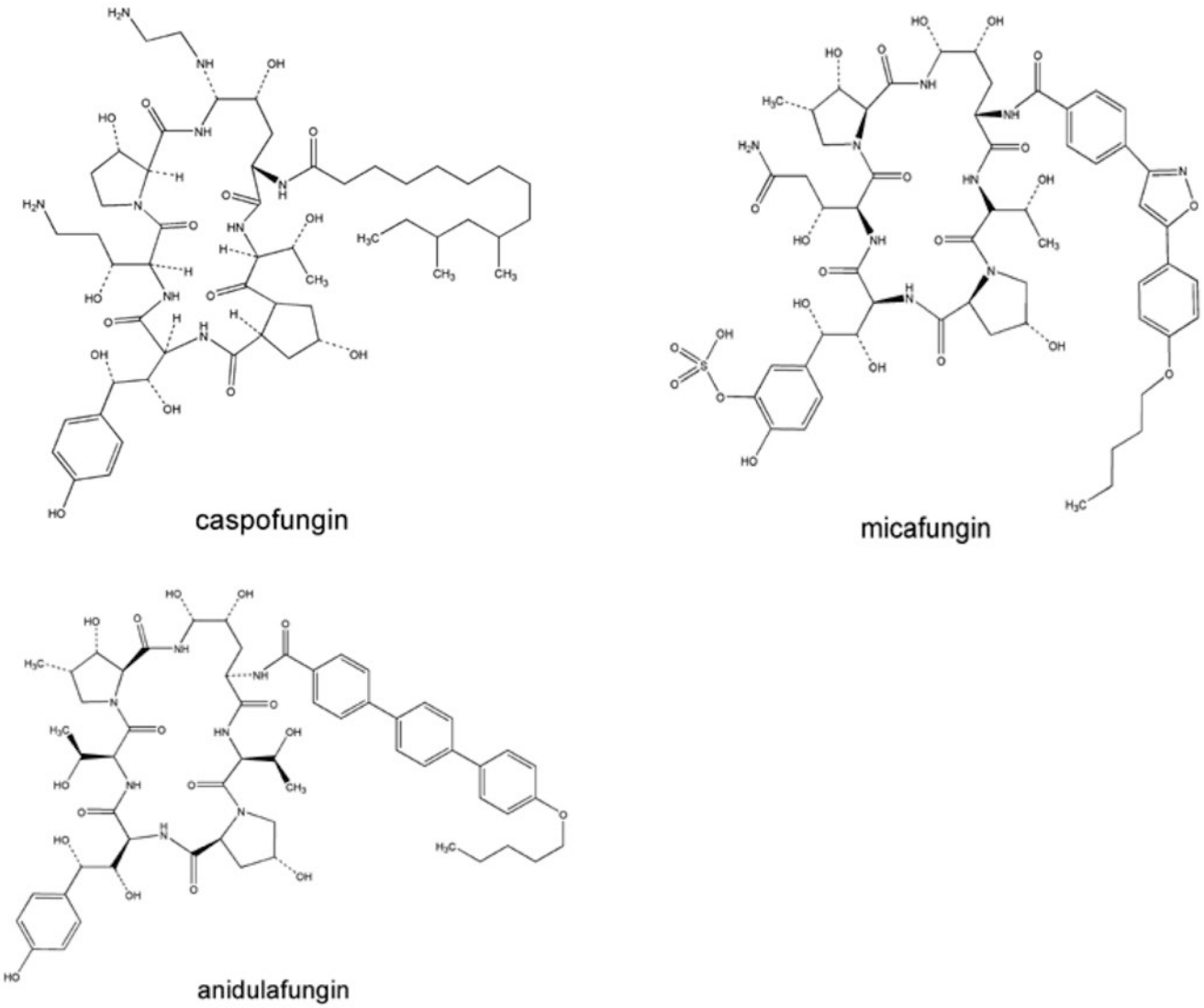


Fig. 30.13 Structures of echinocandins. From http://www.doctorfungus.org/thedrugs/Glucan_synth_inhibitors.htm

derived from echinocandin B produced by *A. nidulans*; Fig. 30.13) [282]. Each inhibits cell wall β-glucan synthesis by inhibiting β-glucan synthetase [283, 284]. They have broad-spectrum antifungicidal activities in vitro and in vivo (reviewed by [285, 286]). Echinocandins are also very promising in that resistant mutants are not cross-resistant to other classes of antifungals, and conversely, clinical isolates that are resistant to other antifungals, notably FLZ, are not typically cross-resistant to caspofungin [287–289].

The lack of cross-resistance of echinocandins results from their inhibition of synthesis of a unique target, the essential (1–3)β-D-glucans in the fungal cell wall. The primary targets in *S. cerevisiae* are β-glucan synthase subunits encoded by FKS1 or a paralogous gene encoded by FKS2. They are presumed to be alternate catalytic subunits; FKS2 expression is activated by calcineurin. Point mutations in either ScFKS gene confer resistance [290–292]. The activity of the synthase is regulated by ScRho1p GTPase [293, 294]. In *C. albicans*, the same target enzyme is encoded by CaFKS1, with no paralog. Four of four independent spontaneous mutants, selected in vitro for resistance to semisynthetic echinocandin L-733,560, showed cross-resistance only to other echinocandins and had in vitro-resistant β-glucan synthase activity [295]. Disruption of the resistant CaFKS1 allele in each mutant, using an integrative plasmid containing a fragment of CaFKS1, negated this resistance. This shows that mutation in either CaFKS1 allele is sufficient for resistance [285]. These caspofungin-resistant mutants were fully virulent in a mouse disseminated candidiasis model. Surprisingly, in this model, even resistant mutants were effectively treated with caspofungin [295]. However, transformants in which the susceptible allele had been disrupted, leaving only one resistant allele, were highly resistant in vivo [285]. Further analysis of

point mutations in CaFKS1 that confer resistance has not been published.

There are other mechanisms of resistance to echinocandins. Recently, CDR2 has been implicated as a resistance mechanism, since its overexpression in *S. cerevisiae* or *Candida* increases the MIC from 0.1 to 1.0 μg/mL [55]. While this increase is significant mechanistically, it should be emphasized that this level of resistance is 30-fold lower than that afforded by point mutations in FKS1 [295]. Other active efflux mechanisms seem incapable of conferring caspofungin resistance [296]. At least at low concentrations, both uptake and efflux of caspofungin by *C. albicans* appear to be mediated by a high-affinity facilitated transporter, not CDR2, that is energy independent [297]. Although mutants in this putative function have not yet been reported, it is another potential resistance mechanism. Another resistance mechanism may underlie the "paradoxical" resistance to caspofungin [298]. In this, 16% of clinical isolates of *C. albicans* are susceptible to caspofungin at low concentrations, but resistant at higher concentrations. Over half of tested clinical isolates are killed at low concentrations but are tolerant at higher concentrations. These effects reverse to wild type upon subculture. This effect was not seen for other echinocandins, or in other species. The authors suggest that the higher concentrations induce or derepress a resistance mechanism, which has yet to be investigated. Alternatively, any inhibition by caspofungin may trigger the adaptive response, but only higher concentrations may inhibit a cryptic second target to inhibit cell division, necessary for killing by caspofungin. This concentration-dependent delay may give time for the adaptive response to confer transient resistance. Global expression analysis of paradoxical strains, exposed to lower versus higher concentrations of caspofungin, should be revealing.

Taking a different approach, one group overexpressed *S. cerevisiae* cDNA clones in *S. cerevisiae* transformants and selected for caspofungin resistance. They isolated a single gene, SBE2, a Golgi protein required for cell wall assembly, that conferred specific resistance when overexpressed, and hypersensitivity when deleted [299]. These early studies suggest that we are potentially many novel mechanisms for resistance to caspofungin. Some of these genes may become important in resistant clinical isolates as caspofungin becomes more widely used.

Genome-scaled functional analysis of caspofungin resistance in *S. cerevisiae* is a predictor of resistance mechanisms that may apply to *C. albicans*. A library of 4787 individual knockout mutations were screened for resistance and hypersusceptibility. Twenty disruptants were hypersusceptible; eleven were involved in the PKC cell integrity pathway, and chitin, mannan, and ergosterol biosynthesis, including the target gene FKS1. Consistently, PKC inhibitor staurosporine was synergistic with caspofungin against *A. fumigatus*, *A. nidulans*, and *A. flavus* isolates that were resistant to caspofungin alone. Nine disruptants were resistant, and five of these encoded cell wall or signal transduction genes [300].

Using the same approach, a different group identified an overlapping set of *S. cerevisiae* genes whose disruption altered susceptibility to caspofungin [301]. Disruption of 53 genes resulted in hypersusceptibility, and another 39 in resistance, using a less stringent definition of altered susceptibility than in the competing study. Notably, deletion of FKS2, but not FKS1, conferred resistance, as did CZR1, the calcineurin-dependent upstream activator of FKS2 [301]. One expects that FKS1 disruptants would be susceptible, since FKS2 is normally not expressed unless FKS1 is deleted, and since Fks2p is more sensitive to caspofungin than Fks1p. It is not clear why FKS2 disruptants would be resistant to caspofungin. In the absence of additional information, it would seem that these strains would have the same susceptibility as wild type, since both express FKS1. From this open question, it seems that there is still much to be learned about the regulation of cell wall biosynthesis and its regulation.

From these two disruption studies in *S. cerevisiae*, it appears that agents that interfere with the PKC cell integrity pathway, and those conditions or mutations which inhibit compensatory changes in cell wall biosynthesis, may act synergistically with caspofungin and allow effective treatment of strains and species that are relatively insensitive to caspofungin alone.

Preliminary data from our *C. albicans* library indicate that overexpression of a gene with no known function or relationship to cell wall biogenesis confers resistance to caspofungin.

7.2 5-Fluorocytosine (FC) and Fluoroorotic Acid (FOA)

These pyrimidine analogs are suicide inhibitors that must be modified by susceptible cells by enzymes in the pyrimidine salvage pathway to be toxic. The pathway for FC, outlined in Fig. 30.14, includes a cytosine-purine permease for uptake, a deaminase which is not present in humans, thereby explaining the basis for fungal specificity, and a uracil phosphoribosyltransferase, to generate the toxic intermediate F-UMP. This is incorporated into RNA via F-UTP, presumably inactivating its template function and also inhibiting RNA synthesis. It is also converted by ribonucleotide reductase to F-dUMP, which inhibits thymidylate synthase and DNA replication [302–304].

FOA has long been used to inhibit orotidylate decarboxylase or orotate phosphoribosyltransferase, encoded by URA3 and URA5, respectively. Resistance to FOA is classically by inactivation of either of these genes, which imposes uridine auxotrophy, and is the basis for negative selection for the

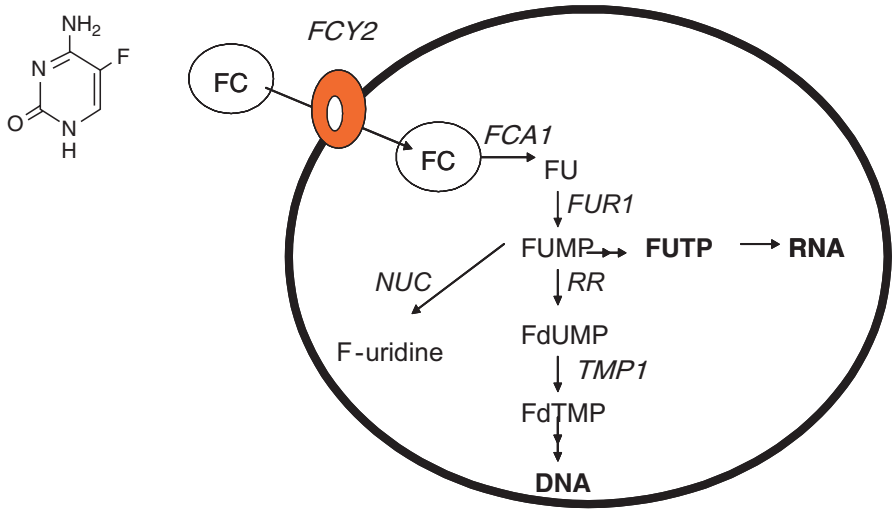


Fig. 30.14 Pyrimidine salvage pathway in *C. albicans* is responsible for susceptibility to FC. Genes: FCY2(1–4) = 4 redundant cytosine-purine permeases; FCA1 = cytosine deaminase; FUR1 = uracil phosphoribosyl transferase (UMP pyrophosphorylase); RR = ribonucleotide reductase; TMP1 = thymidylate synthase; NUC1 = putative nucleotidase (unpublished observation)

deletion of URA3-containing vectors or gene cassettes in *S. cerevisiae* [305] and *C. albicans* [306]. These recessive mutations are not a likely cause of resistance in diploid *C. albicans* except in lineages that are heterozygous when exposed.

Resistance in clinical strains has long been recognized as a frequent occurrence, seen in up to 11–15 %, occasionally 35 % of *C. albicans* isolates, depending on the site of the study and the patient types [307, 308]. This factor alone has limited the use of an otherwise potent fungicidal agent, which is now used mainly in combination with other antifungals or in cases that are refractory to azoles [309–313]. In one study, FC was not effective against *C. lusitaniae* [314], although this is disputed by others [315]. Many isolates of *C. krusei* [316] and *C. tropicalis* show intermediate resistance [315, 317]. In at least three studies, resistant isolates of *C. glabrata* were rare (<4 %) [314–316], although resistant strains are readily isolated in vitro [318].

Early studies suggested, quite insightfully, that resistance in isolates from clinical samples of many clinical isolates of *C. albicans* was due to heterozygosity in a hypothetical FCY gene. Susceptible strains were homozygous FCY/FCY, resistant strains were fcy/fcy, and heterozygous FCY/fcy strains gave rise to resistant colonies at a high frequency [319]. The authors suggested that two different FCY genes were involved and independently capable of conferring FC resistance. FCY1 mutants were deficient in UMP pyrophosphorylase, and FCY2 mutants were deficient in cytosine deaminase activity [320–322]. Since it is now known that four genes encode cytosine permeases, its deficiency in the FCY2 mutants must be due to mutation in a common regulator.

More recent molecular studies have all but confirmed the role of UMP pyrophosphorylase, now called uracil phosphoribosyltransferase. Resistance in most, but not all, clinical strains is most likely due to a mutation in the FUR1 gene, formerly FCY1, encoding this enzyme. The mutation at C301T alters a conserved amino acid, is homozygous in FC-resistant strains, is heterozygous in strains with intermediate levels of resistance, and is confined to a single lineage, Clade I [323–325]. The model is that a defective or deficient Fur1p cannot effectively convert FC to the toxic F-UMP, thereby providing resistance.

We have confirmed this model by introducing wild-type FUR1 into FC-resistant strains containing homozygous C310T mutations, and showing that the transformants were susceptible to FC (unpublished observations).

Why study FC resistance in *C. albicans* instead of the more tractable *S. cerevisiae*? In addition to the staple that *C. albicans* is the more clinically relevant, there are clear indications that the mechanisms of resistance are not the same in the two yeasts. It is clear that point mutations in FUR1 implicate that gene in FC-resistant *C. albicans*. In contrast, after deleting six pyrimidine salvage genes in *S. cerevisiae*, only the permease deletion showed moderate FC resistance, not FUR1 [304].

Evidence that mechanisms other than FUR1 inactivation are operative in *C. albicans* is suggested by early biochemical and genetic studies of *C. albicans* and *C. glabrata*. These studies point to deficiencies in cytosine permease, cytosine deaminase, or alterations in thymidylate synthase activities [302, 318, 326]. One of 25 clinical isolates showing FC resistance had a homozygous mutation in cytosine deaminase FCA1, although no evidence was presented that this was responsible for its resistance [325]. Our observation is that strains that are homozygous at FUR1 C310 still spontaneously mutate to FC resistance at a high frequency, suggesting that some other gene whose loss of activity confers resistance is nonallelic or heterozygous. We have identified a putative nucleotidase gene, termed here NUC1, by selecting for

FC resistance among a library of *C. albicans* transformants overexpressing wild-type *C. albicans* genes, whose overexpression confers resistance to both FC and FOA. These transformants are *not* auxotrophic (unpublished observations). Presumably, this resistance results from depletion of the pool of toxic F-UMP (Fig. 30.14).

Rapamycin inhibits a kinase encoded by TOR1 (target of rapamycin) that plays a pivotal role in nutrient sensing in *S. cerevisiae* [327, 328]. Likewise, in *C. albicans*, rapamycin binds to and promotes binding of FKBP12 to TOR kinase, inhibiting its function. While rapamycin has not been used clinically as an antifungal due to its immunosuppressive effects, non-immunosuppressive analogs are also effective as antifungals [329].

In *S. cerevisiae*, rapamycin-inhibited cells halt protein synthesis and ribosome biogenesis, and undergo cell cycle arrest and autophagy [327]. TOR-dependent resumption of growth upon nutrient restoration may be mediated by the RAS/cAMP pathway, since its overexpression suppresses TOR mutants [330]. Many intermediate genes in *S. cerevisiae* have been implicated in the TOR response, but the big picture is far from clear: For example, is there a critical target of transcriptional activation that is essential for survival? What is clear is that yeast cells must have a functional TOR pathway in order to survive in a nutrient-poor environment and to resume growth when the environment improves.

Despite the complexity of the pathway, overexpression of several genes could indicate whether similar interactions are active in *C. albicans* or implicate unknown genes involved in the TOR response. Some examples include overexpression of MSN2/4, TAP42, a gene normally activated by TOR (Fig. 30.15), or genes in the RAS/cAMP or PKC cell integrity pathways (not shown). TOR inactivates Apg13 to indirectly inactivate protein kinase Apg1, a key kinase responsible for induction of autophagy, explaining the rapamycin induction of autophagy [331]. Overexpression of APG13 may confer tolerance to rapamycin. Some of these gene products could be targets of next-generation antifungals.

Preliminary data from our *C. albicans* overexpressant library indicate that overexpression of several genes with unknown functions are resistant to rapamycin, suggesting that there is much to be discovered in this pathway. Overexpression of another gene may confer TOR-independent activation of RAS-cAMP.

Aureobasidin A is a cyclic depsipeptide, produced by *Aureobasidium pullulans* R106 that inhibits inositol phosphorylceramide (IPC) synthase, which catalyzes a late step in the synthesis of sphingolipids (Fig. 30.16). Sphingolipids IPC, MIPC, or M(IP_2)C are essential for yeast viability, and the downstream forms are required for normal tolerance to calcium. Intermediates, especially ceramide, are potent inhibitors at low concentration. Sphingolipids and select precursors are implicated in heat stress responses, endocytosis, cell integrity pathway, and cell signaling [332]. Polarization of the plasma membrane into ergosterol- and sphingolipid-rich "raft" domains is thought to be a basis for collecting proteins, especially GPI-anchored proteins, ABC transporters, and MFS transporters, which in turn are needed for morphogenesis, hyphal formation, and antifungal resistance in *C. albicans* [333].

The enzyme targeted by aureobasidin A is encoded by the essential gene AUR1 (ISC1); select point mutations in, or overexpression of, this gene result in resistance [334, 335]. This enzyme complexes the ceramide chains to inositol phosphate, rather than to choline phosphate as in mammals,

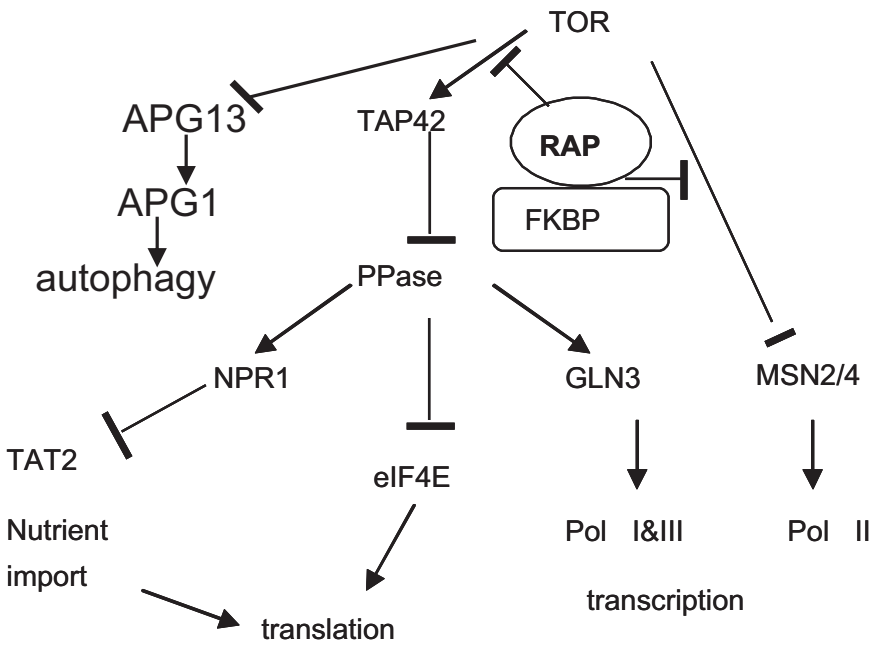


Fig. 30.15 In *S. cerevisiae*, rapamycin inhibits TOR signaling to impair complex responses that allow survival in nutrient-poor environments. Modified from [327]. Clear *C. albicans* orthologs named after *S. cerevisiae* genes exist for all genes. TAT2 is CaO19.12096; FKBP12 is CaO19.6452=RBP12

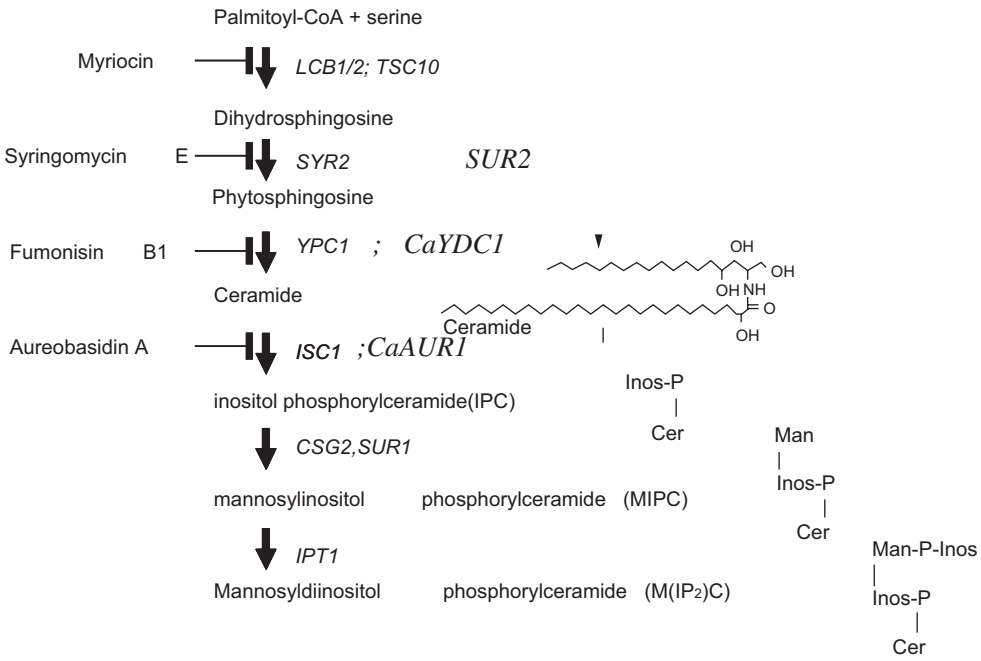


Fig. 30.16 Yeast sphingolipid pathway, genes, and inhibitors. There are clear orthologs for most genes in *S. cerevisiae* and *C. albicans*; in cases where the gene names differ, the *C. albicans* name is noted. There is no apparent *C. albicans* homolog for ScGSG2, encoding the regulatory subunit for MIPC synthase. Two genes in *C. albicans* were named SUR1; this gene refers to CaO19.4077. Modified from [332, 427]

accounting for its specificity [332]. Aureobasidin A is active against many species of *Candida*. In *S. cerevisiae*, it results in cell death by loss of membrane integrity [336]. It also effectively inhibits IPC synthase in *A. fumigatus*, but the organism is resistant, apparently due to CDR1-mediated efflux [337].

Screening of our library of *C. albicans* overexpressants identified CaIPT1, not CaISC1, as a gene capable of conferring resistance to aureobasidin A (unpublished observation). The two genes encode are similar, phospholipase C-like enzymes, and both transfer inositol phosphate, albeit onto different substrates. Perhaps the elevated level of Ipt1p catalyzes sufficient ceramide → IPC synthesis under conditions in which aureobasidin A is inhibiting Isc1p. However, IPT1 is a resistance gene in its own right. Disruption of ScIPT1 confers hypersusceptibility to butoconazole and oligomycin, and resistance to hygromycin B and cycloheximide, but no data is available on its effects on susceptibility to aureobasidin A. Perhaps our CaIPT1-overexpressing transformant has altered membrane permeability, rafting properties, or fluidity that mediates resistance; it does not, however, show resistance to other antifungals (azoles, FC, AmB).

7.3 Peptides

7.3.1 Histatin

Histatins, notably Hst3 and Hst5, are histidine-rich cationic peptides present in human saliva which have antimicrobial, anti-candidal activity (Table 30.6) [338]. The mechanism of action of Hst5 is intriguing, and is not, as one might have expected, due to formation of membrane pores by a complex of the alpha-helical peptides. Instead, Hst 5 binds to the membrane-associated heat-shock protein Ssp1p. This interaction is necessary for most of the killing effect of Hst5 [339]. Binding and activity of Hst5 are inhibited by low concentrations of Ca^{++} present in human saliva, masking its antifungal activity [340]. After binding, Hst5 is internalized and targets the mitochondria [341, 342]. In *C. albicans*, respiring mitochondria are essential for the fungicidal effect of Hst5, since respiration-defective mutants are resistant [343], and since inhibition or uncoupling of oxidative phosphorylation blocks killing by Hst5 [341, 343–345]. Unaccountably, the latter is not true in *S. cerevisiae*, even though respiring cells are more susceptible than fermentative cells [346]. A similar pathway is implicated for Hst3, from which Hst5 is derived

Table 30.6 Candidacidal peptides

Uptake and ATP release:	
His5	DSHAKRHHGYKRKFHEKHHSHRGY
His3	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN
hLF1-11	GRRRRSVQWCA
Membrane disruption:	
Cathelicidin-like	
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIA-NH2
Fn/23	GRGDSPASSKGGGGSRLLLLLLR
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR-NH2
HP-L1 19	AKKVFKRLEKLFSKIWNWK-NH2
Cepropin18	KWKKLLKKPLLKKLLKKL-NH2
Defensins	
HNP1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP2	CYCRIPACIAGERRYGTCIYQGRLWAFCC

[347, 348]. In vitro selection on Hst3 identified resistant mutants that still bound and internalized Hst3, and still released ATP [348]. Hst5-affected mitochondrial membranes are depolarized [341] and release ROS, but this is not the mediator of cell killing [349]. Death mediated by Hst5 depends on the release of K^+ and ATP from the cell [344, 350], probably mediated by Trk1p, the plasma membrane K^+ transporter [351]. However, this release is selective and does not involve cell lysis [344] or classic apoptotic mechanisms [349].

Depletion of intracellular ATP following its efflux is not responsible for cell death, since anaerobically grown cells show similar ATP depletion but are Hst5 resistant [344]. Instead, it is proposed that the extracellular ATP binds to and activates a protein that cross-reacts with human P2X(7) receptor [350]. In humans, this protein may act as a cell death receptor which triggers massive Ca^{++} influx [352]. Hst5-mediated death is partially mimicked by exogenous ATP and its analogues [350]. However, neither form of killing is dependent on extracellular Ca^{++} [350]. The identity and function of the hypothetical *C. albicans* cell death receptor, and whether Hst3-resistant mutants [348] are defective in this receptor, are issues that remain to be established.

7.3.2 Lactoferrin (LF)

LF is another human antimicrobial peptide, present in milk, saliva, and various exocrine secretions and in neutrophils. Its anti-*Candida* effects have been documented in many laboratories, in which it acts synergistically with FLZ or AmB, and is notably more effective on *C. krusei*, which is typically more resistant to antifungals [353–365]. However, LF's mechanism of action is less studied. This mechanism is not likely related to its ability to bind iron, since iron-free LF, and an amino terminal peptide derivative hLF1-11, retains antifungal activity [363, 366]. Despite sequence differences, hLF1-11 has many features in common with histatins, including low-level K^+ release without cell lysis, inhibition by mitochondrial inhibitors and by Ca^{++}, release of ATP, and inhibition of killing by ATP antagonists [366–368]. In its "activated," immobilized form, LF inhibits adhesion of *C. albicans* to epithelial cells [361, 362]. LF may interact with cell wall mannoprotein(s), since their inhibition by preexposure to tunicamycin blocks inhibition [369, 370]. Resistance and resistance mechanisms to LF and derivatives remain to be explored.

7.3.3 Membrane-Disrupting Peptides

Two classes of cationic peptides bind preferentially to microbial membranes, forming intermolecular amphipathic structures that disrupt the membrane. Cathelicidins are a diverse group of peptides that derive from myeloid cells; several of these have antifungal activity [371–373], and derivatives have potent candidacidal activity (Table 30.6 and [374, 375]). Defensins are synthesized in neutrophils where they are concentrated in phagolysosomes or secreted into mucus membranes as an innate defense against microbes; HNP1 and 2 are candidacidal (Table 30.6 and [376]).

Resistance to all candidacidal peptides is understudied. This may be due to a misperception that the nonspecific, lytic mechanism precludes mutation-based resistance. However, *S. aureus* has acquired an innate resistance to defensins via Mpr-F, an enzyme that adds a lysine to membrane phosphatidylglycerol. Presumably the reduced membrane charge prevents its interaction with the cationic defensin [377]. Other bacteria have acquired different charge-related modifications to their membrane, or efflux mechanisms, that confer resistance to these peptides [378]. Potentially, similar mechanisms, or wall alterations that restrict access, might confer resistance in *Candida*.

Both groups of candidacidal peptides share the property that their activity is inhibited by low, physiological concentrations of Ca^{++}. For defensins, this may not be a factor when acting in mucus or in phagolysosomes, but it is a limiting factor for histatins acting in saliva. This suggests that these peptides, applied as extracellular agents, will not be effective in killing fungi in bloodstream infections (4–5 mM Ca^{++}). Thus, their most likely potential seems to be for treatment of OPC and VVC infections.

Plant-derived oils from a variety of sources, notably Thymus, Melaleuca (tea tree) and cinnamon, have rapid cidal effects on human fungal pathogens. Where investigated, these seem to act as membrane-disrupting agents and are likely limited to topical use [379–385]. Whether these oils have mechanisms related to lytic peptides remains to be seen. Resistance to these agents is not documented.

Synthetic peptide libraries have been constructed and screened for fungicidal activity. These have been cationic peptides, to mimic natural antifungal peptides and derivatives. For example, Monk's group made a 1.8 million member D-octapeptide library that contained cationic peptides D-NH2-A-B-X3-*X*2-X1-RRR-CONH2. A peptide that seems to act by inhibiting Pma1p, the major plasma membrane ATPase, was fungicidal and, at lower doses, sensitized *C. albicans* to FLZ [386]. Other synthetic antifungal peptides have also been identified [375, 387–389]. Though promising, the studies in general are limited to in vitro susceptibilities, so their in vivo efficacy is not known. Resistance mechanisms to these peptides have not generally been investigated, yet.

7.4 Amino-acyl tRNA Synthetase Inhibitors

PLD-118 represents another class of antifungal compounds, cyclic β-amino acids (Fig. 30.17), that apparently target aminoacyl synthetases and inhibit growth in vivo. Recently, PLD-118 was shown to be effective in eradicating FLZ-resistant *C. albicans* in a rabbit OPEC model [390]. Preliminary studies suggest that yeasts are susceptible to

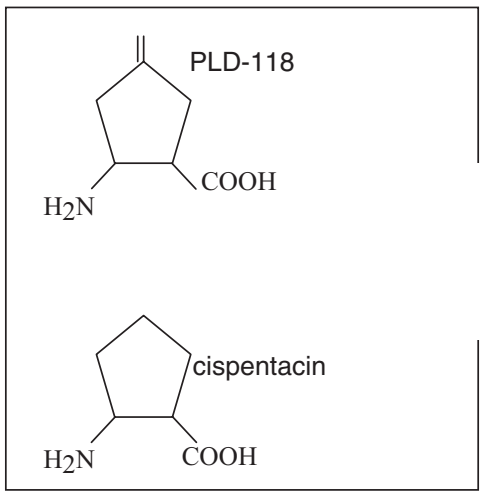


Fig. 30.17 Structures of cyclic amino acid inhibitors

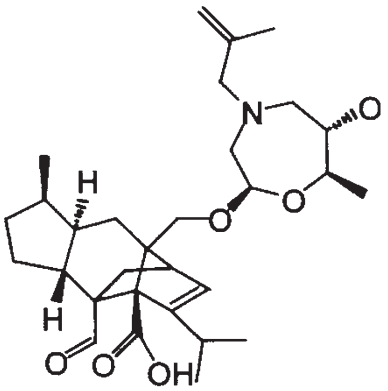


Fig. 30.18 Structure of sordarin derivative R-135853 [407]

PLD-118, formerly BAY10-8888, because of a combination of its ability to accumulate in the cytoplasm, and its ability to inhibit isoleucyl-tRNA synthetase. In vitro studies show that isoleucyl-tRNA synthetase activity is inhibited by 90+% at 10 mM PLD-118, with a corresponding decrease in protein synthesis. Furthermore, adding isoleucine to media precludes inhibition by PLD-118, suggesting, in this model, that isoleucine concentrations compete for both uptake and synthetase [391]. Consistently, but not conclusively, increased expression levels of the synthetase among *Candida* species and mutants of *C. albicans* are correlated with resistance [392]. More convincingly, the levels of accumulation of PLD-118 are much lower in the resistant *C. albicans* mutants, suggesting that uptake is blocked by a defective permease, or that efflux is increased in the mutants.

Because PLD-118 has a different target than clinical antifungals, one would expect an additive or synergistic interaction with azoles, echinocandins, etc., but no data is available. Combination therapy, therefore, is worth considering and testing, to exploit possible synergies and to preclude breakthrough of resistant isolates.

Another cyclic β-amino acid analog, *cispentacin*, or (1R,1S)-2-aminocyclopentane-1-carboxylic acid (Fig. 30.17), is taken up by *C. albicans* via the inducible proline permeases, and probably by other permeases, and accumulates to mM concentrations [393, 394]. Uptake is competitively inhibited by proline, and it inhibits proline tRNA synthetase and protein synthesis. This static agent is effective in a mouse systemic candidiasis model [395].

7.5 CAN-296

A complex polysachharide isolated from Mucor rouxii, CAN-296, has rapid fungicidal effects on many pathogenic yeasts, regardless of susceptibilities to other antifungals, although it is not effective on *Aspergillus* [396]. Based on the premise that its high molecular weight implies a wall or membrane target, a group at Wayne State University showed that CAN-296 inhibits proton pumping (media acidification) in susceptible but not resistant *Candida* isolates. This implicated the membrane H^+-ATPase; however, the H^+-ATPase activity of membrane fractions was not affected by CAN-296 [397]. CAN-296 binds to wall-membrane fractions of *C. albicans*, and the binding is reversibly inhibited by Ca++ [398]. Together, the data suggest that inhibition of proton pumping by CAN-296 is an indirect effect, and therefore that the true target is not yet known. Its in vivo binding may limit its clinical use to topical antifungals, where it is effective [399]. However, identification of its target, whose inhibition is so dramatically lethal, is still worth pursuing.

7.6 Sordarins

Sordarins are natural products from the sordariomycete Graphium putredinis. They inhibit microbial but not human translation elongation factor 2 (eEF2) [400–403]. Derivative forms are effective in vivo against yeasts other than *C. glabrata*, *C. parapsilosis*, and *C. krusei*. In disseminated infections in mice, these derivatives were effective against *C. albicans* but less so against *Aspergillus* [404–406]. A newer derivative (Fig. 30.18) was effective against FLZ-susceptible or -resistant *C. albicans*, *C. glabrata*; *C. guilliermondii*; and *C. neoformans*, but not against *C. parapsilosis*, *C. krusei*, and *Aspergillus* spp. [407]. New sordarin analogs are being identified [408–412], as when these are characterized, the spectrum of susceptible species may be broadened. The existing spectrum is the biggest current problem for sordarins, not specificity or toxicity.

Sordarin binds to *C. albicans* and *S. cerevisiae* eEF2 in vitro and its binding is enhanced by the presence of ribo-

somes, suggesting a complex interaction between eEF2 and ribosomes [402, 413]. Consistently, resistance to sordarin in *S. cerevisiae* is conferred by mutations in eEF2 which result in loss of drug binding in extracts [400, 413, 414]. Resistance is also conferred by alterations (chimeras, site-specific mutagenesis) in ribosomal proteins which interact with eEF2 [415–417]. These alterations are outside the points of contact between eEF2 and the proteins [418], indicating that the interaction is complex and allosteric. Studies of resistance to sordarins are therefore of fundamental interest to the molecular mechanics of translation.

References

1. Loeffler J, Stevens DA. Antifungal drug resistance. Clin Infect Dis. 2003;36:S31–41.
2. Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev. 1999;12:501–17.
3. Joseph-Horne T, Hollomon DW. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol Lett. 1997;149:141–9.
4. Iwata K. Drug resistance in human pathogenic fungi. Eur J Epidemiol. 1992;8(3):407–21.
5. Lamb D, Kelly D, Kelly S. Molecular aspects of azole antifungal action and resistance. Drug Resist Updat. 1999;2:390–402.
6. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev. 1998;11:382–402.
7. Sanglard D. Resistance of human fungal pathogens to antifungal drugs. Curr Opin Microbiol. 2002;5(4):379–85.
8. Vanden Bossche H, Dromer F, Improvisi I, Lozano-Chiu M, Rex JH, Sanglard D. Antifungal drug resistance in pathogenic fungi. Med Mycol. 1998;36 Suppl 1:119–28.
9. Vanden Bossche H, Marichal P, Odds FC. Molecular mechanisms of drug resistance in fungi. Trends Microbiol. 1994;2(10): 393–400.
10. Sanglard D, Bille J. Current understanding of the modes of action of and resistance mechanisms to conventional and emerging antifungal agents for treatment of *Candida* infections. In: Calderon RA, editor. *Candida* and Candidiasis. Washington, DC: ASM Press; 2002. p. 349–83.
11. Casalinuovo IA, Di Francesco P, Garaci E. Fluconazole resistance in *Candida albicans*: a review of mechanisms. Eur Rev Med Pharmacol Sci. 2004;8:69–77.
12. Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis. 2002;2:73–85.
13. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC, Bossche HV. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans* [In Process Citation]. Microbiology. 1999;145:2701–13.
14. Prasad R, Kapoor K. Multidrug resistance in yeast *Candida*. Int Rev Cytol. 2005;242:215–48.
15. Houten SM, Waterham HR. Nonorthologous gene displacement of phosphomevalonate kinase. Mol Genet Metab. 2001;72:273–6.
16. Tsay YH, Robinson GW. Cloning and characterization of ERG8, an essential gene of *Saccharomyces cerevisiae* that encodes phosphomevalonate kinase. Mol Cell Biol. 1991;11:620–31.
17. Roemer T, Jiang B, Davison J, Ketela T, Veillette K, Breton A, Tandia F, Linteau A, Sillaots S, Marta C, et al. Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. Mol Microbiol. 2003;50:167–81.
18. Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Kelly DE. Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. Biochem Biophys Res Commun. 1995;207:910–5.
19. Watson PF, Rose ME, Ellis SW, England H, Kelly SL. Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. Biochem Biophys Res Commun. 1989;164:1170–5.
20. Bard M, Lees ND, Turi T, Craft D, Cofrin L, Barbuch R, Koegel C, Loper JC. Sterol synthesis and viability of erg11 (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. Lipids. 1993;28:963–7.
21. Shimokawa O, Kato Y, Nakayama H. Increased drug sensitivity in *Candida albicans* cells accumulating 14-methylated sterols. J Med Vet Mycol. 1986;24:481–3.
22. Bard M, Lees ND, Barbuch RJ, Sanglard D. Characterization of a cytochrome P450 deficient mutant of *Candida albicans*. Biochem Biophys Res Commun. 1987;147:794–800.
23. Shimokawa O, Nakayama H. A *Candida albicans* mutant conditionally defective in sterol 14 alpha-demethylation. J Med Vet Mycol. 1989;27:121–5.
24. Chau AS, Mendrick CA, Sabatelli FJ, Loebenberg D, McNicholas PM. Application of real-time quantitative PCR to molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. Antimicrob Agents Chemother. 2004;48:2124–31.
25. Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RE, McNicholas PM. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. Antimicrob Agents Chemother. 2004;48:568–74.
26. Fukuoka T, Johnston DA, Winslow CA, de Groot MJ, Burt C, Hitchcock CA, Filler SG. Genetic basis for differential activities of fluconazole and voriconazole against *Candida krusei*. Antimicrob Agents Chemother. 2003;47:1213–9.
27. Ji H, Zhang W, Zhou Y, Zhang M, Zhu J, Song Y, Lu J. A three-dimensional model of lanosterol 14alpha-demethylase of *Candida albicans* and its interaction with azole antifungals. J Med Chem. 2000;43:2493–505.
28. Ji H, Zhang W, Zhang M, Kudo M, Aoyama Y, Yoshida Y, Sheng C, Song Y, Yang S, Zhou Y, et al. Structure-based de novo design, synthesis, and biological evaluation of non-azole inhibitors specific for lanosterol 14alpha-demethylase of fungi. J Med Chem. 2003;46:474–85.
29. Macchiarulo A, Costantino G, Fringuelli D, Vecchiarelli A, Schiaffella F, Fringuelli R. 1,4-Benzothiazine and 1,4-benzoxazine imidazole derivatives with antifungal activity: a docking study. Bioorg Med Chem. 2002;10:3415–23.
30. Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother. 1998;42:241–53.
31. Kontoyiannis DP, Sagar N, Hirschi KD. Overexpression of Erg11p by the regulatable GAL1 promoter confers fluconazole resistance in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. 1999;43(11):2798–800.
32. Marichal P, Vanden Bossche H, Odds FC, Nobels G, Warnock DW, Timmerman V, Van Broeckhoven C, Fay S, Mose-Larsen P. Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob Agents Chemother. 1997;41:2229–37.
33. van den Bossche H, Marichal P, Odds FC, Le Jeune L, Coene MC. Characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob Agents Chemother. 1992;36:2602–10.
34. Perepnikhatka V, Fischer FJ, Niimi M, Baker RA, Cannon RD, Wang YK, Sherman F, Rustchenko E. Specific chromosome alterations in fluconazole-resistant mutants of *Candida albicans*. J Bacteriol. 1999;181:4041–9.

35. Rustad TR, Stevens DA, Pfaller MA, White TC. Homozygosity at the *Candida albicans* MTL locus associated with azole resistance. Microbiology. 2002;148(Pt 4):1061–72.
36. Du W, Coaker M, Sobel JD, Akins RA. Shuttle vectors for *Candida albicans*: control of plasmid copy number and elevated expression of cloned genes. Curr Genet. 2004;45(6):390–8. Epub 2004 Mar 18.
37. Sanglard D, Ischer F, Parkinson T, Falconer D, Bille J. *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother. 2003;47(8):2404–12.
38. Kalb VF, Woods CW, Turi TG, Dey CR, Sutter TR, Loper JC. Primary structure of the P450 lanosterol demethylase gene from *Saccharomyces cerevisiae*. DNA. 1987;6:529–37.
39. Geber A, Hitchcock CA, Swartz JE, Pullen FS, Marsden KE, Kwon-Chung KJ, Bennett JE. Deletion of the *Candida glabrata* ERG3 and ERG11 genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. Antimicrob Agents Chemother. 1995;39:2708–17.
40. Jackson CJ, Lamb DC, Manning NJ, Kelly DE, Kelly SL. Mutations in *Saccharomyces cerevisiae* sterol C5-desaturase conferring resistance to the CYP51 inhibitor fluconazole. Biochem Biophys Res Commun. 2003;309:999–1004.
41. Smith SJ, Crowley JH, Parks LW. Transcriptional regulation by ergosterol in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol. 1996;16:5427–32.
42. Ryder NS, Wagner S, Leitner I. In vitro activities of terbinafine against cutaneous isolates of *Candida albicans* and other pathogenic yeasts. Antimicrob Agents Chemother. 1998;42:1057–61.
43. Ryder NS. Activity of terbinafine against serious fungal pathogens. Mycoses. 1999;42:115–9.
44. Jessup CJ, Ryder NS, Ghannoum MA. An evaluation of the in vitro activity of terbinafine. Med Mycol. 2000;38:161–8.
45. Onyewu C, Blankenship JR, Del Poeta M, Heitman J. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. Antimicrob Agents Chemother. 2003;47(3):956–64.
46. Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol Microbiol. 2003;48(4):959–76.
47. Hiratani T, Yamaguchi H. Cross-resistance of *Candida albicans* to several different families of antifungals with ergosterol biosynthesis-inhibiting activity. Jpn J Antibiot. 1994;47(2):125–8.
48. Klobucnikova V, Kohut P, Leber R, Fuchsbichler S, Schweighofer N, Turnowsky F, Hapala I. Terbinafine resistance in a pleiotropic yeast mutant is caused by a single point mutation in the ERG1 gene. Biochem Biophys Res Commun. 2003;309(3):666–71.
49. Leber R, Fuchsbichler S, Klobucnikova V, Schweighofer N, Pitters E, Wohlfarter K, Lederer M, Landl K, Ruckenstuhl C, Hapala I, et al. Molecular mechanism of terbinafine resistance in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. 2003;47(12):3890–900.
50. Leber R, Landl K, Zinser E, Ahorn H, Spok A, Kohlwein SD, Turnowsky F, Daum G. Dual localization of squalene epoxidase, Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles. Mol Biol Cell. 1998;9(2):375–86.
51. Henry KW, Nickels JT, Edlind TD. Upregulation of ERG genes in *Candida* species by azoles and other sterol biosynthesis inhibitors [In Process Citation]. Antimicrob Agents Chemother. 2000;44:2693–700.
52. Liu W, May GS, Lionakis MS, Lewis RE, Kontoyiannis DP. Extra copies of the *Aspergillus fumigatus* squalene epoxidase gene confer resistance to terbinafine: genetic approach to studying gene dose-dependent resistance to antifungals in *A. fumigatus*. Antimicrob Agents Chemother. 2004;48(7):2490–6.
53. Graminha MA, Rocha EM, Prade RA, Martinez-Rossi NM. Terbinafine resistance mediated by Salicylate 1-Monooxygenase in *Aspergillus nidulans*. Antimicrob Agents Chemother. 2004;48(9):3530–5.
54. Sanglard D, Ischer F, Monod M, Bille J. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology. 1997;143:405–16.
55. Schuetzer-Muehlbauer M, Willinger B, Egner R, Ecker G, Kuchler K. Reversal of antifungal resistance mediated by ABC efflux pumps from *Candida albicans* functionally expressed in yeast. Int J Antimicrob Agents. 2003;22:291–300.
56. Schuetzer-Muehlbauer M, Willinger B, Krapf G, Enzinger S, Presterl E, Kuchler K. The *Candida albicans* Cdr2p ATP-binding cassette (ABC) transporter confers resistance to caspofungin. Mol Microbiol. 2003;48(1):225–35.
57. Sanglard D, Ischer F, Monod M, Bille J. Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob Agents Chemother. 1996;40:2300–5.
58. Wirsching S, Moran GP, Sullivan DJ, Coleman DC, Morschhauser J. MDR1-mediated drug resistance in *Candida dubliniensis*. Antimicrob Agents Chemother. 2001;45:3416–21.
59. Mukhopadhyay K, Prasad T, Saini P, Pucadyil TJ, Chattopadhyay A, Prasad R. Membrane sphingolipid-ergosterol interactions are important determinants of multidrug resistance in *Candida albicans*. Antimicrob Agents Chemother. 2004;48:1778–87.
60. Tsai HF, Bard M, Izumikawa K, Krol AA, Sturm AM, Culbertson NT, Pierson CA, Bennett JE. *Candida glabrata* erg1 mutant with increased sensitivity to azoles and to low oxygen tension. Antimicrob Agents Chemother. 2004;48(7):2483–9.
61. Rogers KM, Pierson CA, Culbertson NT, Mo C, Sturm AM, Eckstein J, Barbuch R, Lees ND, Bard M. Disruption of the *Candida albicans* CYB5 gene results in increased azole sensitivity. Antimicrob Agents Chemother. 2004;48(9):3425–35.
62. Jain P, Akula I, Edlind T. Cyclic AMP signaling pathway modulates susceptibility of *Candida* species and *Saccharomyces cerevisiae* to antifungal azoles and other sterol biosynthesis inhibitors. Antimicrob Agents Chemother. 2003;47:3195–201.
63. Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M. The yeast gene ERG6 is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol Cell Biol. 1989;9(8):3447–56.
64. Kleinhans FW, Lees ND, Bard M, Haak RA, Woods RA. ESR determinations of membrane permeability in a yeast sterol mutant. Chem Phys Lipids. 1979;23:143–54.
65. Bard M, Lees ND, Burrows LS, Kleinhans FW. Differences in crystal violet uptake and cation-induced death among yeast sterol mutants. J Bacteriol. 1978;135:1146–8.
66. Welihinda AA, Beavis AD, Trumbly RJ. Mutations in LIS1 (ERG6) gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae*. Biochim Biophys Acta. 1994;1193:107–17.
67. Jensen-Pergakes KL, Kennedy MA, Lees ND, Barbuch R, Koegel C, Bard M. Sequencing, disruption, and characterization of the *Candida albicans* sterol methyltransferase (ERG6) gene: drug susceptibility studies in erg6 mutants. Antimicrob Agents Chemother. 1998;42:1160–7.
68. Kaur R, Bachhawat AK. The yeast multidrug resistance pump, Pdr5p, confers reduced drug resistance in erg mutants of *Saccharomyces cerevisiae*. Microbiology. 1999;145:809–18.
69. Parks LW, Smith SJ, Crowley JH. Biochemical and physiological effects of sterol alterations in yeast—a review. Lipids. 1995;30:227–30.

70. Crowley JH, Tove S, Parks LW. A calcium-dependent ergosterol mutant of *Saccharomyces cerevisiae*. Curr Genet. 1998;34:93–9.
71. Oh CS, Toke DA, Mandala S, Martin CE. ELO2 and ELO3, homologues of the *Saccharomyces cerevisiae* ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. J Biol Chem. 1997;272:17376–84.
72. Stolz J, Sauer N. The fenpropimorph resistance gene FEN2 from *Saccharomyces cerevisiae* encodes a plasma membrane H^+-pantothenate symporter. J Biol Chem. 1999;274:18747–52.
73. Jia N, Arthington-Skaggs B, Lee W, Pierson CA, Lees ND, Eckstein J, Barbuch R, Bard M. *Candida albicans* sterol C-14 reductase, encoded by the ERG24 gene, as a potential antifungal target site. Antimicrob Agents Chemother. 2002;46:947–57.
74. Gachotte D, Pierson CA, Lees ND, Barbuch R, Koegel C, Bard M. A yeast sterol auxotroph (erg25) is rescued by addition of azole antifungals and reduced levels of heme. Proc Natl Acad Sci U S A. 1997;94:11173–8.
75. Kennedy MA, Johnson TA, Lees ND, Barbuch R, Eckstein JA, Bard M. Cloning and sequencing of the *Candida albicans* C-4 sterol methyl oxidase gene (ERG25) and expression of an ERG25 conditional lethal mutation in *Saccharomyces cerevisiae*. Pediatr Infect Dis J. 2000;19:319–24.
76. Nose H, Fushimi H, Seki A, Sasaki T, Watabe H, Hoshiko S. PF1163A, a novel antifungal agent, inhibit ergosterol biosynthesis at C-4 sterol methyl oxidase. J Antibiot (Tokyo). 2002;55:969–74.
77. Nose H, Seki A, Yaguchi T, Hosoya A, Sasaki T, Hoshiko S, Shomura T. PF1163A and B, new antifungal antibiotics produced by *Penicillium* sp. I. Taxonomy of producing strain, fermentation, isolation and biological activities. J Antibiot (Tokyo). 2000;53:33–7.
78. Bujdakova H, Kral'ova K, Sidoova E. Antifungal and antialgal activity of 3-(2-alkylthio-6-benzothiazolylaminomethyl)-2-benzoxazolinethi ones. Pharmazie. 1995;50:156.
79. Bujdakova H, Kuchta T, Sidoova E, Gvozdjakova A. Anti-*Candida* activity of four antifungal benzothiazoles. FEMS Microbiol Lett. 1993;112:329–33.
80. Kuchta T, Leka C, Farkas P, Bujdakova H, Belajova E, Russell NJ. Inhibition of sterol 4-demethylation in *Candida albicans* by 6-amino-2-n-pentylthiobenzothiazole, a novel mechanism of action for an antifungal agent. Antimicrob Agents Chemother. 1995;39:1538–41.
81. Kuchta T, Bartkova K, Kubinec R. Ergosterol depletion and 4-methyl sterols accumulation in the yeast *Saccharomyces cerevisiae* treated with an antifungal, 6-amino-2-n-pentylthiobenzothiazole. Biochem Biophys Res Commun. 1992;189:85–91.
82. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature. 1990;343:425–30.
83. Basson ME, Thorsness M, Rine J. *Saccharomyces cerevisiae* contains two functional genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Proc Natl Acad Sci U S A. 1986;83:5563–7.
84. Casey WM, Keesler GA, Parks LW. Regulation of partitioned sterol biosynthesis in *Saccharomyces cerevisiae*. J Bacteriol. 1992;174:7283–8.
85. Polakowski T, Stahl U, Lang C. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol. 1998;49:66–71.
86. Bard M, Lees ND, Burnett AS, Parker RA. Isolation and characterization of mevinolin resistant mutants of *Saccharomyces cerevisiae*. J Gen Microbiol. 1988;134:1071–8.
87. Song JL, Lyons CN, Holleman S, Oliver BG, White TC. Antifungal activity of fluconazole in combination with lovastatin and their effects on gene expression in the ergosterol and prenylation pathways in *Candida albicans*. Med Mycol. 2003;41:417–25.
88. Lorenz RT, Parks LW. Effects of lovastatin (mevinolin) on sterol levels and on activity of azoles in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. 1990;34:1660–5.
89. Dimster-Denk D, Rine J, Phillips J, Scherer S, Cundiff P, DeBord K, Gilliland D, Hickman S, Jarvis A, Tong L, et al. Comprehensive evaluation of isoprenoid biosynthesis regulation in *Saccharomyces cerevisiae* utilizing the Genome Reporter Matrix(TM). J Lipid Res. 1999;40:850–60.
90. Song JL, White TC. RAM2: an essential gene in the prenylation pathway of *Candida albicans*. Microbiology. 2003;149:249–59.
91. Brutyan RA, McPhie P. On the one-sided action of amphotericin B on lipid bilayer membranes. J Gen Physiol. 1996;107:69–78.
92. Langlet J, Berges J, Caillet J, Demaret JP. Theoretical study of the complexation of amphotericin B with sterols. Biochim Biophys Acta. 1994;1191:79–93.
93. Vazquez JA, Arganoza MT, Boikov D, Yoon S, Sobel JD, Akins RA. Stable phenotypic resistance of *Candida* species to amphotericin B conferred by preexposure to subinhibitory levels of azoles [In Process Citation]. J Clin Microbiol. 1998;36:2690–5.
94. Vazquez JA, Arganoza MT, Vaishampayan JK, Akins RA. In vitro interaction between amphotericin B and azoles in *Candida albicans*. Antimicrob Agents Chemother. 1996;40:2511–6.
95. Lewis RE, Prince RA, Chi J, Kontoyiannis DP. Itraconazole preexposure attenuates the efficacy of subsequent Amphotericin B therapy in a murine model of acute invasive pulmonary Aspergillosis. Antimicrob Agents Chemother. 2002;46:3208–14.
96. Sokol-Anderson M, Sligh Jr JE, Elberg S, Brajtburg J, Kobayashi GS, Medoff G. Role of cell defense against oxidative damage in the resistance of *Candida albicans* to the killing effect of amphotericin B. Antimicrob Agents Chemother. 1988;32:702–5.
97. Kelly SL, Lamb DC, Kelly DE, Loeffler J, Einsele H. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients [letter]. Lancet. 1996;348:1523–4.
98. Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, Schumacher U, Einsele H. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett. 1997;400:80–2.
99. Nolte FS, Parkinson T, Falconer DJ, Dix S, Williams J, Gilmore C, Geller R, Wingard JR. Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. Antimicrob Agents Chemother. 1997;41(1):196–9.
100. O'Keeffe J, Kavanagh K. Adriamycin alters the expression of drug efflux pumps and confers amphotericin B tolerance in *Candida albicans*. Anticancer Res. 2004;24(2A):405–8.
101. Gale EF, Johnson AM, Kerridge D, Koh TY. Factors affecting the changes in amphotericin sensitivity of *Candida albicans* during growth. J Gen Microbiol. 1975;87:20–36.
102. Gale EF, Ingram J, Kerridge D, Notario V, Wayman F. Reduction of amphotericin resistance in stationary phase cultures of *Candida albicans* by treatment with enzymes. J Gen Microbiol. 1980;117:383–91.
103. Gale EF, Johnson AM, Kerridge D, Wayman F. Phenotypic resistance to miconazole and amphotericin B in *Candida albicans*. J Gen Microbiol. 1980;117:535–8.
104. Cassone A, Kerridge D, Gale EF. Ultrastructural changes in the cell wall of *Candida albicans* following cessation of growth and their possible relationship to the development of polyene resistance. J Gen Microbiol. 1979;110:339–49.
105. Yoon SA, Vazquez JA, Steffan PE, Sobel JD, Akins RA. High-frequency, in vitro reversible switching of *Candida lusitaniae* clinical isolates from amphotericin B susceptibility to resistance. Antimicrob Agents Chemother. 1999;43:836–45.
106. Seo K, Akiyoshi H, Ohnishi Y. Alteration of cell wall composition leads to amphotericin B resistance in *Aspergillus flavus*. Microbiol Immunol. 1999;43(11):1017–25.
107. Barker KS, Crisp S, Wiederhold N, Lewis RE, Bareither B, Eckstein J, Barbuch R, Bard M, Rogers PD. Genome-wide expression profiling reveals genes associated with amphotericin B and

fluconazole resistance in experimentally induced antifungal resistant isolates of *Candida albicans*. J Antimicrob Chemother. 2004;54:376–85.
108. Dimster-Denk D, Thorsness MK, Rine J. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol Biol Cell. 1994;5:655–65.
109. Soustre I, Dupuy PH, Silve S, Karst F, Loison G. Sterol metabolism and ERG2 gene regulation in the yeast *Saccharomyces cerevisiae*. FEBS Lett. 2000;470(2):102–6.
110. Kennedy MA, Barbuch R, Bard M. Transcriptional regulation of the squalene synthase gene (ERG9) in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta. 1999;1445:110–22.
111. Kennedy MA, Bard M. Positive and negative regulation of squalene synthase (ERG9), an ergosterol biosynthetic gene, in *Saccharomyces cerevisiae*. Biochim Biophys Acta. 2001;1517(2):177–89.
112. Arthington-Skaggs BA, Crowell DN, Yang H, Sturley SL, Bard M. Positive and negative regulation of a sterol biosynthetic gene (ERG3) in the post-squalene portion of the yeast ergosterol pathway. FEBS Lett. 1996;392:161–5.
113. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, Nickerson KW. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl Environ Microbiol. 2001;67:2982–92.
114. Hornby JM, Nickerson KW. Enhanced production of farnesol by *Candida albicans* treated with four azoles. Antimicrob Agents Chemother. 2004;48:2305–7.
115. Hornby JM, Kebaara BW, Nickerson KW. Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. Antimicrob Agents Chemother. 2003;47: 2366–9.
116. Pierson CA, Eckstein J, Barbuch R, Bard M. Ergosterol gene expression in wild-type and ergosterol-deficient mutants of *Candida albicans*. Med Mycol. 2004;42:385–9.
117. Smith WL, Edlind TD. Histone deacetylase inhibitors enhance *Candida albicans* sensitivity to azoles and related antifungals: correlation with reduction in CDR and ERG upregulation. Antimicrob Agents Chemother. 2002;46(11):3532–9.
118. Song JL, Harry JB, Eastman RT, Oliver BG, White TC. The *Candida albicans* lanosterol 14-alpha-demethylase (ERG11) gene promoter is maximally induced after prolonged growth with antifungal drugs. Antimicrob Agents Chemother. 2004;48:1136–44.
119. De Backer MD, Ilyina T, Ma XJ, Vandoninck S, Luyten WH, Vanden Bossche H. Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. Antimicrob Agents Chemother. 2001;45:1660–70.
120. Karababa M, Coste AT, Rognon B, Bille J, Sanglard D. Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. Antimicrob Agents Chemother. 2004;48:3064–79.
121. Cowen LE, Nantel A, Whiteway MS, Thomas DY, Tessier DC, Kohn LM, Anderson JB. Population genomics of drug resistance in *Candida albicans*. Proc Natl Acad Sci U S A. 2002;99(14): 9284–9.
122. Rogers PD, Barker KS. Genome-wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in *Candida albicans* clinical isolates. Antimicrob Agents Chemother. 2003;47(4):1220–7.
123. Barker KS, Pearson MM, Rogers PD. Identification of genes differentially expressed in association with reduced azole susceptibility in *Saccharomyces cerevisiae*. J Antimicrob Chemother. 2003;51:1131–40.
124. Silver PM, Oliver BG, White TC. Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell. 2004;3:1391–7.
125. Henry KW, Nickels JT, Edlind TD. ROX1 and ERG Regulation in *Saccharomyces cerevisiae*: implications for antifungal susceptibility. Eukaryot Cell. 2002;1:1041–4.
126. Lo HJ, Wang JS, Lin CY, Chen CG, Hsiao TY, Hsu CT, Su CL, Fann MJ, Ching YT, Yang YL. Efg1 involved in drug resistance by regulating the expression of ERG3 in *Candida albicans*. Antimicrob Agents Chemother. 2005;49:1213–5.
127. Lewis RE, Lo HJ, Raad II, Kontoyiannis DP. Lack of catheter infection by the efg1/efg1 cph1/cph1 double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. Antimicrob Agents Chemother. 2002;46:1153–5.
128. Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. Nonfilamentous *C. albicans* mutants are avirulent. Cell. 1997;90:939–49.
129. Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. 1997;16:1982–91.
130. van den Hazel HB, Pichler H, do Valle Matta MA, Leitner E, Goffeau A, Daum G. PDR16 and PDR17, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. J Biol Chem. 1999;274(4):1934–41.
131. Prasad R, De Wergifosse P, Goffeau A, Balzi E. Molecular cloning and characterization of a novel gene of *Candida albicans*, CDR1, conferring multiple resistance to drugs and antifungals. Curr Genet. 1995;27:320–9.
132. Krishnamurthy S, Chatterjee U, Gupta V, Prasad R, Das P, Snehlata P, Hasnain SE, Prasad R. Deletion of transmembrane domain 12 of CDR1, a multidrug transporter from *Candida albicans*, leads to altered drug specificity: expression of a yeast multidrug transporter in baculovirus expression system. Yeast. 1998;14:535–50.
133. Niimi M, Niimi K, Takano Y, Holmes AR, Fischer FJ, Uehara Y, Cannon RD. Regulated overexpression of CDR1 in *Candida albicans* confers multidrug resistance. J Antimicrob Chemother. 2004;54:999–1006.
134. Bauer BE, Wolfger H, Kuchler K. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim Biophys Acta. 1999;1461:217–36.
135. Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, et al. The diploid genome sequence of *Candida albicans*. Proc Natl Acad Sci U S A. 2004;101:7329–34.
136. Nakamura K, Niimi M, Niimi K, Holmes AR, Yates JE, Decottignies A, Monk BC, Goffeau A, Cannon RD. Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. Antimicrob Agents Chemother. 2001;45:3366–74.
137. Lopez-Ribot JL, McAtee RK, Lee LN, Kirkpatrick WR, White TC, Sanglard D, Patterson TF. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob Agents Chemother. 1998;42:2932–7.
138. White TC. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother. 1997;41:1488–94.
139. Maebashi K, Niimi M, Kudoh M, Fischer FJ, Makimura K, Niimi K, Piper RJ, Uchida K, Arisawa M, Cannon RD, et al. Mechanisms of fluconazole resistance in *Candida albicans* isolates from Japanese AIDS patients. J Antimicrob Chemother. 2001;47(5):527–36.
140. White TC, Holleman S, Dy F, Mirels LF, Stevens DA. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob Agents Chemother. 2002;46(6):1704–13.

141. Anderson JB, Sirjusingh C, Parsons AB, Boone C, Wickens C, Cowen LE, Kohn LM. Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. Genetics. 2003;163(4):1287–98.
142. Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB, Kohn LM. Evolution of drug resistance in experimental populations of *Candida albicans*. J Bacteriol. 2000;182:1580–91.
143. Balan I, Alarco AM, Raymond M. The *Candida albicans* CDR3 gene codes for an opaque-phase ABC transporter. J Bacteriol. 1997;179(23):7210–8.
144. Franz R, Michel S, Morschhäuser J. A fourth gene from the *Candida albicans* CDR family of ABC transporters. Curr Microbiol. 1998;37:359–61.
145. Jha S, Dabas N, Karnani N, Saini P, Prasad R. ABC multidrug transporter Cdr1p of *Candida albicans* has divergent nucleotide-binding domains which display functional asymmetry. FEMS Yeast Res. 2004;5:63–72.
146. Shukla S, Ambudkar SV, Prasad R. Substitution of threonine-1351 in the multidrug transporter Cdr1p of *Candida albicans* results in hypersusceptibility to antifungal agents and threonine-1351 is essential for synergic effects of calcineurin inhibitor FK520. J Antimicrob Chemother. 2004;54:38–45.
147. Egner R, Bauer BE, Kuchler K. The transmembrane domain 10 of the yeast Pdr5p ABC antifungal efflux pump determines both substrate specificity and inhibitor susceptibility. Mol Microbiol. 2000;35:1255–63.
148. Maesaki S, Marichal P, Vanden Bossche H, Sanglard D, Kohno S. Rhodamine 6G efflux for the detection of CDR1-overexpressing azole-resistant *Candida albicans* strains. J Antimicrob Chemother. 1999;44:27–31.
149. Shukla S, Saini P, Smriti JS, Ambudkar SV, Prasad R. Functional characterization of *Candida albicans* ABC transporter Cdr1p. Eukaryot Cell. 2003;2:1361–75.
150. Gauthier C, Weber S, Alarco AM, Alqawi O, Daoud R, Georges E, Raymond M. Functional similarities and differences between *Candida albicans* Cdr1p and Cdr2p transporters. Antimicrob Agents Chemother. 2003;47(5):1543–54.
151. Moosa MY, Sobel JD, Elhalis H, Du W, Akins RA. Fungicidal activity of fluconazole against *Candida albicans* in a synthetic vagina-simulative medium. Antimicrob Agents Chemother. 2004;48:161–7.
152. Wada S, Niimi M, Niimi K, Holmes AR, Monk BC, Cannon RD, Uehara Y. *Candida glabrata* ATP-binding cassette transporters Cdr1p and Pdh1p expressed in a *Saccharomyces cerevisiae* strain deficient in membrane transporters show phosphorylation-dependent pumping properties. J Biol Chem. 2002;277:46809–21.
153. Wada S, Tanabe K, Yamazaki A, Niimi M, Uehara Y, Niimi K, Lamping E, Cannon RD, Monk BC. Phosphorylation of *Candida glabrata* ATP-binding cassette transporter Cdr1p regulates drug efflux activity and ATPase stability. J Biol Chem. 2005;280:94–103.
154. Sauna ZE, Peng XH, Nandigama K, Tekle S, Ambudkar SV. The molecular basis of the action of disulfiram as a modulator of the multidrug resistance-linked ATP binding cassette transporters MDR1 (ABCB1) and MRP1 (ABCC1). Mol Pharmacol. 2004;65:675–84.
155. Loo TW, Clarke DM. Blockage of drug resistance in vitro by disulfiram, a drug used to treat alcoholism. J Natl Cancer Inst. 2000;92:898–902.
156. Shukla S, Sauna ZE, Prasad R, Ambudkar SV. Disulfiram is a potent modulator of multidrug transporter Cdr1p of *Candida albicans*. Biochem Biophys Res Commun. 2004;322:520–5.
157. Krishnamurthy S, Gupta V, Prasad R, Panwar SL. Expression of CDR1, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones. FEMS Microbiol Lett. 1998;160:191–7.
158. Hernaez ML, Gil C, Pla J, Nombela C. Induced expression of the *Candida albicans* multidrug resistance gene CDR1 in response to fluconazole and other antifungals. Yeast. 1998;14:517–26.
159. Puri N, Krishnamurthy S, Habib S, Hasnain SE, Goswami SK, Prasad R. CDR1, a multidrug resistance gene from *Candida albicans*, contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole. FEMS Microbiol Lett. 1999;180:213–9.
160. Gaur NA, Puri N, Karnani N, Mukhopadhyay G, Goswami SK, Prasad R. Identification of a negative regulatory element which regulates basal transcription of a multidrug resistance gene CDR1 of *Candida albicans*. FEMS Yeast Res. 2004;4:389–99.
161. de Micheli M, Bille J, Schueller C, Sanglard D. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Mol Microbiol. 2002;43(5):1197–214.
162. Karnani N, Gaur NA, Jha S, Puri N, Krishnamurthy S, Goswami SK, Mukhopadhyay G, Prasad R. SRE1 and SRE2 are two specific steroid-responsive modules of *Candida* drug resistance gene 1 (CDR1) promoter. Yeast. 2004;21:219–39.
163. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters CDR1 and CDR2. Eukaryot Cell. 2004;3:1639–52.
164. Chen CG, Yang YL, Shih HI, Su CL, Lo HJ. CaNdt80 is involved in drug resistance in *Candida albicans* by regulating CDR1. Antimicrob Agents Chemother. 2004;48:4505–12.
165. Alarco AM, Balan I, Talibi D, Mainville N, Raymond M. AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires FLR1 encoding a transporter of the major facilitator superfamily. J Biol Chem. 1997;272(31):19304–13.
166. Alarco AM, Raymond M. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. J Bacteriol. 1999;181(3):700–8.
167. Delaveau T, Delahodde A, Carvajal E, Subik J, Jacq C. PDR3, a new yeast regulatory gene, is homologous to PDR1 and controls the multidrug resistance phenomenon. Mol Gen Genet. 1994;244(5):501–11.
168. Katzmann DJ, Burnett PE, Golin J, Mahe Y, Moye-Rowley WS. Transcriptional control of the yeast PDR5 gene by the PDR3 gene product. Mol Cell Biol. 1994;14(7):4653–61.
169. Balzi E, Chen W, Ulaszewski S, Capieaux E, Goffeau A. The multidrug resistance gene PDR1 from *Saccharomyces cerevisiae*. J Biol Chem. 1987;262(35):16871–9.
170. Meyers S, Schauer W, Balzi E, Wagner M, Goffeau A, Golin J. Interaction of the yeast pleiotropic drug resistance genes PDR1 and PDR5. Curr Genet. 1992;21(6):431–6.
171. Balzi E, Wang M, Leterme S, Van Dyck L, Goffeau A. PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. J Biol Chem. 1994;269(3):2206–14.
172. Talibi D, Raymond M. Isolation of a putative *Candida albicans* transcriptional regulator involved in pleiotropic drug resistance by functional complementation of a pdr1 pdr3 mutation in *Saccharomyces cerevisiae*. J Bacteriol. 1999;181(1):231–40.
173. Yang X, Talibi D, Weber S, Poisson G, Raymond M. Functional isolation of the *Candida albicans* FCR3 gene encoding a bZip transcription factor homologous to *Saccharomyces cerevisiae* Yap3p. Yeast. 2001;18(13):1217–25.
174. Hallstrom TC, Katzmann DJ, Torres RJ, Sharp WJ, Moye-Rowley WS. Regulation of transcription factor Pdr1p function by an Hsp70 protein in *Saccharomyces cerevisiae*. Mol Cell Biol. 1998;18(3):1147–55.
175. Kim DY, Song WY, Yang YY, Lee Y. The role of PDR13 in tolerance to high copper stress in budding yeast. FEBS Lett. 2001;508:99–102.

176. Hallstrom TC, Moye-Rowley WS. Hyperactive forms of the Pdr1p transcription factor fail to respond to positive regulation by the hsp70 protein Pdr13p. Mol Microbiol. 2000;36(2):402–13.
177. Michimoto T, Aoki T, Toh-e A, Kikuchi Y. Yeast Pdr13p and Zuo1p molecular chaperones are new functional Hsp70 and Hsp40 partners. Gene. 2000;257:131–7.
178. Eisenman HC, Craig EA. Activation of pleiotropic drug resistance by the J-protein and Hsp70-related proteins, Zuo1 and Ssz1. Mol Microbiol. 2004;53:335–44.
179. Aleman C, Annereau JP, Liang XJ, Cardarelli CO, Taylor B, Yin JJ, Aszalos A, Gottesman MM. P-glycoprotein, expressed in multidrug resistant cells, is not responsible for alterations in membrane fluidity or membrane potential. Cancer Res. 2003;63:3084–91.
180. Luker GD, Pica CM, Kumar AS, Covey DF, Piwnica-Worms D. Effects of cholesterol and enantiomeric cholesterol on P-glycoprotein localization and function in low-density membrane domains. Biochemistry. 2000;39:7651–61.
181. Troost J, Lindenmaier H, Haefeli WE, Weiss J. Modulation of cellular cholesterol alters P-glycoprotein activity in multidrug-resistant cells. Mol Pharmacol. 2004;66:1332–9.
182. Ghetie MA, Marches R, Kufert S, Vitetta ES. An anti-CD19 antibody inhibits the interaction between P-glycoprotein (P-gp) and CD19, causes P-gp to translocate out of lipid rafts, and chemosensitizes a multidrug-resistant (MDR) lymphoma cell line. Blood. 2004;104:178–83.
183. Malinska K, Malinsky J, Opekarova M, Tanner W. Distribution of Can1p into stable domains reflects lateral protein segregation within the plasma membrane of living *S. cerevisiae* cells. J Cell Sci. 2004;117:6031–41.
184. Li Y, Prinz WA. ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulated sterol movement from the plasma membrane to the endoplasmic reticulum. J Biol Chem. 2004;279:45226–34.
185. Hearn JD, Lester RL, Dickson RC. The uracil transporter Fur4p associates with lipid rafts. J Biol Chem. 2003;278:3679–86.
186. Bagnat M, Chang A, Simons K. Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. Mol Biol Cell. 2001;12:4129–38.
187. Bagnat M, Keranen S, Shevchenko A, Shevchenko A, Simons K. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc Natl Acad Sci U S A. 2000;97:3254–9.
188. Moffett S, Brown DA, Linder ME. Lipid-dependent targeting of G proteins into rafts. J Biol Chem. 2000;275:2191–8.
189. Malinska K, Malinsky J, Opekarova M, Tanner W. Visualization of protein compartmentation within the plasma membrane of living yeast cells. Mol Biol Cell. 2003;14:4427–36.
190. Dogra S, Krishnamurthy S, Gupta V, Dixit BL, Gupta CM, Sanglard D, Prasad R. Asymmetric distribution of phosphatidylethanolamine in *C. albicans*: possible mediation by CDR1, a multidrug transporter belonging to ATP binding cassette (ABC) superfamily. Yeast. 1999;15(2):111–21.
191. Smriti Krishnamurthy SS, Prasad R. Membrane fluidity affects functions of Cdr1p, a multidrug ABC transporter of *Candida albicans* [published erratum appears in FEMS Microbiol Lett. 1999;176(1):263]. FEMS Microbiol Lett. 1999;173:475–81.
192. Mukhopadhyay K, Kohli A, Prasad R. Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob Agents Chemother. 2002;46:3695–705.
193. Kohli A, Smriti Mukhopadhyay K, Rattan A, Prasad R. In vitro low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. Antimicrob Agents Chemother. 2002;46:1046–52.
194. Sa-Correia I, Tenreiro S. The multidrug resistance transporters of the major facilitator superfamily, 6 years after disclosure of *Saccharomyces cerevisiae* genome sequence. J Biotechnol. 2002;98:215–26.
195. Ben-Yaacov R, Knoller S, Caldwell GA, Becker JM, Koltin Y. *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. Antimicrob Agents Chemother. 1994;38:648–52.
196. Fling ME, Kopf J, Tamarkin A, Gorman JA, Smith HA, Koltin Y. Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. Mol Gen Genet. 1991;227:318–29.
197. Kohli A, Gupta V, Krishnamurthy S, Hasnain SE, Prasad R. Specificity of drug transport mediated by CaMDR1: a major facilitator of *Candida albicans*. J Biosci. 2001;26:333–9.
198. Morschhauser J, Michel S, Staib P. Sequential gene disruption in *Candida albicans* by FLP-mediated site-specific recombination. Mol Microbiol. 1999;32(3):547–56.
199. Calabrese D, Bille J, Sanglard D. A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. Microbiology. 2000;146(Pt 11):2743–54.
200. Gupta V, Kohli A, Krishnamurthy S, Puri N, Aalamgeer SA, Panwar S, Prasad R. Identification of polymorphic mutant alleles of CaMDR1, a major facilitator of *Candida albicans* which confers multidrug resistance, and its in vitro transcriptional activation. Curr Genet. 1998;34:192–9.
201. Wirsching S, Michel S, Kohler G, Morschhauser J. Activation of the multiple drug resistance gene MDR1 in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a trans-regulatory factor. J Bacteriol. 2000;182(2):400–4.
202. Hooshdaran MZ, Barker KS, Hilliard GM, Kusch H, Morschhauser J, Rogers PD. Proteomic analysis of azole resistance in *Candida albicans* clinical isolates. Antimicrob Agents Chemother. 2004;48:2733–5.
203. Kusch H, Biswas K, Schwanfelder S, Engelmann S, Rogers PD, Hecker M, Morschhauser J. A proteomic approach to understanding the development of multidrug-resistant *Candida albicans* strains. Mol Genet Genomics. 2004;271:554–65.
204. Krcmery V, Huttova M, Mateicka F, Laho L, Jurga L, Ondrusova A, Tarekova Z, Kralinsky K, Hanzen J, Liskova A, et al. Breakthrough fungaemia in neonates and infants caused by *Candida albicans* and *Candida parapsilosis* susceptible to fluconazole in vitro. J Antimicrob Chemother. 2001;48:521–5.
205. Sobel JD, Zervos M, Reed BD, Hooton T, Soper D, Nyirjesy P, Heine MW, Willems J, Panzer H. Fluconazole susceptibility of vaginal isolates obtained from women with complicated *Candida* vaginitis: clinical implications. Antimicrob Agents Chemother. 2003;47(1):34–8.
206. Maxwell MJ, Messer SA, Hollis RJ, Boyken L, Tendolkar S, Diekema DJ, Pfaller MA. Evaluation of Etest method for determining fluconazole and voriconazole MICs for 279 clinical isolates of *Candida* species infrequently isolated from blood. J Clin Microbiol. 2003;41:1087–90.
207. Diekema DJ, Messer SA, Brueggemann AB, Coffman SL, Doern GV, Herwaldt LA, Pfaller MA. Epidemiology of candidemia: 3-year results from the emerging infections and the epidemiology of Iowa organisms study. J Clin Microbiol. 2002;40:1298–302.
208. Pfaller MA, Diekema DJ, Jones RN, Messer SA, Hollis RJ. Trends in antifungal susceptibility of *Candida* spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY Antimicrobial Surveillance Program, 1997 to 2000. J Clin Microbiol. 2002;40:852–6.
209. Pfaller MA, Diekema DJ, Jones RN, Sader HS, Fluit AC, Hollis RJ, Messer SA. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program. J Clin Microbiol. 2001;39:3254–9.

210. Pfaller MA, Diekema DJ. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. J Clin Microbiol. 2004;42:4419–31.
211. Pfaller MA, Diekema DJ. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. Clin Microbiol Infect. 2004;10 Suppl 1:11–23.
212. Kovacicova G, Mateicka F, Hanzen J, Liskova A, Sabo A, Szovenyova Z, Chmelik B, Huttova M, Krcmery V. Breakthrough *Candida*emias during empirical therapy with fluconazole in non-cancer and non-HIV adults caused by in vitro-susceptible *Candida* spp.: report of 33 cases. Scand J Infect Dis. 2001;33(10):749–51.
213. Nucci M, Colombo AL. Risk factors for breakthrough candidemia. Eur J Clin Microbiol Infect Dis. 2002;21(3):209–11.
214. Uzun O, Ascioglu S, Anaissie EJ, Rex JH. Risk factors and predictors of outcome in patients with cancer and breakthrough candidemia. Clin Infect Dis. 2001;32(12):1713–7. Epub 2001 May 7.
215. Sobel JD. Treatment of vaginal *Candida* infections. Expert Opin Pharmacother. 2002;3(8):1059–65.
216. Pfaller MA, Rhine-Chalberg J, Redding SW, Smith J, Farinacci G, Fothergill AW, Rinaldi MG. Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. J Clin Microbiol. 1994;32:59–64.
217. Owen DH, Katz DF. A vaginal fluid simulant. Contraception. 1999;59:91–5.
218. Sobel JD, Wiesenfeld HC, Martens M, Danna P, Hooton TM, Rompalo A, Sperling M, Livengood III C, Horowitz B, Von Thron J, et al. Maintenance fluconazole therapy for recurrent vulvovaginal candidiasis. N Engl J Med. 2004;351:876–83.
219. Calvet HM, Yeaman MR, Filler SG. Reversible fluconazole resistance in *Candida albicans*: a potential in vitro model. Antimicrob Agents Chemother. 1997;41:535–9.
220. Marr KA, Lyons CN, Rustad T, Bowden RA, White TC. Rapid, transient fluconazole resistance in *Candida albicans* is associated with increased mRNA levels of CDR [In Process Citation]. Antimicrob Agents Chemother. 1998;42:2645–9.
221. Marr KA, Lyons CN, Rustad TR, Bowden RA, White TC, Rustad T. Rapid, transient fluconazole resistance in *Candida albicans* is associated with increased mRNA levels of CDR [published erratum appears in Antimicrob Agents Chemother. 1999;43(2):438]. Antimicrob Agents Chemother. 1998;42:2584–9.
222. Marr KA, Lyons CN, Ha K, Rustad TR, White TC. Inducible azole resistance associated with a heterogeneous phenotype in *Candida albicans*. Antimicrob Agents Chemother. 2001;45:52–9.
223. Soll DR. High-frequency switching in *Candida albicans*. Clin Microbiol Rev. 1992;5:183–203.
224. Soll DR, Galask R, Isley S, Rao TV, Stone D, Hicks J, Schmid J, Mac K, Hanna C. Switching of *Candida albicans* during successive episodes of recurrent vaginitis. J Clin Microbiol. 1989;27:681–90.
225. Soll DR. High-frequency switching in *Candida albicans* and its relations to vaginal candidiasis. Am J Obstet Gynecol. 1988;158:997–1001.
226. Zhao R, Lockhart SR, Daniels K, Soll DR. Roles of TUP1 in switching, phase maintenance, and phase-specific gene expression in *Candida albicans*. Eukaryot Cell. 2002;1:353–65.
227. Srikantha T, Tsai L, Daniels K, Klar AJ, Soll DR. The histone deacetylase genes HDA1 and RPD3 play distinct roles in regulation of high-frequency phenotypic switching in *Candida albicans*. J Bacteriol. 2001;183:4614–25.
228. Klar AJ, Srikantha T, Soll DR. A histone deacetylation inhibitor and mutant promote colony-type switching of the human pathogen *Candida albicans*. Genetics. 2001;158:919–24.
229. Soll DR. Gene regulation during high-frequency switching in *Candida albicans*. Microbiology. 1997;143(Pt 2):279–88.
230. Soll DR, Morrow B, Srikantha T, Vargas K, Wertz P. Developmental and molecular biology of switching in *Candida albicans*. Oral Surg Oral Med Oral Pathol. 1994;78:194–201.
231. Vargas K, Messer SA, Pfaller M, Lockhart SR, Stapleton JT, Hellstein J, Soll DR. Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. J Clin Microbiol. 2000;38:3595–607.
232. Marr KA, Rustad TR, Rex JH, White TC. The trailing end point phenotype in antifungal susceptibility testing is pH dependent. Antimicrob Agents Chemother. 1999;43:1383–6.
233. Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG, Patterson TF. Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. J Clin Microbiol. 1998;36:153–6.
234. Lee MK, Williams LE, Warnock DW, Arthington-Skaggs BA. Drug resistance genes and trailing growth in *Candida albicans* isolates. J Antimicrob Chemother. 2004;53:217–24.
235. Rex JH, Nelson PW, Paetznick VL, Lozano-Chiu M, Espinel-Ingroff A, Anaissie EJ. Optimizing the correlation between results of testing in vitro and therapeutic outcome in vivo for fluconazole by testing critical isolates in a murine model of invasive candidiasis. Antimicrob Agents Chemother. 1998;42:129–34.
236. Heinisch JJ, Lorberg A, Schmitz HP, Jacoby JJ. The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. Mol Microbiol. 1999;32(4):671–80.
237. Rusnak F, Mertz P. Calcineurin: form and function. Physiol Rev. 2000;80(4):1483–521.
238. Bader T, Bodendorfer B, Schroppel K, Morschhauser J. Calcineurin is essential for virulence in *Candida albicans*. Infect Immun. 2003;71(9):5344–54.
239. Maesaki S, Marichal P, Hossain MA, Sanglard D, Vanden Bossche H, Kohno S. Synergic effects of tactolimus and azole antifungal agents against azole-resistant *Candida albicans* strains. J Antimicrob Chemother. 1998;42(6):747–53.
240. Cruz MC, Goldstein AL, Blankenship JR, Del Poeta M, Davis D, Cardenas ME, Perfect JR, McCusker JH, Heitman J. Calcineurin is essential for survival during membrane stress in *Candida albicans*. EMBO J. 2002;21:546–59.
241. Marchetti O, Moreillon P, Entenza JM, Vouillamoz J, Glauser MP, Bille J, Sanglard D. Fungicidal synergism of fluconazole and cyclosporine in *Candida albicans* is not dependent on multidrug efflux transporters encoded by the CDR1, CDR2, CaMDR1, and FLU1 genes. Antimicrob Agents Chemother. 2003;47:1565–70.
242. Cameron AM, Steiner JP, Roskams AJ, Ali SM, Ronnett GV, Snyder SH. Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca2+ flux. Cell. 1995;83(3):463–72.
243. Onyewu C, Wormley Jr FL, Perfect JR, Heitman J. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of *Candida albicans*. Infect Immun. 2004;72:7330–3.
244. Thevelein JM, de Winde JH. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol Microbiol. 1999;33(5):904–18.
245. Kafadar KA, Cyert MS. Integration of stress responses: modulation of calcineurin signaling in *Saccharomyces cerevisiae* by protein kinase A. Eukaryot Cell. 2004;3:1147–53.
246. do Valle Matta MA, Jonniaux JL, Balzi E, Goffeau A, van den Hazel B. Novel target genes of the yeast regulator Pdr1p: a contribution of the TPO1 gene in resistance to quinidine and other drugs. Gene. 2001;272:111–9.
247. De Deken X, Raymond M. Constitutive activation of the PDR16 promoter in a *Candida albicans* azole-resistant clinical isolate

overexpressing CDR1 and CDR2. Antimicrob Agents Chemother. 2004;48:2700–3.
248. Panwar SL, Krishnamurthy S, Gupta V, Alarco AM, Raymond M, Sanglard D, Prasad R. CaALK8, an alkane assimilating cytochrome P450, confers multidrug resistance when expressed in a hypersensitive strain of *Candida albicans*. Yeast. 2001;18(12): 1117–29.
249. Noel T, Francois F, Paumard P, Chastin C, Brethes D, Villard J. Flucytosine-fluconazole cross-resistance in purine-cytosine permease-deficient *Candida lusitaniae* clinical isolates: indirect evidence of a fluconazole uptake transporter. Antimicrob Agents Chemother. 2003;47(4):1275–84.
250. Albertson GD, Niimi M, Cannon RD, Jenkinson HF. Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob Agents Chemother. 1996;40(12):2835–41.
251. Maebashi K, Kudoh M, Nishiyama Y, Makimura K, Uchida K, Mori T, Yamaguchi H. A novel mechanism of fluconazole resistance associated with fluconazole sequestration in *Candida albicans* isolates from a myelofibrosis patient. Microbiol Immunol. 2002;46(5):317–26.
252. Ghosh M, Shen J, Rosen BP. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A. 1999;96:5001–6.
253. Janbon G, Sherman F, Rustchenko E. Monosomy of a specific chromosome determines L-sorbose utilization: a novel regulatory mechanism in *Candida albicans*. Proc Natl Acad Sci U S A. 1998;95:5150–5.
254. Legrand M, Lephart P, Forche A, Mueller FM, Walsh T, Magee PT, Magee BB. Homozygosity at the MTL locus in clinical strains of *Candida albicans*: karyotypic rearrangements and tetraploid formation. Mol Microbiol. 2004;52:1451–62.
255. Pujol C, Messer SA, Pfaller M, Soll DR. Drug resistance is not directly affected by mating type locus zygosity in *Candida albicans*. Antimicrob Agents Chemother. 2003;47:1207–12.
256. Hersh MN, Ponder RG, Hastings PJ, Rosenberg SM. Adaptive mutation and amplification in *Escherichia coli*: two pathways of genome adaptation under stress. Res Microbiol. 2004;155:352–9.
257. Heidenreich E, Eisler H. Non-homologous end joining dependency of gamma-irradiation-induced adaptive frameshift mutation formation in cell cycle-arrested yeast cells. Mutat Res. 2004;556:201–8.
258. Heidenreich E, Holzmann V, Eisler H. Polymerase zeta dependency of increased adaptive mutation frequencies in nucleotide excision repair-deficient yeast strains. DNA Repair (Amst). 2004;3:395–402.
259. Heidenreich E, Novotny R, Kneidinger B, Holzmann V, Wintersberger U. Non-homologous end joining as an important mutagenic process in cell cycle-arrested cells. EMBO J. 2003;22:2274–83.
260. Loffler J, Kelly SL, Hebart H, Schumacher U, Lass-Florl C, Einsele H. Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol Lett. 1997;151:263–8.
261. Fox TD, Folley LS, Mulero JJ, McMullin TW, Thorsness PE, Hedin LO, Costanzo MC. Analysis and manipulation of yeast mitochondrial genes. Methods Enzymol. 1991;194:149–65.
262. O'Connor RM, McArthur CR, Clark-Walker GD. Respiratory-deficient mutants of Torulopsis *glabrata*, a yeast with circular mitochondrial deoxyribonucleic acid of 6 mu m. J Bacteriol. 1976;126(2):959–68.
263. Kontoyiannis DP. Modulation of fluconazole sensitivity by the interaction of mitochondria and erg3p in *Saccharomyces cerevisiae*. J Antimicrob Chemother. 2000;46:191–7.
264. Sanglard D, Ischer F, Calabrese D, Majcherczyk PA, Bille J. The ATP binding cassette transporter gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents [In Process Citation]. Antimicrob Agents Chemother. 1999;43:2753–65.
265. Brun S, Berges T, Poupard P, Vauzelle-Moreau C, Renier G, Chabasse D, Bouchara JP. Mechanisms of azole resistance in petite mutants of *Candida glabrata*. Antimicrob Agents Chemother. 2004;48(5):1788–96.
266. Defontaine A, Bouchara JP, Declerk P, Planchenault C, Chabasse D, Hallet JN. In-vitro resistance to azoles associated with mitochondrial DNA deficiency in *Candida glabrata*. J Med Microbiol. 1999;48(7):663–70.
267. Hallstrom TC, Moye-Rowley WS. Multiple signals from dysfunctional mitochondria activate the pleiotropic drug resistance pathway in *Saccharomyces cerevisiae*. J Biol Chem. 2000;275(48):37347–56.
268. Bennett JE, Izumikawa K, Marr KA. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. Antimicrob Agents Chemother. 2004;48:1773–7.
269. Zhang X, Moye-Rowley WS. *Saccharomyces cerevisiae* multidrug resistance gene expression inversely correlates with the status of the F(0) component of the mitochondrial ATPase. J Biol Chem. 2001;276(51):47844–52.
270. Sanglard D, Ischer F, Bille J. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. Antimicrob Agents Chemother. 2001;45:1174–83.
271. Kaur R, Castano I, Cormack BP. Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. Antimicrob Agents Chemother. 2004;48:1600–13.
272. Kontoyiannis DP. Efflux-mediated resistance to fluconazole could be modulated by sterol homeostasis in *Saccharomyces cerevisiae*. J Antimicrob Chemother. 2000;46(2):199–203.
273. Geraghty P, Kavanagh K. Disruption of mitochondrial function in *Candida albicans* leads to reduced cellular ergosterol levels and elevated growth in the presence of amphotericin B. Arch Microbiol. 2003;179(4):295–300. Epub 2003 Mar 15.
274. Geraghty P, Kavanagh K. Erythromycin, an inhibitor of mitoribosomal protein biosynthesis, alters the amphotericin B susceptibility of *Candida albicans*. J Pharm Pharmacol. 2003;55:179–84.
275. Gyurko C, Lendenmann U, Troxler RF, Oppenheim FG. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. Antimicrob Agents Chemother. 2000;44:425–7.
276. Arie ZR, Altboum Z, Berdicevsky I, Segal E. Isolation of a petite mutant from a histidine auxotroph of *Candida albicans* and its characterization [In Process Citation]. Mycopathologia. 1998;141:137–42.
277. Arie ZR, Altboum Z, Sandovsky-Losica H, Segal E. Adhesion of *Candida albicans* mutant strains to host tissue. FEMS Microbiol Lett. 1998;163:121–7.
278. Aoki S, Ito-Kuwa S. Induction of petite mutation with acriflavine and elevated temperature in *Candida albicans*. J Med Vet Mycol. 1987;25:269–77.
279. Aoki S, Ito-Kuwa S, Nakamura Y, Masuhara T. Comparative pathogenicity of a wild-type strain and respiratory mutants of *Candida albicans* in mice. Int J Med Microbiol. 1990;273:332–43.
280. Dumitru R, Hornby JM, Nickerson KW. Defined anaerobic growth medium for studying *Candida albicans* basic biology and resistance to eight antifungal drugs. Antimicrob Agents Chemother. 2004;48:2350–4.
281. Ramage G, Bachmann S, Patterson TF, Wickes BL, Lopez-Ribot JL. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. J Antimicrob Chemother. 2002;49(6):973–80.
282. Denning DW. Echinocandin antifungal drugs. Lancet. 2003; 362:1142.
283. Onishi J, Meinz M, Thompson J, Curotto J, Dreikorn S, Rosenbach M, Douglas C, Abruzzo G, Flattery A, Kong L, et al. Discovery of

novel antifungal (1,3)-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother. 2000;44(2):368–77.
284. Kurtz MB, Douglas C, Marrinan J, Nollstadt K, Onishi J, Dreikorn S, Milligan J, Mandala S, Thompson J, Balkovec JM, et al. Increased antifungal activity of L-733,560, a water-soluble, semisynthetic pneumocandin, is due to enhanced inhibition of cell wall synthesis. Antimicrob Agents Chemother. 1994;38:2750–7.
285. Douglas CM, D'Ippolito JA, Shei GJ, Meinz M, Onishi J, Marrinan JA, Li W, Abruzzo GK, Flattery A, Bartizal K, et al. Identification of the FKS1 gene of *Candida albicans* as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother. 1997;41(11):2471–9.
286. Higashiyama Y, Kohno S. Micafungin: a therapeutic review. Expert Rev Anti Infect Ther. 2004;2(3):345–55.
287. Rex JH, Pfaller MA, Walsh TJ, Chaturvedi V, Espinel-Ingroff A, Ghannoum MA, Gosey LL, Odds FC, Rinaldi MG, Sheehan DJ, et al. Antifungal susceptibility testing: practical aspects and current challenges. Clin Microbiol Rev. 2001;14:643–58. table of contents.
288. Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Diekema DJ. Caspofungin activity against clinical isolates of fluconazole-resistant *Candida*. J Clin Microbiol. 2003;41:5729–31.
289. Carver PL. Micafungin. Ann Pharmacother. 2004;38:1707–21.
290. Douglas CM, Foor F, Marrinan JA, Morin N, Nielsen JB, Dahl AM, Mazur P, Baginsky W, Li W, el-Sherbeini M, et al. The *Saccharomyces cerevisiae* FKS1 (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-beta-D-glucan synthase. Proc Natl Acad Sci U S A. 1994;91:12907–11.
291. Douglas CM, Marrinan JA, Li W, Kurtz MB. A *Saccharomyces cerevisiae* mutant with echinocandin-resistant 1,3-beta-D-glucan synthase. J Bacteriol. 1994;176:5686–96.
292. Mazur P, Morin N, Baginsky W, el-Sherbeini M, Clemas JA, Nielsen JB, Foor F. Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. Mol Cell Biol. 1995;15(10):5671–81.
293. Mazur P, Baginsky W. In vitro activity of 1,3-beta-D-glucan synthase requires the GTP-binding protein Rho1. J Biol Chem. 1996;271(24):14604–9.
294. Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, Watanabe T, Levin DE, Ohya Y. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science. 1996;272(5259):279–81.
295. Kurtz MB, Abruzzo G, Flattery A, Bartizal K, Marrinan JA, Li W, Milligan J, Nollstadt K, Douglas CM. Characterization of echinocandin-resistant mutants of *Candida albicans*: genetic, biochemical, and virulence studies. Infect Immun. 1996;64:3244–51.
296. Bachmann SP, Patterson TF, Lopez-Ribot JL. In vitro activity of caspofungin (MK-0991) against *Candida albicans* clinical isolates displaying different mechanisms of azole resistance. J Clin Microbiol. 2002;40:2228–30.
297. Paderu P, Park S, Perlin DS. Caspofungin uptake is mediated by a high-affinity transporter in *Candida albicans*. Antimicrob Agents Chemother. 2004;48:3845–9.
298. Stevens DA, Espiritu M, Parmar R. Paradoxical effect of caspofungin: reduced activity against *Candida albicans* at high drug concentrations. Antimicrob Agents Chemother. 2004;48:3407–11.
299. Osherov N, May GS, Albert ND, Kontoyiannis DP. Overexpression of Sbe2p, a Golgi protein, results in resistance to caspofungin in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. 2002;46(8):2462–9.
300. Markovich S, Yekutiel A, Shalit I, Shadkchan Y, Osherov N. Genomic approach to identification of mutations affecting caspofungin susceptibility in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. 2004;48:3871–6.
301. Lesage G, Sdicu AM, Menard P, Shapiro J, Hussein S, Bussey H. Analysis of beta-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. Genetics. 2004;167:35–49.
302. Waldorf AR, Polak A. Mechanisms of action of 5-fluorocytosine. Antimicrob Agents Chemother. 1983;23(1):79–85.
303. Polak A, Wain WH. The effect of 5-fluorocytosine on the blastospores and hyphae of *Candida albicans*. J Med Microbiol. 1979;12(1):83–97.
304. Kurtz JE, Exinger F, Erbs P, Jund R. New insights into the pyrimidine salvage pathway of *Saccharomyces cerevisiae*: requirement of six genes for cytidine metabolism. Curr Genet. 1999;36(3):130–6.
305. Boeke JD, Trueheart J, Natsoulis G, Fink GR. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. 1987;154:164–75.
306. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics. 1993;134:717–28.
307. Weber S, Polak A. Susceptibility of yeast isolates from defined German patient groups to 5-fluorocytosine. Mycoses. 1992;35(7–8):163–71.
308. Stiller RL, Bennett JE, Scholer HJ, Wall M, Polak A, Stevens DA. Susceptibility to 5-fluorocytosine and prevalence of serotype in 402 *Candida albicans* isolates from the United States. Antimicrob Agents Chemother. 1982;22(3):482–7.
309. Polak A. Combination therapy of experimental candidiasis, cryptococcosis, aspergillosis and wangiellosis in mice. Chemotherapy. 1987;33(5):381–95.
310. Polak A. Combination therapy for systemic mycosis. Infection. 1989;17(4):203–9.
311. Polak A. Combination therapy in systemic mycosis. J Chemother. 1990;2(4):211–7.
312. Polak A. Synergism of polyene antibiotics with 5-fluorocytosine. Chemotherapy. 1978;24(1):2–16.
313. Polak A, Scholer HJ, Wall M. Combination therapy of experimental candidiasis, cryptococcosis and aspergillosis in mice. Chemotherapy. 1982;28(6):461–79.
314. Nobre G, Sobral T, Ferreira AF. In vitro susceptibility to 5-fluorocytosine and nystatin of common clinical yeast isolates. Mycopathologia. 1981;73(1):39–41.
315. Cuenca-Estrella M, Diaz-Guerra TM, Mellado E, Rodriguez-Tudela JL. Flucytosine primary resistance in *Candida* species and Cryptococcus neoformans. Eur J Clin Microbiol Infect Dis. 2001;20(4):276–9.
316. Pfaller MA, Messer SA, Boyken L, Huynh H, Hollis RJ, Diekema DJ. In vitro activities of 5-fluorocytosine against 8,803 clinical isolates of *Candida* spp.: global assessment of primary resistance using National Committee for Clinical Laboratory Standards susceptibility testing methods. Antimicrob Agents Chemother. 2002;46(11):3518–21.
317. Barchiesi F, Arzeni D, Caselli F, Scalise G. Primary resistance to flucytosine among clinical isolates of *Candida* spp. J Antimicrob Chemother. 2000;45(3):408–9.
318. Fasoli MO, Kerridge D, Morris PG, Torosantucci A. 19F nuclear magnetic resonance study of fluoropyrimidine metabolism in strains of *Candida glabrata* with specific defects in pyrimidine metabolism. Antimicrob Agents Chemother. 1990;34(10): 1996–2006.
319. Whelan WL, Beneke ES, Rogers AL, Soll DR. Segregation of 5-fluorocytosine-resistance variants by *Candida albicans*. Antimicrob Agents Chemother. 1981;19(6):1078–81.
320. Whelan WL, Kerridge D. Decreased activity of UMP pyrophosphorylase associated with resistance to 5-fluorocytosine in *Candida albicans*. Antimicrob Agents Chemother. 1984;26(4):570–4.
321. Whelan WL. The genetic basis of resistance to 5-fluorocytosine in *Candida* species and Cryptococcus neoformans. Crit Rev Microbiol. 1987;15(1):45–56.
322. Whelan WL, Markie D, Kwon-Chung KJ. Complementation analysis of resistance to 5-fluorocytosine in *Candida albicans*. Antimicrob Agents Chemother. 1986;29(5):726–9.
323. Dodgson AR, Dodgson KJ, Pujol C, Pfaller MA, Soll DR. Clade-specific flucytosine resistance is due to a single nucleotide change

in the FUR1 gene of *Candida albicans*. Antimicrob Agents Chemother. 2004;48(6):2223–7.
324. Pujol C, Pfaller MA, Soll DR. Flucytosine resistance is restricted to a single genetic clade of *Candida albicans*. Antimicrob Agents Chemother. 2004;48(1):262–6.
325. Hope WW, Tabernero L, Denning DW, Anderson MJ. Molecular mechanisms of primary resistance to flucytosine in *Candida albicans*. Antimicrob Agents Chemother. 2004;48:4377–86.
326. Montplaisir S, Drouhet E, Mercier-Soucy L. Sensitivity and resistance of pathogenic yeasts to 5-fluoropyrimidines. II.—mechanisms of resistance to 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) (author's transl). Ann Microbiol (Paris). 1975;126B(1):41–9.
327. Schmelzle T, Hall MN. TOR, a central controller of cell growth. Cell. 2000;103(2):253–62.
328. Rohde JR, Cardenas ME. Nutrient signaling through TOR kinases controls gene expression and cellular differentiation in fungi. Curr Top Microbiol Immunol. 2004;279:53–72.
329. Cruz MC, Goldstein AL, Blankenship J, Del Poeta M, Perfect JR, McCusker JH, Bennani YL, Cardenas ME, Heitman J. Rapamycin and less immunosuppressive analogs are toxic to *Candida albicans* and *Cryptococcus neoformans* via FKBP12-dependent inhibition of TOR. Antimicrob Agents Chemother. 2001;45(11):3162–70.
330. Schmelzle T, Beck T, Martin DE, Hall MN. Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. Mol Cell Biol. 2004;24(1):338–51.
331. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol. 2000;150:1507–13.
332. Obeid LM, Okamoto Y, Mao C. Yeast sphingolipids: metabolism and biology. Biochim Biophys Acta. 2002;1585(2–3):163–71.
333. Martin SW, Konopka JB. Lipid raft polarization contributes to hyphal growth in *Candida albicans*. Eukaryot Cell. 2004;3(3):675–84.
334. Hashida-Okado T, Ogawa A, Endo M, Yasumoto R, Takesako K, Kato I. AUR1, a novel gene conferring aureobasidin resistance on *Saccharomyces cerevisiae*: a study of defective morphologies in Aur1p-depleted cells. Mol Gen Genet. 1996;251(2):236–44.
335. Heidler SA, Radding JA. The AUR1 gene in *Saccharomyces cerevisiae* encodes dominant resistance to the antifungal agent aureobasidin A (LY295337). Antimicrob Agents Chemother. 1995;39(12):2765–9.
336. Endo M, Takesako K, Kato I, Yamaguchi H. Fungicidal action of aureobasidin A, a cyclic depsipeptide antifungal antibiotic, against *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. 1997;41(3):672–6.
337. Zhong W, Jeffries MW, Georgopapadakou NH. Inhibition of inositol phosphorylceramide synthase by aureobasidin A in *Candida* and *Aspergillus* species. Antimicrob Agents Chemother. 2000;44(3):651–3.
338. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. J Biol Chem. 1988;263:7472–7.
339. Li XS, Reddy MS, Baev D, Edgerton M. *Candida albicans* Ssa1/2p is the cell envelope binding protein for human salivary histatin 5. J Biol Chem. 2003;278:28553–61.
340. Dong J, Vylkova S, Li XS, Edgerton M. Calcium blocks fungicidal activity of human salivary histatin 5 through disruption of binding with *Candida albicans*. J Dent Res. 2003;82:748–52.
341. Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weterings E, Oomen LC, Veerman EC, Amerongen AV, Abee T. The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. J Biol Chem. 1999;274:7286–91.
342. Ruissen AL, Groenink J, Helmerhorst EJ, Walgreen-Weterings E, Van't Hof W, Veerman EC, Nieuw Amerongen AV. Effects of histatin 5 and derived peptides on *Candida albicans*. Biochem J. 2001;356:361–8.
343. Gyurko C, Lendenmann U, Troxler RF, Oppenheim FG. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. Antimicrob Agents Chemother. 2000;44:348–54.
344. Koshlukova SE, Lloyd TL, Araujo MW, Edgerton M. Salivary histatin 5 induces non-lytic release of ATP from *Candida albicans* leading to cell death. J Biol Chem. 1999;274:18872–9.
345. De Smet K, Eberhardt I, Reekmans R, Contreras R. Bax-induced cell death in *Candida albicans*. Yeast. 2004;21:1325–34.
346. Smet KD, Reekmans R, Contreras R. Role of oxidative phosphorylation in histatin 5-induced cell death in *Saccharomyces cerevisiae*. Biotechnol Lett. 2004;26:1781–5.
347. Xu Y, Ambudkar I, Yamagishi H, Swaim W, Walsh TJ, O'Connell BC. Histatin 3-mediated killing of *Candida albicans*: effect of extracellular salt concentration on binding and internalization. Antimicrob Agents Chemother. 1999;43:2256–62.
348. Fitzgerald DH, Coleman DC, O'Connell BC. Binding, internalisation and degradation of histatin 3 in histatin-resistant derivatives of *Candida albicans*. FEMS Microbiol Lett. 2003;220:247–53.
349. Wunder D, Dong J, Baev D, Edgerton M. Human salivary histatin 5 fungicidal action does not induce programmed cell death pathways in *Candida albicans*. Antimicrob Agents Chemother. 2004;48:110–5.
350. Koshlukova SE, Araujo MW, Baev D, Edgerton M. Released ATP is an extracellular cytotoxic mediator in salivary histatin 5-induced killing of *Candida albicans*. Infect Immun. 2000;68:6848–56.
351. Baev D, Rivetta A, Vylkova S, Sun JN, Zeng GF, Slayman CL, Edgerton M. The TRK1 potassium transporter is the critical effector for killing of *Candida albicans* by the cationic protein, Histatin 5. J Biol Chem. 2004;279:55060–72.
352. James G, Butt AM. P2Y and P2X purinoceptor mediated Ca2+ signalling in glial cell pathology in the central nervous system. Eur J Pharmacol. 2002;447:247.
353. Xu YY, Samaranayake YH, Samaranayake LP, Nikawa H. In vitro susceptibility of *Candida* species to lactoferrin. Med Mycol. 1999;37:35–41.
354. Wakabayashi H, Abe S, Teraguchi S, Hayasawa H, Yamaguchi H. Inhibition of hyphal growth of azole-resistant strains of *Candida albicans* by triazole antifungal agents in the presence of lactoferrin-related compounds. Antimicrob Agents Chemother. 1998;42:1587–91.
355. Wakabayashi H, Abe S, Okutomi T, Tansho S, Kawase K, Yamaguchi H. Cooperative anti-*Candida* effects of lactoferrin or its peptides in combination with azole antifungal agents. Microbiol Immunol. 1996;40:821–5.
356. Ueta E, Tanida T, Osaki T. A novel bovine lactoferrin peptide, FKCRRWQWRM, suppresses *Candida* cell growth and activates neutrophils. J Pept Res. 2001;57:240–9.
357. Soukka T, Tenovuo J, Lenander-Lumikari M. Fungicidal effect of human lactoferrin against *Candida albicans*. FEMS Microbiol Lett. 1992;69:223–8.
358. Samaranayake YH, Samaranayake LP, Wu PC, So M. The antifungal effect of lactoferrin and lysozyme on *Candida krusei* and *Candida albicans*. Apmis. 1997;105:875–83.
359. Samaranayake YH, Samaranayake LP, Pow EH, Beena VT, Yeung KW. Antifungal effects of lysozyme and lactoferrin against genetically similar, sequential *Candida albicans* isolates from a human immunodeficiency virus-infected southern Chinese cohort. J Clin Microbiol. 2001;39:3296–302.
360. Nikawa H, Samaranayake LP, Tenovuo J, Pang KM, Hamada T. The fungicidal effect of human lactoferrin on *Candida albicans* and *Candida krusei*. Arch Oral Biol. 1993;38:1057–63.

361. Naidu AS, Chen J, Martinez C, Tulpinski J, Pal BK, Fowler RS. Activated lactoferrin's ability to inhibit *Candida* growth and block yeast adhesion to the vaginal epithelial monolayer. J Reprod Med. 2004;49:859–66.
362. Naidu AS, Fowler RS, Martinez C, Chen J, Tulpinski J. Activated lactoferrin and fluconazole synergism against *Candida albicans* and *Candida glabrata* vaginal isolates. J Reprod Med. 2004;49:800–7.
363. Kuipers ME, de Vries HG, Eikelboom MC, Meijer DK, Swart PJ. Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical *Candida* isolates. Antimicrob Agents Chemother. 1999;43:2635–41.
364. Bellamy W, Wakabayashi H, Takase M, Kawase K, Shimamura S, Tomita M. Killing of *Candida albicans* by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin. Med Microbiol Immunol (Berl). 1993;182:97–105.
365. Kuipers ME, Beljaars L, Van Beek N, De Vries HG, Heegsma J, Van Den Berg JJ, Meijer DK, Swart PJ. Conditions influencing the in vitro antifungal activity of lactoferrin combined with antimycotics against clinical isolates of *Candida*. Impact on the development of buccal preparations of lactoferrin. Apmis. 2002;110:290–8.
366. Lupetti A, Paulusma-Annema A, Welling MM, Senesi S, van Dissel JT, Nibbering PH. *Candida*cidal activities of human lactoferrin peptides derived from the *N* terminus. Antimicrob Agents Chemother. 2000;44:3257–63.
367. Viejo-Diaz M, Andres MT, Fierro JF. Effects of human lactoferrin on the cytoplasmic membrane of *Candida albicans* cells related with its *Candida*cidal activity. FEMS Immunol Med Microbiol. 2004;42:181–5.
368. Viejo-Diaz M, Andres MT, Fierro JF. Modulation of in vitro fungicidal activity of human lactoferrin against *Candida albicans* by extracellular cation concentration and target cell metabolic activity. Antimicrob Agents Chemother. 2004;48:1242–8.
369. Nikawa H, Samaranayake LP, Tenovuo J, Hamada T. The effect of antifungal agents on the in vitro susceptibility of *Candida albicans* to apo-lactoferrin. Arch Oral Biol. 1994;39:921–3.
370. Nikawa H, Samaranayake LP, Hamada T. Modulation of the anti-*Candida* activity of apo-lactoferrin by dietary sucrose and tunicamycin in vitro. Arch Oral Biol. 1995;40:581–4.
371. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol. 2004;75:39–48.
372. Ramanathan B, Davis EG, Ross CR, Blecha F. Cathelicidins: microbicidal activity, mechanisms of action, and roles in innate immunity. Microbes Infect. 2002;4:361–72.
373. Lehrer RI, Ganz T. Cathelicidins: a family of endogenous antimicrobial peptides. Curr Opin Hematol. 2002;9:18–22.
374. Guthmiller JM, Vargas KG, Srikantha R, Schomberg LL, Weistroffer PL, McCray Jr PB, Tack BF. Susceptibilities of oral bacteria and yeast to mammalian cathelicidins. Antimicrob Agents Chemother. 2001;45:3216–9.
375. Klotz SA, Gaur NK, Rauceo J, Lake DF, Park Y, Hahm KS, Lipke PN. Inhibition of adherence and killing of *Candida albicans* with a 23-Mer peptide (Fn/23) with dual antifungal properties. Antimicrob Agents Chemother. 2004;48:4337–41.
376. Lehrer RI, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu Rev Immunol. 1993;11:105–28.
377. Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, et al. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. J Exp Med. 2001;193:1067–76.
378. Peschel A. How do bacteria resist human antimicrobial peptides? Trends Microbiol. 2002;10:179–86.
379. Hammer KA, Carson CF, Riley TV. Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*. J Antimicrob Chemother. 2004;53:1081–5.
380. Pina-Vaz C, Goncalves Rodrigues A, Pinto E, Costa-de-Oliveira S, Tavares C, Salgueiro L, Cavaleiro C, Goncalves MJ, Martinez-de-Oliveira J. Antifungal activity of Thymus oils and their major compounds. J Eur Acad Dermatol Venereol. 2004;18:73–8.
381. Salgueiro LR, Pinto E, Goncalves MJ, Pina-Vaz C, Cavaleiro C, Rodrigues AG, Palmeira A, Tavares C, Costa-de-Oliveira S, Martinez-de-Oliveira J. Chemical composition and antifungal activity of the essential oil of *Thymbra capitata*. Planta Med. 2004;70:572–5.
382. Shin S, Kim JH. Antifungal activities of essential oils from *Thymus quinquecostatus* and *T. magnus*. Planta Med. 2004;70:1090–2.
383. Shin S, Lim S. Antifungal effects of herbal essential oils alone and in combination with ketoconazole against Trichophyton spp. J Appl Microbiol. 2004;97:1289–96.
384. Simic A, Sokovic MD, Ristic M, Grujic-Jovanovic S, Vukojevic J, Marin PD. The chemical composition of some *Lauraceae* essential oils and their antifungal activities. Phytother Res. 2004;18:713–7.
385. Chami N, Chami F, Bennis S, Trouillas J, Remmal A. Antifungal treatment with carvacrol and eugenol of oral candidiasis in immunosuppressed rats. Braz J Infect Dis. 2004;8:217–26.
386. Monk BC, Niimi K, Lin S, Knight A, Kardos TB, Cannon RD, Parshot R, King A, Lun D, Harding DR. Surface-active fungicidal d-Peptide inhibitors of the plasma membrane proton pump that block azole resistance. Antimicrob Agents Chemother. 2005;49:57–70.
387. Klotz SA, Gaur NK, Lake DF, Chan V, Rauceo J, Lipke PN. Degenerate peptide recognition by *Candida albicans* adhesins Als5p and Als1p. Infect Immun. 2004;72:2029–34.
388. Nikawa H, Fukushima H, Makihira S, Hamada T, Samaranayake LP. Fungicidal effect of three new synthetic cationic peptides against *Candida albicans*. Oral Dis. 2004;10:221–8.
389. Park Y, Lee DG, Hahm KS. HP(2-9)-magainin 2(1-12), a synthetic hybrid peptide, exerts its antifungal effect on *Candida albicans* by damaging the plasma membrane. J Pept Sci. 2004;10:204–9.
390. Petraitis V, Petraitiene R, Kelaher AM, Sarafandi AA, Sein T, Mickiene D, Bacher J, Groll AH, Walsh TJ. Efficacy of PLD-118, a novel inhibitor of *Candida* isoleucyl-tRNA synthetase, against experimental oropharyngeal and esophageal candidiasis caused by fluconazole-resistant *C. albicans*. Antimicrob Agents Chemother. 2004;48:3959–67.
391. Ziegelbauer K, Babczinski P, Schonfeld W. Molecular mode of action of the antifungal beta-amino acid BAY 10-8888. Antimicrob Agents Chemother. 1998;42(9):2197–205.
392. Ziegelbauer K. Decreased accumulation or increased isoleucyl-tRNA synthetase activity confers resistance to the cyclic beta-amino acid BAY 10-8888 in *Candida albicans* and *Candida tropicalis*. Antimicrob Agents Chemother. 1998;42(7):1581–6.
393. Capobianco JO, Zakula D, Coen ML, Goldman RC. Anti-*Candida* activity of cispentacin: the active transport by amino acid permeases and possible mechanisms of action. Biochem Biophys Res Commun. 1993;190:1037–44.
394. Jethwaney D, Hofer M, Khaware RK, Prasad R. Functional reconstitution of a purified proline permease from *Candida albicans*: interaction with the antifungal cispentacin. Microbiology. 1997;143(Pt 2):397–404.
395. Konishi M, Nishio M, Saitoh K, Miyaki T, Oki T, Kawaguchi H. Cispentacin, a new antifungal antibiotic. I. Production, isolation, physico-chemical properties and structure. J Antibiot (Tokyo). 1989;42:1749–55.
396. Ben-Josef AM, Manavathu EK, Platt D, Sobel JD. In vitro antifungal activity of CAN-296: a naturally occurring complex carbohydrate. J Antibiot (Tokyo). 1997;50:937–43.
397. Ben-Josef AM, Manavathu EK, Platt D, Sobel JD. Involvement of calcium inhibitable binding to the cell wall in the fungicidal activity of CAN-296. J Antimicrob Chemother. 1999;44:217–22.

398. Ben-Josef AM, Manavathu EK, Platt D, Sobel JD. Proton translocating ATPase mediated fungicidal activity of a novel complex carbohydrate: CAN-296. Eur J Med Res. 2000;5:126.
399. Ben-Josef AM, Cutright JL, Manavathu EK, Sobel JD. CAN-296-P is effective against cutaneous candidiasis in guinea pigs. Int J Antimicrob Agents. 2003;22:168–71.
400. Capa L, Mendoza A, Lavandera JL, Gomez de las Heras F, Garcia-Bustos JF. Translation elongation factor 2 is part of the target for a new family of antifungals. Antimicrob Agents Chemother 1998;42:2694–9.
401. Dominguez JM, Kelly VA, Kinsman OS, Marriott MS, Gomez de las Heras F, Martin JJ. Sordarins: a new class of antifungals with selective inhibition of the protein synthesis elongation cycle in yeasts. Antimicrob Agents Chemother 1998;42:2274–8.
402. Dominguez JM, Martin JJ. Identification of elongation factor 2 as the essential protein targeted by sordarins in *Candida albicans*. Antimicrob Agents Chemother. 1998;42:2279–83.
403. Dominguez JM, Gomez-Lorenzo MG, Martin JJ. Sordarin inhibits fungal protein synthesis by blocking translocation differently to fusidic acid. J Biol Chem. 1999;274:22423–7.
404. Martinez A, Aviles P, Jimenez E, Caballero J, Gargallo-Viola D. Activities of sordarins in experimental models of candidiasis, aspergillosis, and pneumocystosis. Antimicrob Agents Chemother. 2000;44:3389–94.
405. Martinez A, Ferrer S, Santos I, Jimenez E, Sparrowe J, Regadera J, De Las Heras FG, Gargallo-Viola D. Antifungal activities of two new azasordarins, GW471552 and GW471558, in experimental models of oral and vulvovaginal candidiasis in immunosuppressed rats. Antimicrob Agents Chemother. 2001;45:3304–9.
406. Martinez A, Regadera J, Jimenez E, Santos I, Gargallo-Viola D. Antifungal efficacy of GM237354, a sordarin derivative, in experimental oral candidiasis in immunosuppressed rats. Antimicrob Agents Chemother. 2001;45:1008–13.
407. Kamai Y, Kakuta M, Shibayama T, Fukuoka T, Kuwahara S, Jorgensen R, Yates SP, Teal DJ, Nilsson J, Prentice GA, et al. Antifungal activities of R-135853, a sordarin derivative, in experimental candidiasis in mice. Antimicrob Agents Chemother. 2005;49:52–6.
408. Hall RM, Dawson MJ, Jones CA, Roberts AD, Sidebottom PJ, Stead P, Taylor NL. The production of novel sordarin analogues by biotransformation. J Antibiot (Tokyo). 2001;54:948–57.
409. Tanaka M, Moriguchi T, Kizuka M, Ono Y, Miyakoshi S, Ogita T. Microbial hydroxylation of zofimarin, a sordarin-related antibiotic. J Antibiot (Tokyo). 2002;55:437–41.
410. Serrano-Wu MH, St Laurent DR, Chen Y, Huang S, Lam KR, Matson JA, Mazzucco CE, Stickle TM, Tully TP, Wong HS, et al. Sordarin oxazepine derivatives as potent antifungal agents. Bioorg Med Chem Lett. 2002;12:2757–60.
411. Serrano-Wu MH, St Laurent DR, Mazzucco CE, Stickle TM, Barrett JF, Vyas DM, Balasubramanian BN. Oxime derivatives of sordaricin as potent antifungal agents. Bioorg Med Chem Lett. 2002;12:943–6.
412. Daferner M, Mensch S, Anke T, Sterner O. Hypoxysordarin, a new sordarin derivative from *Hypoxylon croceum*. Z Naturforsch C. 1999;54:474–80.
413. Shastry M, Nielsen J, Ku T, Hsu MJ, Liberator P, Anderson J, Schmatz D, Justice MC. Species-specific inhibition of fungal protein synthesis by sordarin: identification of a sordarin-specificity region in eukaryotic elongation factor 2. Microbiology. 2001;147:383–90.
414. Justice MC, Hsu MJ, Tse B, Ku T, Balkovec J, Schmatz D, Nielsen J. Elongation factor 2 as a novel target for selective inhibition of fungal protein synthesis. J Biol Chem. 1998;273:3148–51.
415. Justice MC, Ku T, Hsu MJ, Carniol K, Schmatz D, Nielsen J. Mutations in ribosomal protein L10e confer resistance to the fungal-specific eukaryotic elongation factor 2 inhibitor sordarin. J Biol Chem. 1999;274:4869–75.
416. Gomez-Lorenzo MG, Garcia-Bustos JF. Ribosomal P-protein stalk function is targeted by sordarin antifungals. J Biol Chem. 1998;273:25041–4.
417. Santos C, Rodriguez-Gabriel MA, Remacha M, Ballesta JP. Ribosomal P0 protein domain involved in selectivity of antifungal sordarin derivatives. Antimicrob Agents Chemother. 2004;48:2930–6.
418. Lalioti VS, Perez-Fernandez J, Remacha M, Ballesta JPG. Characterization of interaction sites in the *Saccharomyces cerevisiae* ribosomal stalk components. Mol Microbiol. 2002;46:719–92.
419. Kelly SL, Lamb DC, Kelly DE. Y132H substitution in *Candida albicans* sterol 14alpha-demethylase confers fluconazole resistance by preventing binding to haem. FEMS Microbiol Lett. 1999;180:171–5.
420. Lamb D, Kelly DE, Hagen-Schunck W, Shyadehi AZ, Akhtar M, Lowe DJ, Baldwin BC, Kelly SL. The mutation T315A in *Candida albicans* sterol 14a-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. J Biol Chem. 1997;272:5682–8.
421. Lamb DC, Kelly DE, White TC, Kelly SL. The R467K amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes drug resistance through reduced affinity. Antimicrob Agents Chemother. 2000;44:63–7.
422. White T. The presence of R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14a-demethylase in *Candida albicans*. Antimicrob Agents Chemother. 1997;41:1488–94.
423. Kelly SL, Lamb DC, Loeffler J, Einsele H, Kelly DE. The G464S amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. Biochem Biophys Res Commun. 1999;262:174–9.
424. Loffler J, Kelly SL, Hebart H, Schumacher U, Lass-Florl C, Einsele H. Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. Gene. 1997;192:235–40.
425. Favre B, Didmon M, Ryder NS. Multiple amino acid substitutions in lanosterol 14alpha-demethylase contribute to azole resistance in *Candida albicans* [In Process Citation]. Microbiology. 1999;145:2715–25.
426. Franz R, Michel S, Morschhauser J. A fourth gene from the *Candida albicans* CDR family of ABC transporters. Gene. 1998;220(1–2):91–8.
427. Dickson RC, Lester RL. Sphingolipid functions in *Saccharomyces cerevisiae*. Biochim Biophys Acta. 2002;1583(1):13–25.

Part V

Viral Drug Resistance: Mechanisms

Mechanisms of Resistance of Antiviral Drugs Active Against the Human Herpes Virus

31

Clyde S. Crumpacker II

1 Introduction

The antiviral drugs against the human herpesviruses provided pioneering insights, which have led to the development of the field of antiviral therapy. The first successful use of antiviral drugs to treat any life-threatening viral infection was vidarabine (adenine arabinoside) in 1977 [1]. This was followed by the development of acyclovir as the first specific antiviral drug which required a viral enzyme (thymidine kinase, TK) for activation to a nucleoside triphosphate, which inhibited the viral DNA polymerase and was a chain-terminator of viral DNA elongation [2, 3]. When tested against clinical viral isolates, acyclovir was most effective against those herpesviruses which established latency in neuronal tissue, (HSV-1, HSV-2, VZV) [4] with some activity against EBV, and very little against clinical isolates of CMV in a plaque reduction assay [4, 5]. With the possible exception of influenza A virus and amantadine, this marked the beginnings of antiviral therapy.

From the earliest times, studies of resistance to acyclovir and other antiviral drugs played an essential role in defining the mechanisms of action of antiviral drugs and elucidated key features of the targets of antiviral therapy. This was especially true of the viral DNA polymerase enzyme, since all of the clinically approved drugs against the herpesvirus act on the viral DNA polymerase as the final target. New antiviral drugs directed against other viral targets such as the HSV helicase–primase complex (Pritelivir), and the CMV UL97 phosphoprotein (Maribavir), and terminase (UL56) (Letermovir) are in development. The lipid-associated analogue of cidofovir (Brincidofovir or CMX 001), which targets the CMV DNA polymerase (UL54), is being developed to prevent CMV disease in transplant recipients. In this chapter, we review the mechanisms of resistance of the current antiviral drugs against the human herpesviruses. This will also include experimental drugs, which are currently in development, but are not yet approved for clinical use.

2 Thymidine Kinase Herpes Simplex Virus Type 1 and Type 2

Two viral encoded proteins, the viral thymidine kinase (TK) and DNA polymerase (pol), are the only targets for the acyclic nucleoside analogue of guanosine, acyclovir, and resistance mutations in the genes for these two proteins account for all of the resistance to acyclovir observed in vitro or in a clinical use of acyclovir [6]. Acyclovir is an acyclic nucleoside of guanosine, which is preferentially phosphorylated by the herpes simplex virus thymidine kinase to form acyclovir monophosphate. Human cellular TK enzymes have very little ability to add the initial phosphate group to acyclovir. The human thymidylate kinase enzyme, however, readily adds the second and third phosphate to acyclovir monophosphate to form acyclovir triphosphate. Resistance to acyclovir which is mediated by the viral TK occurs by three mechanisms: (1) selection of a thymidine kinase-deficient mutant; (2) selection of a TK-low producer mutant of herpes simplex; and (3) selection of a mutant which produces an altered thymidine kinase, which is capable of phosphorylation of thymidine, but no longer phosphorylates acyclovir [7, 8].

In clinical use, selection of TK-deficient mutants is the most common mechanism for development of acyclovir-resistant HSV. This was the mechanism described in the first human example of resistance to acyclovir in a human patient in 1982 [9]. Mutations which result in thymidine kinase deficiency or low-producing thymidine kinase mutants can occur in almost any part of the viral thymidine kinase enzyme. The herpes TK gene contains a run of cytosines (c-cord) and guanosine (g-string), which are essential for function, and mutations in

C.S. Crumpacker II, M.D. (✉)
Division of Infectious Diseases, Harvard Medical School, Beth Israel Deaconess Medical Center, 330 Brookline Ave, DA 617, Boston, MA 02215, USA

Harvard Medical School, Boston, MA USA
e-mail: ccrumpac@bidmc.harvard.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_31

this region of the gene occur commonly in clinical isolates of herpes simplex. These homopolymers result in mutational hot spots that mediate thymidine kinase deficiency and resistance to acyclovir [10]. This results in a truncated TK protein with little ability to phosphorylate thymidine. An analysis of the electrophoretic mobility of the herpes simplex TK enzyme obtained from 13 acyclovir-resistant HSV isolates from patients with AIDS revealed that only one of the TK proteins was of full length and others were truncated and severely shortened [11]. The mutant TK, which was of full length, contained a single mutation in a region of the herpes TK known to contain an alpha helix structure and the proline point mutation is likely to break the alpha helix [11]. None of the TK proteins produced any significant thymidine kinase activity. Since a mutation in any part of the HSV TK gene may result in an enzyme, which is able to confer resistance to acyclovir, nucleotide sequencing of the entire viral thymidine kinase gene is required to detect resistance. The report on an altered substrate specificity as a mechanism for acyclovir resistance showed that prolonged acyclovir treatment of mice selected for a mutant was not able to phosphorylate radiolabelled acyclovir, but was clearly able to phosphorylate thymidine to form thymidine monophosphate [8]. The initial demonstration of resistance to acyclovir mediated by the viral TK and DNA polymerase genes was shown in vitro [12, 13] and in mice treated with acyclovir [14]. Single-nucleotide mutations, additions, or deletions in the viral TK can confer resistance to acyclovir by novel mechanisms. A frame-shift mutation resulting from a single C deletion from the homopolymer stretch of 4 C residues in the open reading frame (nucleotides 1061–1064) resulted in a TK polypeptide with a longer amino acid sequence (407 aa). This was isolated from an immunocompromised child and conferred resistance to acyclovir [15]. Earlier it had been shown that a net 1+ frame shift in TK would permit synthesis of thymidine kinase from an ACV-resistant HSV mutant and restore sensitivity of the virus for acyclovir [16]. A complete listing of all the mutation sites in the viral TK which confer resistance to acyclovir is presented in a review article of acyclovir resistance [17].

3 Herpes Simplex Virus DNA Polymerase

The herpes simplex viral DNA polymerase gene encodes a 1235-amino acid peptide, which is able to carry out synthesis of the herpes virus DNA from an origin of replication located in the long, unique region of the herpesvirus genome. The herpes genome contains two origins of replication, one in the long, unique nucleotide sequence region (ORIL), and the other in the short unique sequence region (ORIS), but the origin of replication at ORIL is considered to be the main origin which functions on reactivation from latency [18]. The HSV DNA pol was first cloned and expressed in vitro in a rabbit reticulocyte system and the single pol peptide was functionally able to carry out synthesis of HSV viral DNA by itself [19, 20]. A second protein, the product of gene UL42, is a polymerase accessory protein, which greatly enhances the DNA-synthesizing activity of the HSV pol [21]. The UL42 pol accessory protein binds directly to the HSV DNA polymerase and acts to increase the processivity of the HSV pol [22]. In addition to the HSV DNA polymerase (UL54) and the polymerase-accessory protein (UL42), forming the functional pol complex, five other virally encoded proteins are necessary for replication at the fork of HSV DNA [23]. These include the origin binding protein (OBP, UL9), which binds to the origin of replication ORIL and initiates viral DNA synthesis, the UL30 protein, a single-strand binding protein which keeps the DNA in a single strand from enabling the pol complex to make a complementary strand of HSV DNA. A helicase primase complex consists of three viral proteins, UL5, UL8, and UL52 carrying out the unwinding at the fork of the newly replicating viral DNA [24]. Although these seven viral proteins appear crucial in HSV DNA synthesis, when an antiviral drug such as acyclovir is used to treat herpes infection in either tissue culture or in patients, resistance has only been documented in the viral DNA polymerase gene UL54. The first direct evidence that a drug resistance mutation conferring acyclovir and phosphonoacetic acid resistance in HSV was due to an altered HSV DNA polymerase function was obtained with HSV-1/HSV-2 intertypic recombinant viruses [25]. This study showed that the purified viral DNA polymerase from a drug-resistant virus had greatly altered kinetics for incorporation of nucleotide triphosphate compared to the drug-sensitive HSV polymerase. The cells infected with both sensitive and resistant recombinant viruses produced similar amounts of acyclovir triphosphate, which excluded the viral TK as a source of resistance and indicated that the altered viral DNA polymerase was the cause of the acyclovir and PAA resistance. The complete nucleotide sequence of the HSV DNA polymerase gene was independently reported by two groups [26, 27]. The nucleotide sequence analysis of the HSV DNA polymerase has also revealed the location of amino acids, which are involved in substrate and drug recognition [28].

The herpes simplex polymerase peptide and the polymerase of all the herpesviruses contain an exonuclease domain in the polymerase peptide. This is an important editing function, which enhances the fidelity of viral DNA replication and is able to remove falsely incorporated nucleotides. This editing function plays a major role in the decreased mutation rate of the human herpesviruses compared to the mutation rate observed in RNA viruses, like HIV-1 and influenza A. This exonuclease also contributes to the highly conserved genomes of the human herpesviruses, compared to RNA viruses. In a

project which involved the nucleotide sequence analysis of the CMV DNA polymerase gene from 40 clinical isolates of HCMV from four different locations in the United States, only a 4% incidence of polymorphisms in the CMV DNA polymerase was observed [29]. The viral DNA polymerase therefore is the preferred target of all clinically approved antiviral therapies for the human herpesviruses.

The herpes simplex DNA polymerase gene was also cloned and expressed in yeast [30]. The polymerase expressed in yeast had functional activity, and could be inhibited by the antiviral drug, acyclovir. The herpesvirus DNA polymerase is a member of the class of alpha DNA polymerases, which includes the human DNA polymerase alpha, and the bacteriophage S6 polymerase. All of the human herpesvirus DNA polymerases are closely related and the enzymes possess clusters of highly conserved amino acids [31]. The conserved residues are not randomly distributed but are clustered at specific regions. These domains also appear to have strong sequence homology with domains in the DNA polymerases of vaccinia virus and adenovirus type 2 and bacteriophages Ø29 as well [26, 27, 31–33]. These conserved regions provide a compelling case for their functional importance and they are considered major sites for nucleotide binding and pyrophosphate exchange. The three most highly conserved regions I–III are located in the same linear arrangement on each polypeptide and the distance between the consensus sequences is remarkably similar at around 100 amino acid residues in each case [34].

These regions are designated by roman numerals I through VII. The most highly conserved region I consists of six invariant amino acid residues YGDTDS (884–889), including the aspartate residues DTD, which are essential for nucleotide binding in all RNA and DNA polymerases. To avoid being lethal for viral replication resistance mutations usually occur at sites that are not directly involved in catalysis such as region I. A study employing site-specific mutagenesis of an in vitro-cloned and -expressed active HSV DNA polymerase surprisingly showed that the amino acid glycine adjacent to the DTD complex could be changed to serine, G885S, and still result in an active enzyme [35]. Any change in the DTD amino acids of region I resulted in an inactive enzyme. Another mutation S889A resulted in an acyclovir-resistant polymerase [36].

The drugs phosphonoacetic acid (PAA) and phosphonoformic acid (PFA) are pyrophosphate analogues and very similar in structure (Fig. 31.3). They work by a similar mechanism as competitive inhibitors of pyrophosphate exchange and they bind directly to the viral DNA polymerase [37]. They are not incorporated into elongating DNA and they do not require activation by any viral enzyme. When drug resistance mutations conferring resistance and hypersensitivity to PAA were mapped by marker rescue five of six mutations mapped in regions II and III of the herpes simplex DNA polymerase [34]. These were Ala 719 Val and Ser 724 Asn in region II, and Asn 815 Ser and Gly 841 Ser in region III. Resistance to acyclovir was conferred by mutations in regions II and III and cross-resistance to both PAA and acyclovir was conferred by mutations in region II (Ser 724 Asn). The regions II and III have important functional significance because each of these regions contains the sites of mutations, which confer resistance to acyclovir. All acyclovir-resistant mutations are found in conserved regions of the DNA polymerase, designated I, II, III, V, VII, and A.

The three-dimensional crystal structure of the herpes simplex I DNA polymerase at a 2.7 Å resolution was described for the first time in 2006 [38]. The HSV-1 DNA polymerase has a structural similarity to other alpha polymerases and has permitted construction of high-confidence models of a replication complex of the polymerase and the DNA chain termination of acyclovir. The analysis of the HSV pol structure provides valuable insight into domain functions, the conformational changes required for catalysis, and an enhanced understanding of herpesvirus DNA replication. The structure also permits increased understanding of the relationship of the highly conserved regions of the amino acids to each other. The structure reveals that HSV pol is composed of six structural domains. These six structural domains are a pre-NH_2 domain, and NH_2 domain, $3^{1}5^{1}$ exonuclease domain, and the polymerase palm, finger, and thumb domains. The polymerase exonuclease domain is essential as an editing function for herpes DNA replication to remove falsely incorporated nucleotides and containing conserved regions exo-I, exo-II (region IV), and exo-III (C region). The highly conserved regions III and IV belong to the finger sub-domain; regions I, II, and VII are located in the palm sub-domain; and the thumb sub-domain contains conserved region V. These domains are assembled to form a disklike shape around the central hole with the NH_2 and C termini at opposite sides of the protein. In the crystal structure of the herpes DNA polymerase, the two main regions conferring resistance to acyclovir are region III, the finger sub-domain, and region II, located in the palm sub-domain [34, 38]. The most highly conserved catalytic region I residues 884–889 are also in the palm sub-domain (Fig. 31.1, DNA Pol).

To avoid being lethal, mutations that confer resistance to acyclovir and other nucleosides usually occur at sites that are not directly involved in catalysis such as the invariant YGDTD8 (884–889) of region I. Since acyclovir monophosphate incorporation into the DNA duplex alone does not inhibit HSV pol strongly, it has been postulated that the strong inhibition of HSV pol and formation of a "dead-end suicide complex" are only observed when the next incoming nucleotide is bound to the acyclovir monophosphate-terminated DNA duplex [39]. The side chains of conserved residues in region II (Y722) and region III (T887) are suggested to limit modifications permitting incorporation of

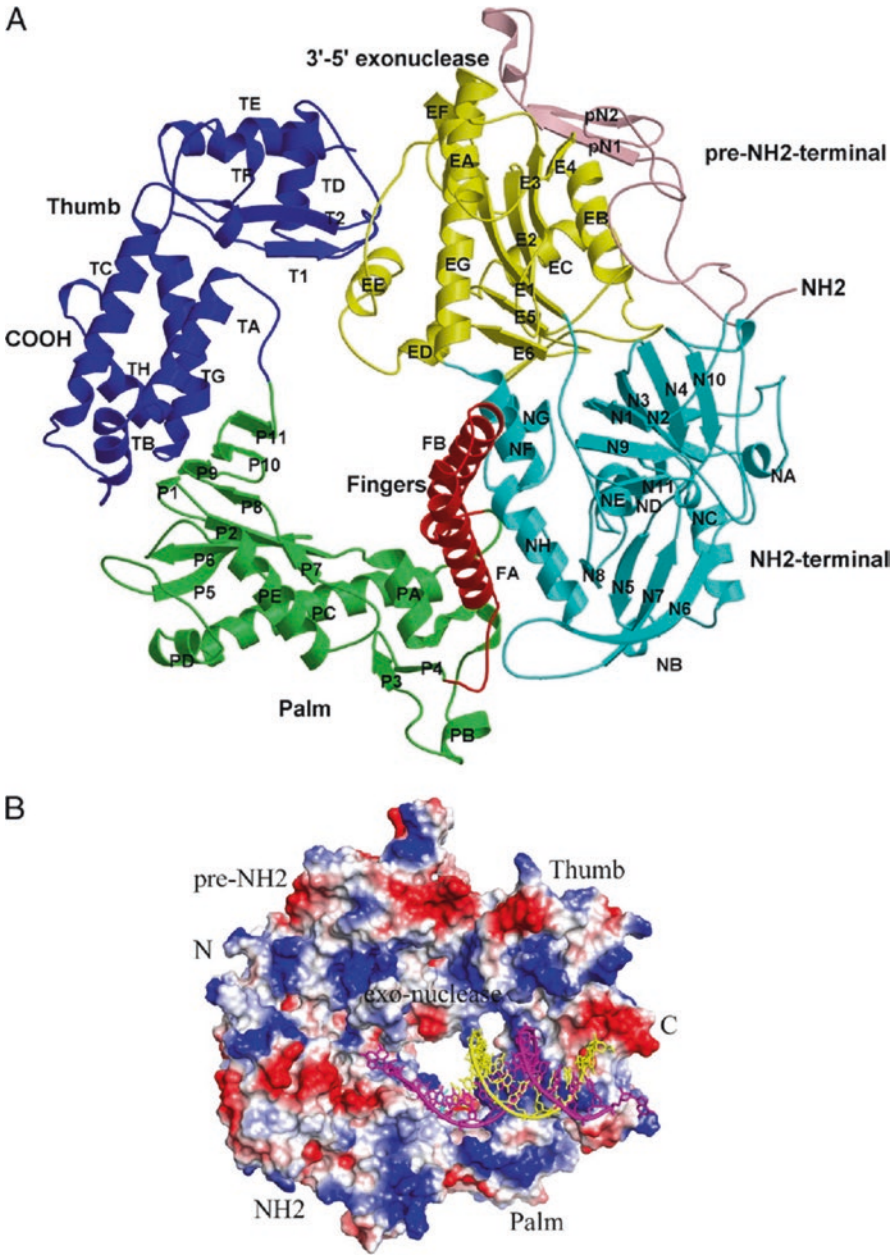


Fig. 31.1 Structure of HSV-1 DNA polymerase in ribbon diagram (**a**) showing the six domains of the polymerase. Finger sub-domain comprises amino acids 767–825 and includes conserved regions III and VI. Palm sub-domian comprises amino acids 701–766 and 826–956 and includes regions I, II, and VIII. Thumb sub-domain comprises amino acids 957–1197 and includes region V, diagram (**b**), which is the back of figure (**a**) [38]

acyclovir monophosphate [38]. Therefore, mutations in regions II and III which confer resistance to acyclovir are likely to prevent incorporation of the acyclovir monophosphate and block the formation of the "dead-end complex" which terminates DNA chain elongation. The 3′→5′ exonuclease domain of HSV contains three highly conserved sequence motifs (Exo I, EX II, Exo III) which maps to the N-terminal half of the enzyme [17]. This 3′→5′ exonuclease has proofreading functions to improve replication fidelity. Mutant polymerase with defective exonuclease activity can have a high mutation frequency. Laboratory strains containing mutated residues (Y577H and D 581A) within the conserved Exo III motif of the polymerase gene were defective in 3′→5′ exonuclease activity and exhibited very high mutation frequency [40]. These mutants also demonstrated higher resistance to PAA and greater sensitivity to ACV and ganciclovir than wild-type virus [41]. These results suggest that the Exo III motif of HSV DNA polymerase may play an important role in maintaining the proper structure of the catalytic site for polymerase activity, in addition to its role in exonuclease activity. A detailed list of all mutations in the HSV DNA polymerase which confer nucleotide analogue resistance is presented in a review article on mechanisms of resistance [17].

4 Penciclovir and Famvir

Penciclovir is a guanosine analogue with a broken sugar ring similar to acyclovir. The oxygen at the two position in the broken sugar ring has been replaced by a carbon and two CH_2OH groups are attached at the end of the broken ring, instead of only one as in acyclovir (Fig. 31.3). The mechanism of action of penciclovir is very similar to acyclovir [42]. The CH_2OH group on the broken sugar ring is phosphorylated by the HSV thymidine kinase. Cellular enzymes add additional phosphate groups to form penciclovir triphosphate. The penciclovir triphosphate binds to the viral DNA polymerase and it is a competitive inhibitor for the incorporation of guanosine triphosphate into elongating DNA. Penciclovir monophosphate is incorporated into elongating DNA and penciclovir is not a chain-terminating drug. Since penciclovir requires a competent viral thymidine kinase for phosphorylation the most common mechanisms of resistance are by selection of thymidine kinase-deficient mutants, which are not able to phosphorylate penciclovir. There is almost complete cross-resistance of thymidine kinase-deficient mutants of HSV to acyclovir and penciclovir. Since the final target of penciclovir triphosphate is the viral DNA polymerase, resistance mutations in the viral DNA polymerase also confer resistance to penciclovir. These resistance mutations in the viral DNA polymerase confer almost complete cross-resistance to both acyclovir and penciclovir, with rare exceptions. Therefore, resistance to acyclovir and penciclovir exhibits a high degree of cross-resistance due to both thymidine kinase and DNA polymerase mutations. Penciclovir is not orally bioavailable, but when it is complexed with two acetate esters, it becomes readily bioavailable to 68 % [37, 43]. This compound is called famciclovir (famvir) and is the oral form of penciclovir. It is readily converted to penciclovir in the plasma by the action of the two esterases, one in the intestinal mucosa of the human small intestine and the other in the liver [42]. Following absorption in the small bowel and one pass through the liver via the portal vein, famvir results in high blood levels of penciclovir. The resistance mechanisms for famvir are identical to penciclovir and are mediated by the viral thymidine kinase and DNA polymerase. One potential antiviral advantage of penciclovir over acyclovir is the high intracellular concentration of penciclovir triphosphate. This concentration persists longer than acyclovir triphosphate and the half time (T ½) of penciclovir triphosphate is 8.5 h compared to 2.5 h for acyclovir triphosphate [42]. The clinical advantage of this persistent high concentration is not clear.

5 Inhibiting the Helicase–Primase Complex

The aminothiazole phenyl-based drug, pritelivir (BAY 57-1293: AIC 316) made by A.I. Curis, is a first-in-class inhibitor of the HSV helicase–primase complex. Pritelivir binds to the complex composed of the gene products of UL5, UL8, and UL52. The helicase–primase inhibitor represents a novel class of HSV inhibitors that are selective in tissue culture [44] and efficacious in animal infection models [45]. Pritelivir, unlike the nucleoside analogues, does not require phosphorylation to be activated and it is protective in uninfected cells. Pritelivir has potent activity in vitro against HSV-1 and HSV-2 and against strains that are resistant to

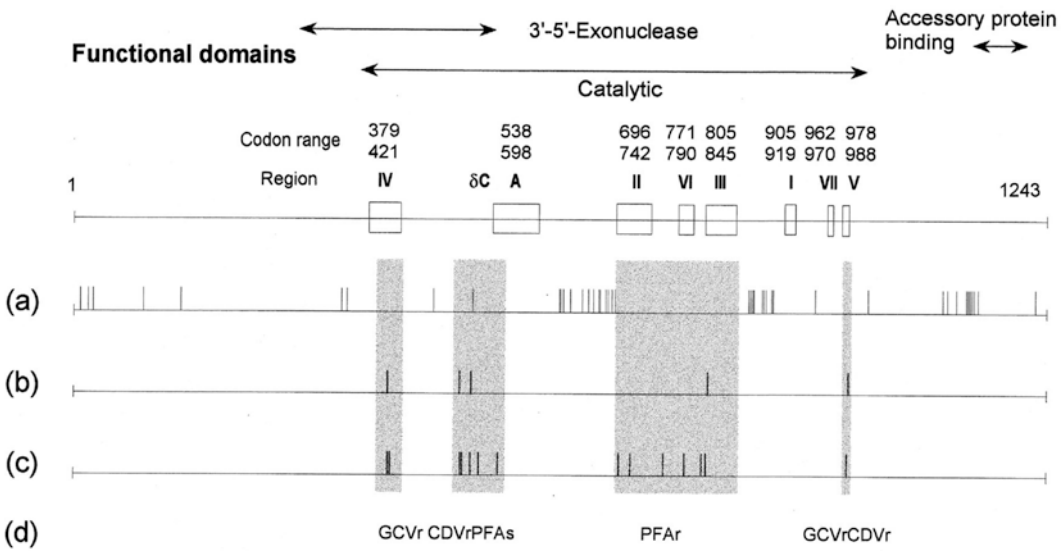


Fig. 31.2 Map of CMV DNA polymerase. CMV DNA polymerase showing functional domains and highly conserved regions of DNA nucleotide sequence (I–VII). *Shaded regions* are associated with drug resistance phenotype. Codons mapped to resistance in clinical isolates are shown as bars. *Abbreviation*: *CDV* cidofovir, *GCV* ganciclovir, *PFA* foscarnet [29]

treatment with nucleoside analogues. HSV DNA replication requires the two proteins comprising the DNA polymerase (UL54 and UL42) and prior to the action of polymerase, a heterotrimeric group of proteins comprising helicase (UL5) and primase (UL8) and an accessory protein (UL52). These proteins act on the HSV double-stranded DNA to open the strands for synthesis of the RNA primase prior to the action of DNA polymerase.

A phase II Clinical Trial of Pritelivir at four doses was compared with placebo for 28 days for the reduction of genital HSV shedding [46]. The primary endpoint was genital HSV DNA shedding and secondary end points included frequency of genital lesions, subclinical shedding, and HSV DNA quantity. The study showed that pritelivir significantly reduced the frequency of genital HSV DNA shedding and lesions in healthy men and women with genital HSV-2 infection. The effect was dose related, with 75 mg daily dose resulting in greatest antiviral effect. Pritelivir also reduced the quantity of HSV in breakthrough shedding by greater than 100-fold. Pritelivir was safe and well tolerated in this 4-week study. No clinical or laboratory abnormalities were observed with daily treatment.

A high frequency of spontaneous helicase–primase drug-resistant variants was reported among laboratory isolates of HSV-1 [47]. Sequence analysis revealed that the majority of the pritelivir resistant variants had amino acid substitutions located close to and downstream of the functional domain IV in the UL5 gene (amino acids 342–350) [47]. Common amino acid substitutions associated with resistance were K356T, K356N, G352V, and M355T. Detection of HSV-1-resistant clinical isolates was also detected in the UL5 gene of two of ten clinical isolates [48]. Both resistant HSV-1 mutants contained the K356N mutation in UL5 and exhibited 5000-fold resistance to pritelivir. The pritelivir-resistant mutants exhibit cross-resistance to another helicase–primase inhibitor (Bils 22 BJ) but remain sensitive to acyclovir.

6 Human Cytomegalovirus

The human cytomegalovirus (HCMV) is the largest virus to infect humans that contains 180–220 open reading frames and is a significant cause of disease in immunocompromised patients. The virus encodes a DNA polymerase enzyme, like all herpesviruses, and has functional domains similar to herpes simplex virus. Ganciclovir, a nucleoside analogue of guanosine, is the mainstay of treatment for cytomegalovirus. Ganciclovir and the highly absorbed prodrug, valganciclovir, are the only orally useful drugs to treat CMV. Ganciclovir is phosphorylated by a viral protein kinase (UL97) and cellular kinases convert this to ganciclovir triphosphate, the active inhibitor of CMV DNA synthesis. Ganciclovir monophosphate is incorporated into elongating CMV DNA, but unlike acyclovir, it is not a chain terminator; CMV DNA synthesis continues at a slow rate and small fragments of CMV DNA encoding the origin of replication in ORIL continue to be made, but the synthesis of full-length CMV DNA is greatly inhibited [49, 50].

7 Resistance to Ganciclovir

Resistant mutations conferring resistance to ganciclovir are found in two viral genes, the viral protein kinase (UL97), which phosphorylates ganciclovir, and the viral polymerase (UL54), which is inhibited by the ganciclovir triphosphate as a competitive inhibitor for nucleotide incorporation into the growing CMV DNA strand. Multiple incorporations of ganciclovir monophosphate near the origin of viral replication greatly slow the action of the viral DNA polymerase. Unlike acyclovir, ganciclovir is not a chain terminator and CMV DNA elongation does not stop completely with ganciclovir monophosphate incorporation [49, 50]. Mutations in the UL97 protein kinase gene in a region of the gene extending from codons 590 to 607 and in two other regions of the gene encoded by the codon 460 or 520 introduce amino acid changes which confer resistance to ganciclovir by blocking phosphorylation [51]. These regions of the protein kinase are probably where ganciclovir binds and is in close approximation to where ATP binds, enabling a phosphate moiety to be transferred from the ATP to the nucleoside analogue ganciclovir. The large majority of resistance with the clinical use of ganciclovir occurs due to mutations in the UL97 gene, which are either single-amino acid mutations at codon 460 or 520 or short deletions in codons from 590 to 607. This results in a protein kinase, which does not effectively phosphorylate ganciclovir. The UL97 protein is an important protein in CMV replication. It is able to participate in phosphorylation of other viral proteins and phosphorylates the UL 44 product, a processivity subunit of the CMV DNA polymerase complex [52]. The UL97 protein is essential for CMV DNA synthesis because it phosphorylates the processivity factor UL44.

8 Maribavir

The UL97 protein is also the target for the antiviral drug maribavir, an L-ribofuranosyl nucleoside, which is a potent inhibitor of CMV replication by inhibiting CMV DNA synthesis (Fig. 31.2). Maribavir strongly inhibits the kinase activity of the viral UL97 [53]. Maribavir also inhibits phosphorylation and accumulation of EBV early antigen D, an essential cofactor in EBV replication [54]. UL97 also phosphorylates a serine in the cell nuclear membrane, causing the

nuclear membrane to develop gaps allowing CMV to exit the cell [55].

When CMV develops resistance to maribavir, mutations are found in the UL97 protein, but in regions, which are distinct from mutations conferring ganciclovir resistance. Passage of laboratory strains of HCMV in the presence of maribavir resulted in a mutation L397R in UL97, which was associated with high-level MBV resistance. Recently, passage of two clinical HCMV isolates in the presence of maribavir, beginning at 0.3 μM and increasing to 15 μM, resulted in two maribavir-resistant viruses with mutations at T409M and V353A of UL97 and a 20-fold increase in the IC50 concentration needed to inhibit CMV replication [43]. When the T409M and V353A mutations were transferred to a CMV laboratory strain, the recombinant viruses also showed a 15- and 80-fold increase, respectively, in maribavir resistance. The mutations at V353A,L397R, T409M and H411Y that influence maribavir binding and susceptibility and confer resistance to maribavir appear to be located upstream of those involved in ganciclovir resistance. These four mutations do not affect ganciclovir susceptibility. The maribavir binding mutations are located in the ATP-binding region of UL97 [56].

9 CMV DNA Polymerase

The human CMV DNA polymerase is the target of all of the licensed drugs for the treatment and prevention of HCMV infection. The crystal structure of the HCMV DNA polymerase has not been determined but it is probably highly similar to the recently elucidated structure for the HSV DNA polymerase. This is because the HCMV DNA polymerase has similar regions of highly conversed amino acids, arranged in a strictly similar relationship to each other, as are observed in the DNA polymerase of the herpes simplex viruses. Following the initial phosphorylation of ganciclovir to ganciclovir monophosphate by the UL97 enzymes, cellular enzymes convert this to ganciclovir triphosphate. Ganciclovir triphosphate is the active competitive inhibitor of the CMV DNA polymerase (UL54).

In the presence of ganciclovir, CMV elongation is greatly slowed, but short fragments of CMV DNA from the origin of replication (ORIL) continue to be synthesized [49, 50]. Ganciclovir monophosphate is incorporated into these short segments and a slow rate of replication continues. The site of binding of ganciclovir monophosphate to CMV DNA polymerase is not clear, but it appears to be distinct from the binding site of phosphonoacetic acid (PAA). In studies on recombinant HSV DNA polymerase, it was observed that ganciclovir and PAA were synergistic against drug-resistant mutants, indicating that these two drugs were able to bind to different regions in the HSV DNA polymerase [43]. Synergistic activity of ganciclovir and foscarnet against CMV has been shown in vitro [57].

The mutations in the CMV DNA polymerase, which confer resistance to ganciclovir, are, with one exception, located in the highly conserved regions of the polymerase enzyme. This is also true for resistance to cidofovir and phosphonoformic acid (PFA), the two other polymerase inhibitors approved for treatment of CMV disease. To assess whether resistance to antiviral drugs is only associated with mutations in these regions or if the mutations in these conserved regions might be attributed to genetic polymorphisms in these regions, a series of 40 clinical isolates of HCMV all sensitive to ganciclovir were analyzed by nucleotide sequencing of the CMV DNA polymerase gene (UL54) [29]. The results showed that there was only a 4% variation in the nucleotide sequence of the CMV polymerase gene. No mutations were detected in the highly conserved regions of the CMV DNA polymerase. Therefore, although a very small amount of genetic polymorphisms are observed in the CMV DNA polymerase, they were not observed in the highly conserved regions of the enzyme. If a mutation is detected in one of the highly conserved regions following use of an antiviral drug, the mutation is almost certainly associated with resistance to the antiviral drug. Therefore, in the use of nucleotide sequence analysis of the CMV DNA polymerase genes to detect drug resistance mutations, a strategy of nucleotide sequencing, which is focused on direct sequencing of these highly conserved regions, provides a rapid approach to detecting drug resistance mutations in human specimens. Cross-resistance to several antiviral drugs, which act on the CMV DNA polymerase protein, can occur with a single mutation in one of the conserved regions of the polymerase peptide. Specifically, this has been noted with resistance to ganciclovir and cidofovir with mutations in conserved regions VII in the CMV DNA polymerase [37, 58]. Cross-resistance to several antiviral drugs can have clinical significance and require phenotypic assays of resistance to reliably determine which alternate antiviral drug should be employed in patients who develop primary resistance to an antiviral drug (Fig. 31.2).

Cross-resistance between ganciclovir and foscarnet has not been observed. In the clinical use of foscarnet (PFA) to treat CMV retinitis in AIDS patients, resistance to PFA has been associated with clinical failure. Resistance mutations to PFA were observed in the clinical isolates from these patients in nucleotides located in regions II, VI, and III of the CMV DNA polymerase [59]. This included foscarnet resistance mutations E756Q (region VI) and V787L (region VI), which were confirmed by marker rescue. All of the foscarnet resistance mutations occur in the shaded region marked PFA_R in Fig. 31.2. Resistance mutations were also observed which conferred resistance to ganciclovir and cidofovir, but not to foscarnet. When a clinical isolate of CMV is highly resistant

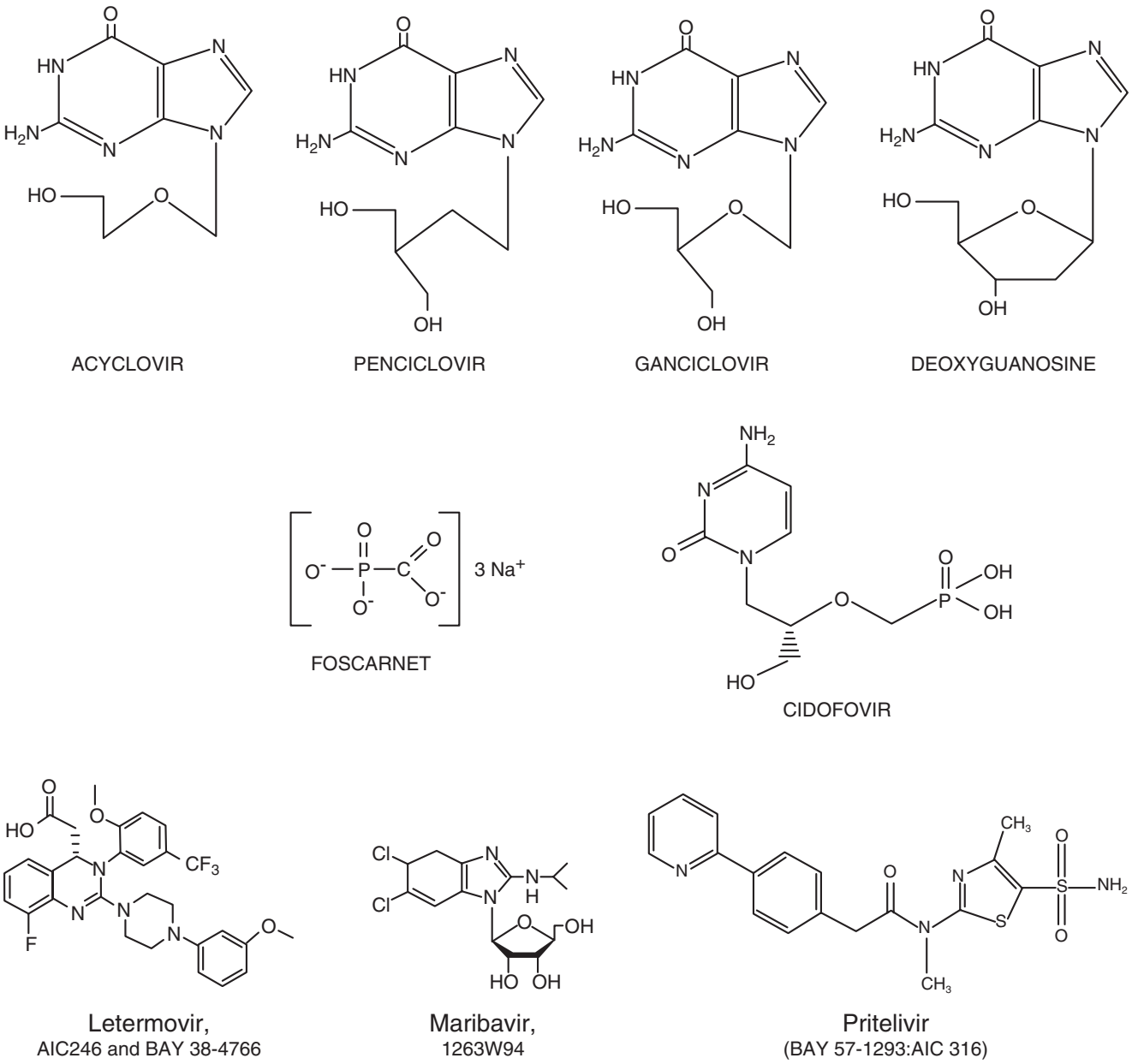


Fig. 31.3 Chemical structure of acyclovir, penciclovir, ganciclovir, the nucleoside deoxyguanosine, foscarnet, cidofovir, maribavir, letermovir, and pritelivir

to ganciclovir (ID50 > 30 μM) and contains mutations in both UL97 and the polymerase genes, cross-resistance to cidofovir may also be observed [52, 58]. These isolates remain sensitive to foscarnet.

10 Letermovir

Letermovir, previously known as AIC246, is a new potent anti-CMV drug with a novel mechanism of action directed at the viral terminase subunit UL56, a component of the terminase complex involved in DNA genome cleavage and packaging [60]. The CMV terminase enzyme has no equivalent target in the human body and this drug should be very safe in humans.

A Phase II study of letermovir prophylaxis in 131 CMV-seropositive transplant recipients of allogeneic hematopoietic cell transplants showed that only 29 % of those who received 240 mg ($N = 234$) daily letermovir for 12 weeks had evidence of CMV replication and virologic failure compared to 64 % who received placebo. The safety profile of letermovir was similar to placebo and no evidence of hematologic

toxicity or nephrotoxicity was observed. If patients were excluded who tested positive for CMV DNA or antigen at screening or at day 1, then no cases of virologic failure occurred in the 240 mg of letermovir daily ($N=30$) group compared to 24 % in the placebo group [61].

A once-daily dose of 120 and 240 mg, when compared with placebo, was effective in preventing CMV replication in recipients of allogeneic hematopoietic cell transplants. In this study, the incidence of virologic failure was much lower in the 240 mg group (6 %), then in the 120 mg group (19 %), the 60 mg group (21 %), and the placebo group (36 %). A follow-up report of this Phase II study showed that the 60 mg per day dose was associated with virologic failure and selected for drug resistance mutations in UL56, the CMV terminase gene, which is the target for letermovir [62]. At a dose of 240 mg per day of letermovir, complete suppression of CMV viremia was noted and emergence of letermovir resistance genotype was restricted. In the patients who received the 60 mg dose, six amino acid mutations were detected in five patients. One subject had the known letermovir resistance mutation V236M and the remaining five sequence varriants (L134V, S227I, Q228H, R410G, and D414N) were shown to be neutral and represented natural polymorphism.

11 Brincidofovir (CMX001)

Brincidofovir (hexadecyloxypropyl cidofovir) is also known as CMX001 (Chimerix). It is a lipid conjugate of cidofovir, acyclic nucleoside phosphonate, that is orally bioavailable, readily absorbed in the small intestine, and transported throughout the body as the phospholipid. It crosses target cell membranes by facilitated and passive diffusion and after cleavage of the lipid moiety it is phosphorylated by cellular kinases to the triphosphate, cidofovir diphosphate. Cidofovir diphospate is a potent inhibitor of CMV DNA polymerase (UL54). CMX001 differs from cidofovir as it is not a substrate for organic ion transporter 1, is not concentrated in proximal renal tubules, and is unlikely to have renal toxicity [63].

In a Phase II study of CMX001 to prevent cytomegalovirus disease in hematopoietic cell transplantation, 230 patients from 27 centers received oral CMX001 or placebo in a dose-escalating, double-blind design for 9–11 weeks after engraftment until week 13 after transplantation. CMV DNA in plasma by PCR analysis was performed weekly, and in patients in whom CMV DNA was detected at a high level, the study drug was discontinued and patients received preemptive treatment against CMV infection. The results showed that patients who received CMX001 at a dose of 100 mg twice weekly had a significantly lower incidence of CMV disease than among patients who received placebo (10 % vs. 37 %, $P=0.002$). Diarrhea was the most common adverse event in patients receiving CMX001 at doses of 200 mg weekly or higher and was dose limiting at a dose of 200 mg twice weekly. Myelosuppression and nephrotoxicity were not observed [64].

To measure resistance to CMX001 in this study, plasma CMV DNA was detected in 30 patients who received CMX001 at doses of 100 mg per week or higher. Nucleic acid sequencing detected a R1052C mutation in the UL54 gene in specimens obtained from three patients. One of these mutations was present before CMX001 exposure. No mutations were detected in UL97. These patients had a response to subsequent preemptive treatment against CMV disease after discontinuation of CMX001.

12 Conclusion

The nucleoside analogues, which inhibit replication of the human herpesviruses, are able to utilize viral encoded kinases to phosphorylate the nucleoside analogue to the monophosphate. This is most notable with acyclovir and the thymidine kinase of herpes simplex virus, varicella zoster virus, and Epstein–Barr virus and ganciclovir and the protein phosphokinase (UL97) of cytomegalovirus and Kaposi's sarcoma herpesvirus. This viral specific kinase provides a great deal of specificity for these nucleoside analogues and prevents cellular toxicity. Cellular kinases convert the monophosphate to the triphosphate of acyclovir or ganciclovir, and the triphosphates are the active inhibitors of viral DNA polymerase. In the clinical use of these nucleoside analogues, the most common mechanism of resistance is the selection of mutants which are defective in the function of HSV viral thymidine kinase TK or the protein phosphokinases (UL97) of cytomegalovirus.

The analysis of the crystal structure of the herpes simplex viral DNA polymerase at a 2.7 Å resolution provides new insights into the mechanisms of resistance to acyclovir. The herpes simplex virus DNA polymerase has a structure with finger and palm domains, remarkably similar to the HIV-1 RT p66 subunit in the "right-hand" model of HIV-1 RT [37]. Acyclovir resistance mutations are found in both the finger sub-domain (region III), similar to the resistance mutations to the nucleosides (AZT, ddI, d4T) in the HIV-1 RT, and in the palm sub-domain (region II), similar to the non-nucleoside RT inhibitors (efavirenz and nevirapine) against HIV-1. Therefore acyclovir monophosphate appears to bind in a significant way to both the finger and the palm domains of the HSV DNA polymerase. A clear function associated with the resistance mutations to acyclovir, such as the enhanced excision of AZT monophosphate with K215Y mutation in HIV RT, has not been shown for acyclovir resistance mutations. The solution of the HSV DNA polymerase crystal structure and the mapping of the acyclovir resistance mutations on the structure reveal the remarkable similarities between the $\propto$ family of DNA polymerases found in all the human herpesviruses

and the HIV-1 RT structure mutations. Ganciclovir and foscarnet resistance in the CMV DNA polymerase occur in different regions of the CMV DNA polymerase, indicating that these drugs bind to different regions of CMV DNA pol. Cross-resistance to ganciclovir and foscarnet has not been observed in clinical treatment of CMV disease.

Resistance to the new drug to prevent CMV disease in the transplant population, maribavir, is located in the ATP-binding region of CMV UL97 [56]. Maribavir inhibits CMV DNA synthesis by preventing phosphorylation of the CMV pol accessory protein UL44 and maribavir blocks the kinase function of UL97.

Resistance mutations to the experimental drug letermovir, to prevent CMV disease in transplant recipients, occurs in the CMV terminase enzyme, UL56, and resistance mutation to the lipid containing analogue of cidofovir, CMX001, or brincidofovir, occurs in the CMV DNA polymerase.

References

1. Whitley RJ, Soong SJ, Dolin R, Galasso GJ, Ch'ien LT, Alford CA. Adenine arabinoside therapy of biopsy-proved herpes simplex encephalitis. National Institute of Allergy and Infectious Diseases collaborative antiviral study. N Engl J Med. 1977;297(6):289–94.
2. Elion GB, Furman PA, Fyfe JA, de Miranda P, Beauchamp L, Schaeffer HJ. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. Proc Natl Acad Sci U S A. 1977;74(12):5716–20.
3. Furman PA, St Clair MH, Fyfe JA, Rideout JL, Keller PM, Elion GB. Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl) guanine and its triphosphate. J Virol. 1979;32(1):72–7.
4. Crumpacker CS, Schnipper LE, Zaia JA, Levin MJ. Growth inhibition by acycloguanosine of herpesviruses isolated from human infections. Antimicrob Agents Chemother. 1979;15(5):642–5.
5. Biron KK, Elion GB. In vitro susceptibility of varicella-zoster virus to acyclovir. Antimicrob Agents Chemother. 1980;18(3):443–7.
6. Dorsky DI, Crumpacker CS. Drugs five years later: acyclovir. Ann Intern Med. 1987;107(6):859–74.
7. Harris W, Collins P, Fenton RJ, Snowden W, Sowa M, Darby G. Phenotypic and genotypic characterization of clinical isolates of herpes simplex virus resistant to aciclovir. J Gen Virol. 2003;84(Pt 6):1393–401.
8. Darby G, Field HJ, Salisbury SA. Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature. 1981;289(5793):81–3.
9. Crumpacker CS, Schnipper LE, Marlowe SI, Kowalsky PN, Hershey BJ, Levin MJ. Resistance to antiviral drugs of herpes simplex virus isolated from a patient treated with acyclovir. N Engl J Med. 1982;306(6):343–6.
10. Sasadeusz JJ, Tufaro F, Safrin S, Schubert K, Hubinette MM, Cheung PK, et al. Homopolymer mutational hot spots mediate herpes simplex virus resistance to acyclovir. J Virol. 1997;71(5):3872–8.
11. Chatis PA, Crumpacker CS. Analysis of the thymidine kinase gene from clinically isolated acyclovir-resistant herpes simplex viruses. Virology. 1991;180(2):793–7.
12. Coen DM, Schaffer PA. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc Natl Acad Sci U S A. 1980;77(4):2265–9.
13. Schnipper LE, Crumpacker CS. Resistance of herpes simplex virus to acycloguanosine: role of viral thymidine kinase and DNA polymerase loci. Proc Natl Acad Sci U S A. 1980;77(4):2270–3.
14. Field HJ, Darby G, Wildy P. Isolation and characterization of acyclovir-resistant mutants of herpes simplex virus. J Gen Virol. 1980;49(1):115–24.
15. Saijo M et al. Nucleotide sequence of thymidine kinase gene of sequential acyclovir-resistant herpes simplex virus type 1 isolates recovered from a child with Wiskott–Aldrich syndrome: evidence for reactivation of acyclovir-resistant herpes simplex virus. J Med Virol. 1999;58(4):387–93.
16. Hwang CB et al. A net +1 frameshift permits synthesis of thymidine kinase from a drug-resistant herpes simplex virus mutant. Proc Natl Acad Sci. 1994;91(12):5461–5.
17. Piret J. Boivin G Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrob Agents Chemother. 2011;55(2):459–72.
18. Hardwicke MA, Schaffer PA. Differential effects of nerve growth factor and dexamethasone on herpes simplex virus type 1 oriL- and oriS-dependent DNA replication in PC12 cells. J Virol. 1997;71(5):3580–7.
19. Dorsky D, Chatis P, Crumpacker C. Functional expression of a cloned herpes simplex virus type 1 DNA polymerase gene. J Virol. 1987;61(5):1704–7.
20. Dorsky DI, Crumpacker CS. Expression of herpes simplex virus type 1 DNA polymerase gene by in vitro translation and effects of gene deletions on activity. J Virol. 1988;62(9):3224–32.
21. Gallo ML, Dorsky DI, Crumpacker CS, Parris DS. The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. J Virol. 1989;63(12):5023–9.
22. Gottlieb J, Marcy AI, Coen DM, Challberg MD. The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. J Virol. 1990;64(12):5976–87.
23. Challberg MD. A method for identifying the viral genes required for herpesvirus DNA replication. Proc Natl Acad Sci U S A. 1986;83(23):9094–8.
24. Crute JJ, Mocarski ES, Lehman IR. A DNA helicase induced by herpes simplex virus type 1. Nucleic Acids Res. 1988;16(14A):6585–96.
25. Knopf KW, Kaufman ER, Crumpacker C. Physical mapping of drug resistance mutations defines an active center of the herpes simplex virus DNA polymerase enzyme. J Virol. 1981;39(3):746–57.
26. Quinn JP, McGeoch DJ. DNA sequence of the region in the genome of herpes simplex virus type 1 containing the genes for DNA polymerase and the major DNA binding protein. Nucleic Acids Res. 1985;13(22):8143–63.
27. Gibbs JS, Chiou HC, Hall JD, Mount DW, Retondo MJ, Weller SK, et al. Sequence and mapping analyses of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. Proc Natl Acad Sci U S A. 1985;82(23):7969–73.
28. Gibbs JS, Chiou HC, Bastow KF, Cheng YC, Coen DM. Identification of amino acids in herpes simplex virus DNA polymerase involved in substrate and drug recognition. Proc Natl Acad Sci U S A. 1988;85(18):6672–6.
29. Chou S, Lurain NS, Weinberg A, Cai GY, Sharma PL, Crumpacker CS. Interstrain variation in the human cytomegalovirus DNA polymerase sequence and its effect on genotypic diagnosis of antiviral drug resistance. Adult AIDS Clinical Trials Group CMV Laboratories. Antimicrob Agents Chemother. 1999;43(6):1500–2.
30. Haffey ML, Stevens JT, Terry BJ, Dorsky DI, Crumpacker CS, Wietstock SM, et al. Expression of herpes simplex virus type 1 DNA

polymerase in Saccharomyces cerevisiae and detection of virus-specific enzyme activity in cell-free lysates. J Virol. 1988;62(12):4493–8.
31. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, et al. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature. 1984;310(5974):207–11.
32. Davison AJ, Scott JE. The complete DNA sequence of varicella-zoster virus. J Gen Virol. 1986;67(Pt 9):1759–816.
33. Kouzarides T, Bankier AT, Satchwell SC, Weston K, Tomlinson P, Barrell BG. Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J Virol. 1987;61(1):125–33.
34. Larder BA, Kemp SD, Darby G. Related functional domains in virus DNA polymerases. EMBO J. 1987;6(1):169–75.
35. Dorsky DI, Crumpacker CS. Site-specific mutagenesis of a highly conserved region of the herpes simplex virus type 1 DNA polymerase gene. J Virol. 1990;64(3):1394–7.
36. Dorsky DI, Plourde C. Resistance to antiviral inhibitors caused by the mutation S889A in the highly-conserved 885-GDTDS motif of the herpes simplex virus type 1 DNA polymerase. Virology. 1993;195(2):831–5.
37. Crumpacker C. Antiviral therapy, Chap 15, p393–434 in Fields Virology, 4th edition 2001 Editors David M. Knipe and Peter M. Howley, by Lippincott, Wiliams and Wilkins, Philadelphia, USA.
38. Liu S, Knafels JD, Chang JS, Waszak GA, Baldwin ET, Deibel Jr MR, et al. Crystal structure of the herpes simplex virus 1 DNA polymerase. J Biol Chem. 2006;281(26):18193–200.
39. Reardon JE, Spector T. Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate. J Biol Chem. 1989;264(13):7405–11.
40. Hwang YT et al. Effects of mutations in the Exo III motif of the herpes simplex virus DNA polymerase gene on enzyme activities, viral replication, and replication fidelity. J Virol. 1997;71(10):7791–8.
41. Hwang YT et al. Mutations in the Exo III motif of the herpes simplex virus DNA polymerase gene can confer altered drug sensitivities. Virology. 1998;246(2):298–305.
42. Vere-Hodges R. Famciclovir and penciclovir: the mode of action of famciclovir including its conversion to penciclovir. Antivir Chem Chemother. 1993;4:67–84.
43. Crumpacker CS, Kowalsky PN, Oliver SA, Schnipper LE, Field AK. Resistance of herpes simplex virus to 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine: physical mapping of drug synergism within the viral DNA polymerase locus. Proc Natl Acad Sci U S A. 1984;81(5):1556–60.
44. Kleymann G et al. New helicase-primase inhibitors as drug candidates for the treatment of herpes simplex disease. Nat Med. 2002;8(4):392–8.
45. Crute JJ et al. Herpes simplex virus helicase-primase inhibitors are active in animal models of human disease. Nat Med. 2002;8(4):386–91.
46. Wald A, Corey L, Timmler B, et al. Helicase-primer inhibitor priteliivir for HSV-2 infection N. Eng. J. Med. 2014;370:201–210
47. Biswas S, Swift M, Field HJ. High Frequency of Spontaneous Helicaseprimase Inhibitor (BAY 57–1293) Drug-Resistant Variants in Certain Laboratory Isolates of HSV-1. Antiviral Chem Chemother. 2007;18(1):13–23.
48. Biswas S, Smith C, Field HJ. Detection of HSV-1 variants highly resistant to the helicase–primase inhibitor BAY 57-1293 at high frequency in 2 of 10 recent clinical isolates of HSV-1. J Antimicrob Chemother. 2007;60(2):274–9.
49. Hamzeh F, Lietman P. Intranuclear accumulation of subgenomic noninfectious human cytomegalovirus DNA in infected cells in the presence of ganciclovir. Antimicrob Agents Chemother. 1991;35(9):1818–23.
50. Hamzeh F, Lietman P, Gibson W, Hayward G. Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination. J Virol. 1990;64(12):6184.
51. Chou S, Waldemer RH, Senters AE, Michels KS, Kemble GW, Miner RC, et al. Cytomegalovirus UL97 phosphotransferase mutations that affect susceptibility to ganciclovir. J Infect Dis. 2002;185(2):162–9.
52. Cherrington JM, Fuller MD, Lamy PD, Miner R, Lalezari JP, Nuessle S, et al. In vitro antiviral susceptibilities of isolates from cytomegalovirus retinitis patients receiving first- or second-line cidofovir therapy: relationship to clinical outcome. J Infect Dis. 1998;178(6):1821–5.
53. Williams SL, Hartline CB, Kushner NL, Harden EA, Bidanset DJ, Drach JC, et al. In vitro activities of benzimidazole D- and L-ribonucleosides against herpesviruses. Antimicrob Agents Chemother. 2003;47(7):2186–92.
54. Gershburg E, Pagano JS. Phosphorylation of the Epstein-Barr virus (EBV) DNA polymerase processivity factor EA-D by the EBV-encoded protein kinase and effects of the L-riboside benzimidazole 1263W94. J Virol. 2002;76(3):998–1003.
55. Hamirally S et al. Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress. PLoS Pathog. 2009;5(1):e1000275.
56. Chou S, Wechel LC, Marousek GI. Cytomegalovirus UL97 kinase mutations that confer maribavir resistance. J Infect Dis. 2007;196(1):91–4.
57. Manischewitz JF, Quinnan Jr GV, Lane HC, Wittek AE. Synergistic effect of ganciclovir and foscarnet on cytomegalovirus replication in vitro. Antimicrob Agents Chemother. 1990;34(2):373–5.
58. Cihlar T, Fuller MD, Cherrington JM. Characterization of drug resistance-associated mutations in the human cytomegalovirus DNA polymerase gene by using recombinant mutant viruses generated from overlapping DNA fragments. J Virol. 1998;72(7):5927–36.
59. Weinberg A, Jabs DA, Chou S, Martin BK, Lurain NS, Forman MS, et al. Mutations conferring foscarnet resistance in a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis. J Infect Dis. 2003;187(5):777–84.
60. Goldner T, Hewlett G, Ettischer N, et al. The novel anticytomegalovirus compound AIC246 (Letermovir) inhibits human cytomegalovirus replication through a specific antiviral mechanism that involves the viral terminase. J Virol. 2011;85:10884–93.
61. Chemaly RF, Ullmann AJ, Stoelben S, et al. Letermovir for cytomegalovirus prophylaxis in hematopoietic-cell transplantation. N Engl J Med. 2014;370:1781–9.
62. Lischka P, Michel D, Zimmermann H. Characterization of cytomegalovirus breakthrough events in a phase 2 prophylaxis trial of letermovir (AIC246, MK 8228). J Infect Dis. 2016;213:23–30.
63. Aldern KA, Ciesla SL, Winegarden KL, Hostetler KY. Increased antiviral activity of 1-O-hexadecyloxypropyl-[2-14C] cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol Pharmacol. 2003;63:678–81.
64. Marty FM et al. CMX001 to prevent cytomegalovirus disease in hematopoietic-cell transplantation. N Engl J Med. 2013;369:1227–36.

Resistance to Influenza Neuraminidase Inhibitors

32

Hui-Ling Yen

1 Introduction

Four competitive NA inhibitors, whose designs are based on the transition state analog Neu5Ac2en at the conserved NA enzyme site, have been approved for prophylaxis and treatment of influenza A and B infections. The structural based design of the NA inhibitors warrants their specific interactions with the highly conserved NA residues critical for the enzymatic activity and viral fitness. Since the approval of oseltamivir and zanamivir in 1999, the drug-resistant variants have been detected at low rate except during 2007–2009 when an oseltamivir-resistant seasonal H1N1 influenza spread globally. To date, resistance variants have been isolated from in vitro studies after serial passages in the presence of NA inhibitors, from patients treated with NA inhibitors, or from patients without prior treatment histories. Mutations that confer resistance to the NA inhibitors were predominantly found at the conserved catalytic or framework residues. Due to differential interactions of conserved NA residues with the modified C4 and C6 side chains on the four inhibitors, some mutations that confer resistance to one drug may not confer resistance to the other.

Global surveillance identified seasonal H1N1 or A(H1N1) pdm09 viruses with the H274Y mutation, H3N2 viruses with the R292K or E119V mutation, and influenza B viruses with the R152K, D198E, or H274Y mutation exhibiting reduced inhibition to one or more NA inhibitors. Epistatic NA mutations may restore NA functionality and viral fitness of resistant variants. Host factors including age and immune status associated with viral load also play important roles in the emergence of NA inhibitor-resistant variants clinically.

H.-L. Yen (✉)
School of Public Health, LKS Faculty of Medicine,
The University of Hong Kong, Pokfulam, Hong Kong
e-mail: hyen@hku.hk

2 Influenza A and B Viruses

Influenza A and B viruses are major causes for respiratory infections in adults and children. In the United States, annual seasonal influenza infections have been associated with an average of 294,128 respiratory and circulatory illness hospitalizations during 1978–2001 [1] and 23,607 influenza-associated death during 1976–2007 [2]. Influenza A viruses have a wide range of hosts and can be classified into different subtypes based on the hemagglutinin (HA) and NA surface glycoproteins. While 16 HA subtypes and 9 NA subtypes have been isolated from the wild aquatic birds [3] and nucleic acid sequences of H17–H18 and N10–N11 being identified from bats [4, 5], pandemic influenza viruses in humans have been restricted to H1N1, H2N2, and H3N2 subtypes since last century. In addition, zoonotic infections by avian (H5N1, H7N9, H9N2, H6N1, H10N8, H5N6) or swine influenza viruses have been reported sporadically at the human–animal interface (WHO). In contrast, influenza B virus predominantly infects humans and has been isolated from seals [6]; there are two lineages (Yamagata and Victoria) that co-circulate under different selection pressures among humans [7].

Influenza A and B viruses are members of the *Orthomyxoviridae* with segmented single-stranded negative-sense RNA genomes. Viral replication requires attachment of HA to terminal sialic acid residues linked to galactose via α2,3- or α2,6-linkages [8] followed by entry of influenza viruses via clathrin-dependent endocytosis and macropinocytosis [9]. Under acidic pH inside the endosome, the HA protein undergoes conformational changes to expose the fusion peptide, which mediates fusion between viral envelope and endosome membrane allowing the release of viral ribonucleoproteins (RNPs) [10]. The M2 ion channels mediate proton flow from the endosome into the interior of the virion to dissociate RNPs from the M1 proteins. The RNPs are transported into the nucleus for viral mRNA synthesis and viral genome replication [11] mediated by the RNA-dependent RNA polymerase PB1 protein; the cap-binding

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_32

activity of the PB2 protein and the endonuclease activity of the PA protein are critical for cap-snatching and mRNA synthesis. The mRNAs are transported to the cytoplasm for protein synthesis and newly synthesized PB1, PB2, PA, NP proteins, and genome segments are packed as RNPs. The HA, NA, and M2 proteins are synthesized at the endoplasmic reticulum and are transported via the trans-Golgi network to the apical cell membrane. The M1 and NEP proteins mediate nucleus exportation of the RNPs to the cytoplasm followed by final assembly and budding from the apical cell membrane [12].

Existing control measures for influenza rely on annual vaccination and two types of antiviral drugs. Adamantanes (amantadine and rimantadine) target the homo-tetrameric proton-conducting ion channel formed by the M2 integral membrane protein of the influenza A viruses. Currently, adamantanes are not recommended for clinical use due to global spread of H3N2-resistant variants reaching 96.4 % in 2005 [13] and the presence of S31N mutation in the M2 protein of the A(H1N1)pdm09 viruses emerged in 2009 that confers resistance to adamatanes [14]. Neuraminidase inhibitors (zanamivir, oseltamivir, peramivir, and laninamivir) effective against both influenza A and B viruses are the mainstay antiviral compounds used clinically. A new viral RNA polymerase inhibitor (favipiravir) has been approved in 2014 in Japan against novel or reemerging influenza virus infections under conditions that other anti-influenza virus drugs are ineffective.

3 Influenza NA as an Antiviral Target

The NA of influenza A and B viruses are homo-tetramers of 240 kDa with sialidase activity that hydrolizes the α-ketosidic linkage between sialic acid and the adjacent oligosaccharide at acidic pH (optimal range of 5.5–6.5) [15]. Each monomer has a mushroom-shaped morphology and contains the N-terminal cytoplasmic sequence, a transmembrane domain, a stalk region, and the globular head domain where the conserved enzymatic site is located [15]. Calcium ions found within each of the active site and at the fourfold axis of the tetramer are needed for the enzymatic activity and stability [15]. In addition to the enzymatic site, avian influenza viruse NA protein may possess a secondary sialic binding site, which was first discovered using purified N9 proteins with hemadsorption activity [16]. Conventionally, the sialidase activity is believed to facilitate the release of newly formed virion. This is supported by the observation that influenza virus with massive deletion in the NA-coding sequence may complete the replication cycle and produce virus progeny but would form aggregates unless exogenous bacterial NA were supplemented in MDCK cells [17]. The sialidase activity has been shown to enhance the HA-binding activity by removing the sialic acids from the newly formed HA [18]. Experimental evidence suggests that the sialidase activity is also needed during the initiation of infection in the human airway epithelium cells due to the presence of mucin [19].

Since the HA and NA proteins both recognize sialyl receptors but with counteracting activities, functional HA–NA balance has been shown to be critical for viral fitness [20]. The HA glycoproteins of influenza A viruses determine host range by exhibiting different binding preferences for α2,3- or α2,6-linked terminal sialic acids; however, the NA glycoprotein of influenza A viruses generally shows higher activity over α2,3-linked sialyl glycans [15]. There is limited knowledge on the substrate specificity of influenza B NA protein.

The NA proteins of influenza A and B viruses share 30 % amino acid sequence homology but are similar in the overall folding and structure [21]. Among N1–N9 proteins of influenza A viruses that possess sialidase activity, phylogenetic analysis allows further separation into structurally similar groups: Group 1 contains N1, N4, N5, and N8, while Group 2 contains N2, N3, N6, N7, and N9 NA proteins [22]. Similar to other sialiadase, the enzymatic site is highly conserved at amino acid residues that directly interact with the sialic acid subtrate (R118, D151, R152, R224, E276, R292, R371, and Y406, N2 numbering); in addition, influenza A and B viruses share conserved amino acids (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, E425) that support the framework of the enzymatic cavity [23]. The α-anomer sialic acid (Neu5Ac) is bound within the enzyme active site in a boat configuration [21, 24]. The carboxylate moiety of the sialic acid interacts with the residues R118, R292, and R371 at the enzyme site; the C4-hydroxyl group interacts with E119; the methyl of the C5 acetamido group makes hydrophobic contacts W178 and I222 while the oxygen forms a hydrogen bond with R152; and the glycerol side chain C8 and C9 form hydrogen bonds to E276 [25].

4 NA Inhibitors

The early development of NA inhibitors initiated in the 1970s when the detailed structural info was not yet available with modifications on the natural inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en, DANA), which is a transition state analog of influenza NA with a K_i of approximately 1 μM. However, these early derivatives of DANA did not possess significantly increased potency [26, 27]. 2-Deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) was shown to inhibit influenza multi-cycle replication in vitro [28] but did not show potent efficacy in vivo [26].

The detailed structural data on the highly conserved active site of Group 2 NA proteins interacting with the sialic acid and Neu5Ac2en allowed the structural based design of the NA inhibitors [29–31]. To improve binding of Neu5Ac2en,

basic substitutions at the four-position were introduced to replace the 4-hydroxyl group leading to the synthesis of 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en (zanamivir) [32]. Substitution of the 4-hydroxyl group by an amino group produced a significant increase in the overall binding interaction due to a salt bridge formation with the side-chain carboxylic acid group of E119. The replacement of the 4-hydroxyl group with the more basic guanidino group produced a tighter affinity due to lateral binding through the terminal nitrogens of the guanidino group with E119 and E227 [25]. In NA enzyme assays, both inhibitors were potent competitive inhibitors with inhibition constants of 5×10^{-8} M for the 4-amino-Neu5Ac2en and 2×10^{-10} M for zanamivir [32]. Zanamivir is administered by oral inhalation due to its poor oral bioavailability. Binding of zanamivir to the NA of different influenza subtypes including A (Groups 1 and 2) and B strains is considered to be similar [22, 33]. Further studies showed zanamivir to possess similar potency to all nine NA subtypes including different human subtypes [34] and against different subtypes of avian origin [34, 35].

The rational drug design approach lead to the development of a second series of potent NA inhibitors that aimed to improve oral bioavailability and are based on noncarbohydrate templates [25] including cyclohexenes such as oseltamivir carboxylate (GS4071) [36], and cyclopentanes such as peramivir (BCX-1812, RWJ-270201) [37]. Oseltamivir was the first orally active NA inhibitor; it is the prodrug that would be metabolized by endogenous esterase to the active form of oseltamivir carboxylate [25]. Oseltamivir carboxylate differs from zanamivir in having a cyclohexene ring structure, a bulky hydrophobic pentyl ether side chain that replaced the glycerol side chain at the six-position, and the 4-amino group [36]. Due to the presence of the hydrophobic substitution at the six-position, residue E276 needs to reorient and form a salt bridge with R224 and results in the formation of a hydrophobic pocket to accommodate the substituent at the six-position [38]. Due to the different drug design, N1 and influenza B NA are more sensitive to zanamivir with lower IC50 values while N2 strains are more sensitive to oseltamivir under enzyme-based NA inhibition assays [39]. The 4-guanidino group has been shown to result in higher potency against Group-1 viruses possessing the cavity formed by the 150-loop [40]. Zanamivir and oseltamivir have been approved for prophylaxis and treatment of acute uncomplicated illness due to influenza A and B infections since 1999.

Peramivir differs from zanamivir and oseltamivir carboxylate by possessing a cyclopentane ring; in addition, it possesses a 4-guanidino group that resembles zanamivir, and a hydrophobic side chain that resembles oseltamivir carboxylate [41]; the design leads to multiple binding interactions of peramivir at the NA enzyme site. Peramivir shows potent inhibitory effect in vitro [42, 43] and efficacy in the mouse and ferret models [44–46]. In humans, the bioavailability via oral dosing was poor and the parenteral formulations via intramuscular (IM) or intravenous (IV) injections were pursued. It was approved in 2010 in Japan and Korea and subsequently approved in 2014 by FDA for treatment of acute uncomplicated influenza in adults 18 years and older given by IV injection [47].

Laninamivir (R-125489) is a long-acting NA inhibitor containing a 4-guanidino group and a 7-methoxy group. The prodrug laninamivir octanoate (CS-8958 or R-118958) is processed into laninamivir in the lungs after being administered through inhalation [48]. A high retention time of the laninamivir in the lungs allows long-lasting anti-influenza activity after a single nasal administration [49]. Laninamivir has been approved in Japan since 2010 for the treatment of influenza A and B infection.

Overall, four competitive NA inhibitors are currently approved for the treatment of influenza infections (Fig. 32.1). They were designed based on the transition state analog of influenza NA, Neu5Ac2en, with different side-chain modifications to improve bioavailability and binding to the NA active site. While these four inhibitors share some common interactions as seen with the Neu5Acen at the NA active site, the differential side-chain modification at C4 and C6 would affect their interactions to some conserved NA residues. This would further affect the resistance profile observed between different inhibitors.

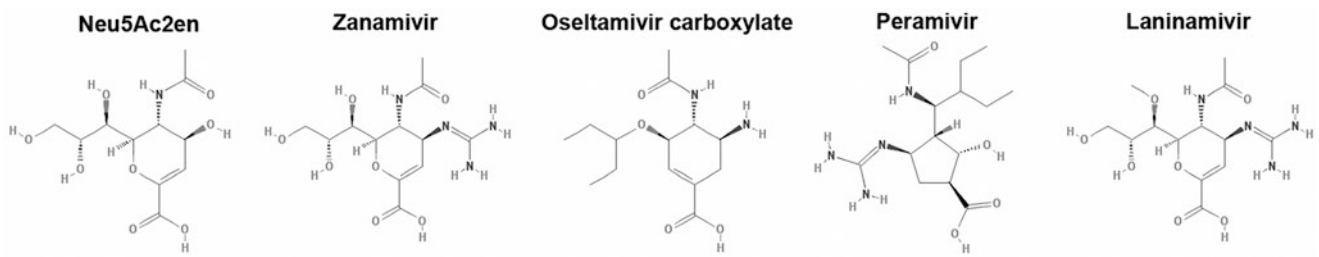


Fig. 32.1 Chemical structures Neu5Ac2en and approved NA inhibitors for treatment of influenza infections. *Source of the images*: PubChem Compound Database, National Center for Biotechnology Information

5 Mechanisms of Resistance

The structural based design of the NA inhibitors warrants their specific binding to highly conserved NA residues critical for the enzymatic activity and viral fitness [31, 32]; this approach aimed to establish the fitness constraints for influenza viruses on the development of resistance to these drugs. To date, resistance variants have been isolated from in vitro studies after serial passages in the presence of NA inhibitors, from patients treated with NA inhibitors, or from patients without prior treatment histories.

Early research activities following on the discovery of zanamivir and oseltamivir focused on assessing the potential for resistance development in vitro. Variants with reduced inhibition to zanamivir or oseltamivir in vitro emerged after serial passages under increasing levels of the inhibitors [50–58]. However, while resistant variants to amantadine or rimantadine can be generated within a few passages in vitro, resistant variants to NA inhibitors require multiple serial passages under increasing concentrations of the drugs. Only one study evaluated the development of resistance to zanamivir in comparison with amantadine in vivo; resistance to amantadine developed rapidly within 6 days during a single course of treatment in ferrets, similar to that observed in the clinic, but no resistance to zanamivir was detected from ferrets after two passages over 18-day treatment [59]. Mapping of the mutations that confer to reduced inhibition to NA inhibitors in vitro found changes in both HA and NA genes. In HA, mutations were found at the proximity of the receptor-binding domain which led to reduced binding affinity of the HA to the sialyl receptors and thereby reduced the viral dependence for the NA activity [55, 58, 60]. While the HA mutations do not confer resistance to NA inhibitors clinically [54], they may still play a role in maintaining a functional balance with NA mutations that are clinically relevant [61].

The NA mutations that confer resistance to the inhibitors were predominantly found at the conserved catalytic or framework residues that determine the interactions with the inhibitors [53, 62]. Due to differential interactions of conserved NA residues with the modified C4 and C6 side chains on the four NA inhibitors, some mutations that confer resistance to one drug may not confer resistance to the other [63]. Generally, mutations at the conserved NA residues that commonly interact with all NA inhibitors may lead to cross-resistance to multiple NA inhibitors; however, this is also subject to the fitness constraint of the particular residue. Although the NA enzymatic site is highly conserved between influenza A Group 1 (N1, N4, N5, N7), Group 2 (N2, N3, N6, N8, N9), and influenza B NA proteins, they differed in the sensitivity to the NA inhibitors. Surveillance studies that routinely monitor global of influenza sensitivity to NA inhibitors suggest that the NA proteins of H1N1 and influenza B viruses are more susceptible for zanamivir than oseltamivir carboxylate by the enzyme-based NA inhibition assay while H3N2 viruses are more susceptible for oseltamivir carboxylate than zanamivir [64–66]. Furthermore, influenza B viruses are generally less susceptible than H1N1 or H3N2 viruses to both zanamivir and oseltamivir [64–66]. It is therefore not surprising that many mutations conferring resistance NA inhibitors are type/subtype specific.

To assess the sensitivity of the virus to NA inhibitors, enzyme-based NA inhibition assay that determines the concentrations of the NA inhibitors that inhibit the NA activity by 50% (IC_{50}) as well as genetic analysis that monitors NA mutations associated with resistance can be applied [67]. The NA inhibition assay may apply methyl umbelliferone N-acetylneuraminic acid (MUNANA) as the substrate for the fluorescent assay or a 1,2-dioxetane derivative of neuraminic acid as substrate for the chemiluminescent assay [67]. Based on the fold changes between the clinical isolate and the subtype specific median IC_{50} values, viral susceptibility to NA inhibitors can be categorized as normal inhibition (<10-fold increase for influenza A and <5-fold for influenza B), reduced inhibition (10–100-fold increase for influenza A and 5–50-fold for influenza B), and highly reduced inhibition (>100-fold increase for influenza A and >50-fold increase for influenza B) [68].

Prior to the introduction of the NA inhibitors into the clinic, no naturally occurring resistant variants were observed between 1996 and 1999 [64]. Following the introduction of zanamivir and oseltamivir in 1999, oseltamivir-resistant variants have been detected in 0.33 % among those ≥13 years old and in 4 % among those ≤12 years old from 1999 to 2004 from clinical trial samples [39]. The resistance rates among clinical isolates were generally low until the emergence and global spread of the A/Brisbane/59/2007-like seasonal H1N1 variants carrying the H274Y mutation during the 2007–2008; however, this strain has disappeared since the emergence of the A(H1N1)pdm09 virus. Most recent surveillance reports from WHO collaborating centers during 2012–2013 and 2013–2014 reported 0.2 % (27/11,387) and 2 % (172/10,641) isolates exhibiting highly reduced inhibition against one or more NA inhibitors.

In addition to the viral-inhibitor interactions, host factors may also contribute to the emergence of resistant variants. Resistant variants were reported at higher detection rates in pediatric patients (5.5 % in outpatients and 16–18 % in both inpatients and outpatients) who tend to shed higher amount of viral load during the course of infection when compared to the adults (0.4 % in outpatients) [63, 69–72]. In addition, multiple resistant variants as well as multidrug-resistant variants were often reported from immunocompromised patients with limited ability for viral clearance and are often receiving prolonged treatments of NA inhibitors [73–81].

5.1 Group 1 NA

The H274Y mutation is the most frequently reported mutation clinically that confers resistance to NA inhibitors; this

mutation results in highly reduced inhibition to oseltamivir and peramivir but would not change viral sensitivity to zanamivir. To accommodate the bulky hydrophobic side chain of the oseltamivir carboxylate, the E276 needs to reorient and form hydrogen bond with R224; the H274Y mutation would block this hydrogen bond formation between E276 and R224 and thereby confer resistance to oseltamivir. Since binding of zanamivir does not require the hydrogen bond formation between E276 and R224, the H274Y mutant remains susceptible for zanamivir [82].

During human challenge studies, the H274Y NA mutation was first documented in 2 (3.7 %) out of 54 human volunteers during the course of oseltamivir treatment after challenged with the A/Texas/36/91 (H1N1) virus [83]. The H274Y mutation was also reported from H5N1 patients after receiving oseltamivir treatment [84, 85]. In one H5N1-infected patient, mixed population of H274Y and N294S mutations were observed after treatment, with the H274Y conferring to highly reduced inhibition and the N294S conferring to reduced change in IC50 to oseltamivir carboxylate [85]. The N294S mutation that confers 57–138-fold change in IC50 to oseltamivir has also been isolated from an H5N1 patient prior to receiving oseltamivir treatment in Egypt [86]. During 2007–2008, an H275Y variant of A/Brisbane/59/2007-like seasonal H1N1 virus emerged and spread globally. Studies showed that epistatic NA mutations emerged in chronological order prior to (V233M, R221Q, K328E, D343N) and post to (D353G) the acquisition of the H275Y mutation facilitated the restoration of the NA function [87–90]. The A/Brisbane/59/2007-like viruses carrying the H274Y mutation only circulated 2 years and have disappeared since the emergence of the A(H1N1)pdm09 virus. During the 2009 pandemic, the H274Y was also detected from a household contact that received prophylactic oseltamivir treatment [91] or from immune-compromised patients after receiving oseltamivir treatment [92]. Community clusters of A(H1N1)pdm09 viruses carrying the H274Y mutation have been reported in Australia in 2011 as well as in Japan and the USA in 2013–2014 [93–95]; in addition to the H274Y mutation and V240I and N368K mutations that were commonly found in the virus isolated these outbreaks may have epistasic effect to promote the fitness of the H274Y mutant [94–96]. It should be noted that the A(H1N1)pdm09 virus did not possess the V240I and N368K mutations when it emerged in 2009 but >97 % of the A(H1N1)pdm09 viruses circulating in 2012–2013 were reported to possess these two mutations [65]. A(H1N1)pdm09 viruses with H274Y mutation have been reported to accompany I222R, I222V, I222K, or S246N [97–100]; among which the I222R or I222K in combination with the H274Y may confer multidrug resistance to all four drugs. The I222R mutation has been isolated from immunocompetent without prior NA inhibitor treatment history [101] and from immunocompromised patients after receiving oseltamivir and zanamivir treatments [98, 102]. The I222R mutation alone can lead to reduced inhibition to zanamivir (~10-fold increase in IC_{50}) and oseltamivir (10–40-fold increase in IC_{50}) and minimal resistance to peramivir (<10-fold increase in IC_{50}) [101–103]. The combination of I222R and H274Y mutations would lead to highly reduced inhibition to oseltamivir and peramivir (>100-fold increase in IC_{50}) as well as reduced inhibition to zanamivir and laninamivir (>10-fold increase in IC_{50}) [103]. Combination of I222K and H274Y mutations has also been detected from patients under oseltamivir treatment with reduced susceptibility to zanamivir and laninamivir (11-fold increase in IC_{50}) and highly reduced inhibition (>1000-fold increase in IC_{50}) to oseltamivir and peramivir [99]. A single N294S mutation in A(H1N1)pdm09 isolated during 2012–2013 surveillance led to highly reduced sensitivity to oseltamivir [65]; a single-amino acid substitution of D198E, I222K, I222T, I222R, or S246G isolated during 2013–2014 would lead to reduced inhibition to oseltamivir with >10-fold increase in IC_{50} [103].

A Q136K mutation that leads to highly reduced inhibition to zanamivir (with >100-fold increase in IC_{50}) and peramivir (60–100-fold increase in IC_{50}) but not to oseltamivir was first reported from seasonal H1N1 influenza viruses during the 2006–2008 surveillance [104]. However, this mutation was not detected from the primary clinical specimens and was only found in in vitro-passaged samples [104]. The Q136K mutation was also detected from seasonal H1N1 influenza virus isolated from a subject who participated in a zanamivir postapproval efficacy study in 2007–2008, prior to receiving zanamivir treatment. Similar to the previous study, the Q136K mutation confers highly reduced susceptibility to zanamivir (with ~300-fold increase in IC_{50}) but not to oseltamivir and the mutation was only detected in the cultured samples but not from the original swab samples [105]. A study that serial passaged A(H1N1)pdm09 virus under increasing concentrations of zanamivir also yielded the Q136K mutation that led to reduced susceptibility (86-fold increase in IC_{50}) for zanamivir [106]. The Q136K mutant showed reduced NA activity and surface expression, compromised replication in MDCK-SIAT1 cells, and reduced transmissibility in guinea pigs [106]. Global surveillance in 2012–2013 detected A(H1N1)pdm09 virus carrying a single Q136K or Q136R mutation; both conferred highly reduced inhibition to zanamivir and peramivir (>100-fold increase in IC_{50}) and reduced inhibition to laninamivir (>30-fold increase in IC_{50}) but remained susceptible to oseltamivir [65]. In N1 structure (derived from H5N1 NA) complexed with zanamivir, Q136 interacts with R156, which also interacts with D151. As R156 interacts with zanamivir via van der Waals interactions and D151 forms hydrogen bonds with the 4-guanidino group of zanamivir, the Q136K change would indirectly interfere the binding of R156 and D151 to zanamivir and peramivir, which also possess a 4-guanidino group [104].

5.2 Group 2 NA

The R292K mutation is the most commonly reported mutation among human H3N2 influenza that confers resistance to NA inhibitors; it results in highly reduced inhibition to oseltamivir and peramivir and leads to reduced inhibition to zanamivir. The E119V mutation is also frequently observed among H3N2 viruses with highly reduced inhibition to oseltamivir but remains inhibition to zanamivir. The overall detection rate of the R292K or E119V mutations through global surveillance remains low during 2012–2013 and 2013–2014 seasons (4/22,028 for E119V and 1/22,028 for R292K) [65, 103]. The N294S mutation that results in highly reduced inhibition to oseltamivir has been reported from paediatric patients in Japan [69].

The fitness and transmissibility of the E119V and R292K mutant viruses have been shown to differ in the laboratory setting [107]. The R292 is one of the conserved catalytic residues that interact with the carboxylate of sialic acid and all NA inhibitors. The mechanism for R292K mutation to confer resistance is similar to that described for the H274Y mutation by blocking the hydrogen bond formation between E276 and R224. Residue E119 interacts with the 4-guanido group of zanamivir; therefore, the mechanism for E119V mutation to selectively confer resistance to oseltamivir but not to zanamivir is not well studied [41]. Although early in vitro studies have identified the E119A/D/G mutations in N2 and the E119G mutation in N9 that lead to highly reduced inhibition to zanamivir (E119A/D/G) and peramivir (E119D) [108], these mutations have been shown to affect the stability of the NA protein and compromise viral fitness and they have not been reported from clinical isolates [51–53].

The E119V mutation can be maintained within an immunocompromised patient for 7 weeks in the absence of the drug [109]. From a separate immunocompromised patient who received oseltamivir, amantadine, and zanamivir treatment, the I222V mutation emerged after the emergence of the E119V mutation enhanced the level of oseltamivir resistance [73]. The specimen collected from the same patient 3 months after the detection of the E119V + I222V mutation identified a combination of E119V, N146K, and S219T, and deletion of residues 245–248, which showed highly reduced inhibition to oseltamivir but remained sensitive for zanamivir and peramivir. Recombinant proteins with different mutations were made to confirm that the deletions at 245–248 alone may confer resistance to oseltamivir. The deletion was stably maintained after four passages in vitro [110].

Surveillance studies have identified D151V/D amino acid changes in H3N2 viruses exhibited highly reduced inhibition for zanamivir but remained sensitive to oseltamivir [64, 66]. In addition, the Q136K mutation reported in H1N1 viruses was also detected from H3N2 viruses isolated in Myanmar in 2007 and 2008; the Q136K mutation conferred resistance to zanamivir (with 20–50-fold increase in IC_{50}) but not to oseltamivir [111]. Mixed Q136K/Q alone or in combination with mixed D151G/D were isolated in 2013–2014 with reduced inhibition to zanamivir [103]. In addition, a single mutation of T148K, N329K, or S331R was associated with ~10 fold reduction in inhibition to zanamivir (T148K and N329K) or oseltamivir (N329K and S331R) in 2013–2014 [103]. It should be noted that the D151 and T148I mutations are commonly acquired by seasonal H3N2 influenza viruses after passaged in MDCK cells; D151G mutation was shown to increase binding to α2,3-linked sialyl receptors [112, 113] while the T148I mutation alone confers six-fold increased IC_{50} to zanamivir but would interfere the inhibition profile while in combination with an E119V variant [114].

Human infections by the H7N9 avian influenza viruses have been reported since Spring 2013. Mutations that confer resistance to NA inhibitors have been reported from patients who received treatments. The R292K mutation was the most commonly reported mutation among the H7N9 patients [115, 116]. In one patient, mixed populations of E119V, I222K, I222R, or R292K single substitutions have been observed [117]; the R292K mutation leads to highly reduced inhibition to oseltamivir and peramivir while the I222K, I222R, and E119V lead to reduced inhibition to oseltamivir.

5.3 Influenza B

Clinically, influenza B variant with an R152K NA mutation was first isolated in 1998 from an immunocompromised child who received aerosolized ribavirin followed by nebulized zanamivir (under approval of FDA); the patient continued to shed virus while receiving zanamivir and died 2 days after the treatment was discontinued [76]. The R152K mutation was first detected on day 12 post-treatment and was accompanied by an HA-T198I mutation that reduced the HA-binding affinity. The R152K mutation was later confirmed to confer cross-resistance to zanamivir, oseltamivir, and peramivir [108]. Direct competition assay in ferrets suggests lower fitness of the R152K mutant than the wild-type virus in the absence of zanamivir [76]. The D198N mutation that emerged from an immunocompromised patient after prolonged oseltamivir treatment was found to confer cross-resistance to oseltamivir (10-fold increase in IC_{50}) and zanamivir (~10-fold increase in IC_{50}) [80] but remain sensitive for peramivir [108]. Recently, the I222L mutation that confers resistance to both oseltamivir (>100-fold increase in IC_{50}) and zanamivir (~10-fold increase in IC_{50}) was detected from an immunocompromised patient after prolonged oseltamivir treatment [74].

In addition to the detection of the R152K, D198N, and I222L mutations from immunocompromised patients after

receiving prolonged NA inhibitor treatment, influenza B viruses carrying D198N, I222T, and S250G mutations have been detected from 1.7 % of (7/422) pediatric outpatients prior to oseltamivir treatment, although many of them had household contacts with influenza B patients under NA inhibitor treatment [118]. In the same study, G402S-resistant variant was also detected in 1.4 % (1/74) patients after oseltamivir treatment [118]. The D198E/Y mutations have also been isolated from surveillance studies that led to ten-fold reduced inhibition to zanamivir (D198E/Y) and oseltamivir (D198Y) [119–122]. The N294S mutation has been isolated from a pediatric inpatient with acute lymphoblastic leukemia without NA inhibitor treatment history [123]. The H274Y mutation has been detected from an adult patient without known prior treatment history [124]. Global surveillance during 2012–2013 and 2013–2014 has also identified the H274Y mutation from both B/Victoria and B/Yamagata lineages that lead to highly reduced inhibition to peramivir and <10-fold reduced inhibition to oseltamivir [65, 103]. The E110K mutation that showed reduced inhibition to zanamivir and laninamivir as well as highly reduced inhibition to peramivir was selected after in vitro passage but at below detection level in the original specimen [125]. Interestingly, E110 is localized at the monomer–monomer interface of the NA tetramer, which is different from the other mutations that are localized at the enzyme site. The mechanism of resistance could be through destabilizing the NA tetrameric form [125].

Large-scale surveillance studies in 2004–2008 have identified the R371K mutation as an extreme outlier that leads to highly reduced inhibition to oseltamivir (407-fold increased IC_{50}) and reduced inhibition to zanamivir (29-fold increased IC_{50}) [66]. Multiple single mutations have been detected during 2012–2013 and 2013–2014 surveillance with <10-fold increase to NA inhibitors [65, 103]. Previously, the E119A mutation was reported to result in reduced or highly reduced inhibition to zanamivir and oseltamivir in B/Illinois/03/2008 [121]; an E119G mutation was detected from a B/Victoria-lineage isolate during the 2013–2014 surveillance with highly reduced inhibition to zanamivir (>1000-fold increased IC_{50}), peramivir (>2000-fold increased IC_{50}), and laninamivir (>600-fold increased IC_{50}) as well as reduced inhibition to oseltamivir (>10-fold increased IC_{50}) [103].

6 Investigational NA Inhibitors

The global spread of the seasonal H1N1 influenza viruses carrying the H274Y mutation that confer resistance to oseltamivir in 2007–2009 highlights the need for improving current NA inhibitors and developing additional antiviral strategies. Novel advancement has been the development of the 2,3 difluorosialic acid (DFSA)-based NA inhibitors that form stabilized covalent intermediate in the influenza neuraminidase enzyme [126]. DFSA analogs with 4-amino (Am) or 4-guanidino (Gu) side chains and the three-fluoro in either the axial (Fax) or the equatorial (Feq) orientations have been tested against influenza A (H1N1 with H274Y and H3N2 with E119V) and B (D198E) variants resistant to oseltamivir and zanamivir; the FeqGu showed the lowest IC_{50} while compared to the other analogs. The FaxGu and FeqGu also exhibited comparable efficacy as zanamivir against the challenge of A/Hong Kong/1/68 (H3N2) in the mouse model.

7 Concluding Remarks

NA inhibitors have been the only effective antiviral option against influenza A and B viruses since 2009. The isolation of drug-resistant variants among individuals without prior treatment history demonstrated the uncompromised viral fitness and transmissibility of some NA-resistant variants. There have been increasing numbers of cell culture-derived variants as well as amino acid changes at non-conserved NA residues associated with reduced inhibition to NA inhibitors. Understanding the biological function and the mechanisms for the emergence of these mutations should be a priority for future research.

References

1. Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, Fukuda K. Influenza-associated hospitalizations in the United States. JAMA. 2004;292:1333–40.
2. Centers for Disease C, Prevention. Estimates of deaths associated with seasonal influenza—United States, 1976–2007. MMWR Morb Mortal Wkly Rep. 2010;59:1057–62.
3. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56:152–79.
4. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A. 2012;109:4269–74.
5. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO. New world bats harbor diverse influenza A viruses. PLoS Pathog. 2013;9:e1003657.
6. Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA. Influenza B virus in seals. Science. 2000;288:1051–3.
7. Vijaykrishna D, Holmes EC, Joseph U, Fourment M, Su YC, Halpin R, Lee RT, Deng YM, Gunalan V, Lin X, Stockwell TB, Fedorova NB, Zhou B, Spirason N, Kuhnert D, Boskova V, Stadler T, Costa AM, Dwyer DE, Huang QS, Jennings LC, Rawlinson W, Sullivan SG, Hurt AC, Maurer-Stroh S, Wentworth DE, Smith GJ, Barr IG. The contrasting phylodynamics of human influenza B viruses. Elife. 2015;4:e05055.
8. Gamblin SJ, Skehel JJ. Influenza hemagglutinin and neuraminidase membrane glycoproteins. J Biol Chem. 2010;285:28403–9.

9. van der Vries E, Veldhuis Kroeze EJ, Stittelaar KJ, Linster M, Van der Linden A, Schrauwen EJ, Leijten LM, van Amerongen G, Schutten M, Kuiken T, Osterhaus AD, Fouchier RA, Boucher CA, Herfst S. Multidrug resistant 2009 A/H1N1 influenza clinical isolate with a neuraminidase I223R mutation retains its virulence and transmissibility in ferrets. PLoS Pathog. 2011;7:e1002276.
10. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem. 2000;69:531–69.
11. Resa-Infante P, Jorba N, Coloma R, Ortin J. The influenza virus RNA synthesis machine: advances in its structure and function. RNA Biol. 2011;8:207–15.
12. Nayak DP, Balogun RA, Yamada H, Zhou ZH, Barman S. Influenza virus morphogenesis and budding. Virus Res. 2009;143:147–61.
13. Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, Shu Y, Gubareva LV, Cox NJ, Klimov AI. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J Infect Dis. 2007;196:249–57.
14. Gubareva LV, Trujillo AA, Okomo-Adhiambo M, Mishin VP, Deyde VM, Sleeman K, Nguyen HT, Sheu TG, Garten RJ, Shaw MW, Fry AM, Klimov AI. Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. Antivir Ther. 2010;15:1151–9.
15. Air GM. Influenza neuraminidase. Influenza Other Respi Viruses. 2012;6:245–56.
16. Laver WG, Colman PM, Webster RG, Hinshaw VS, Air GM. Influenza virus neuraminidase with hemagglutinin activity. Virology. 1984;137:314–23.
17. Liu C, Air GM. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. Virology. 1993;194:403–7.
18. Ohuchi M, Feldmann A, Ohuchi R, Klenk HD. Neuraminidase is essential for fowl plague virus hemagglutinin to show hemagglutinating activity. Virology. 1995;212:77–83.
19. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol. 2004;78:12665–7.
20. Wagner R, Matrosovich M, Klenk HD. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol. 2002;12:159–66.
21. Burmeister WP, Ruigrok RW, Cusack S. The 2.2 A resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J. 1992;11:49–56.
22. Russell RJ, Haire LF, Stevens DJ, Collins PJ, Lin YP, Blackburn GM, Hay AJ, Gamblin SJ, Skehel JJ. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature. 2006;443:45–9.
23. Colman PM, Hoyne PA, Lawrence MC. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J Virol. 1993;67:2972–80.
24. Varghese JN, McKimm-Breschkin JL, Caldwell JB, Kortt AA, Colman PM. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins. 1992;14:327–32.
25. von Itzstein M. The war against influenza: discovery and development of sialidase inhibitors. Nat Rev Drug Discov. 2007;6:967–74.
26. Colman PM. Neuraminidase inhibitors as antivirals. Vaccine. 2002;20 Suppl 2:S55–8.
27. Meindl P, Bodo G, Palese P, Schulman J, Tuppy H. Inhibition of neuraminidase activity by derivatives of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. Virology. 1974;58:457–63.
28. Palese P, Compans RW. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. J Gen Virol. 1976;33:159–63.
29. Colman PM. Influenza virus neuraminidase: structure, antibodies, and inhibitors. Protein Sci. 1994;3:1687–96.
30. Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature. 1983;303:41–4.
31. Varghese JN, Laver WG, Colman PM. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. Nature. 1983;303:35–40.
32. von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Van Phan T, Smythe ML, White HF, Oliver SW, et al. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature. 1993;363:418–23.
33. Varghese JN, Epa VC, Colman PM. Three-dimensional structure of the complex of 4-guanidino-Neu5Ac2en and influenza virus neuraminidase. Protein Sci. 1995;4:1081–7.
34. Woods JM, Bethell RC, Coates JA, Healy N, Hiscox SA, Pearson BA, Ryan DM, Ticehurst J, Tilling J, Walcott SM, et al. 4-Guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses in vitro. Antimicrob Agents Chemother. 1993;37:1473–9.
35. Gubareva LV, Penn CR, Webster RG. Inhibition of replication of avian influenza viruses by the neuraminidase inhibitor 4-guanid ino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid. Virology. 1995;212:323–30.
36. Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver WG, Stevens RC. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J Am Chem Soc. 1997;119:681–90.
37. Babu YS, Chand P, Bantia S, Kotian P, Dehghani A, El-Kattan Y, Lin TH, Hutchison TL, Elliott AJ, Parker CD, Ananth SL, Horn LL, Laver GW, Montgomery JA. BCX-1812 (RWJ-270201): discovery of a novel, highly potent, orally active, and selective influenza neuraminidase inhibitor through structure-based drug design. J Med Chem. 2000;43:3482–6.
38. Varghese JN, Smith PW, Sollis SL, Blick TJ, Sahasrabudhe A, McKimm-Breschkin JL, Colman PM. Drug design against a shifting target: a structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase. Structure. 1998;6:735–46.
39. Samson M, Pizzorno A, Abed Y, Boivin G. Influenza virus resistance to neuraminidase inhibitors. Antiviral Res. 2013;98:174–85.
40. Vavricka CJ, Li Q, Wu Y, Qi J, Wang M, Liu Y, Gao F, Liu J, Feng E, He J, Wang J, Liu H, Jiang H, Gao GF. Structural and functional analysis of laninamivir and its octanoate prodrug reveals group specific mechanisms for influenza NA inhibition. PLoS Pathog. 2011;7:e1002249.
41. McKimm-Breschkin JL. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. Influenza Other Respi Viruses. 2013;7 Suppl 1:25–36.
42. Bantia S, Parker CD, Ananth SL, Horn LL, Andries K, Chand P, Kotian PL, Dehghani A, El-Kattan Y, Lin T, Hutchison TL, Montgomery JA, Kellog DL, Babu YS. Comparison of the anti-influenza virus activity of RWJ-270201 with those of oseltamivir and zanamivir. Antimicrob Agents Chemother. 2001;45:1162–7.
43. Govorkova EA, Leneva IA, Goloubeva OG, Bush K, Webster RG. Comparison of efficacies of RWJ-270201, zanamivir, and oseltamivir against H5N1, H9N2, and other avian influenza viruses. Antimicrob Agents Chemother. 2001;45:2723–32.
44. Bantia S, Arnold CS, Parker CD, Upshaw R, Chand P. Anti-influenza virus activity of peramivir in mice with single intramuscular injection. Antiviral Res. 2006;69:39–45.
45. Boltz DA, Ilyushina NA, Arnold CS, Babu YS, Webster RG, Govorkova EA. Intramuscularly administered neuraminidase inhibitor peramivir is effective against lethal H5N1 influenza virus in mice. Antiviral Res. 2008;80:150–7.

46. Sidwell RW, Smee DF, Huffman JH, Barnard DL, Bailey KW, Morrey JD, Babu YS. In vivo influenza virus-inhibitory effects of the cyclopentane neuraminidase inhibitor RJW-270201. Antimicrob Agents Chemother. 2001;45:749–57.
47. McLaughlin MM, Skoglund EW, Ison MG. Peramivir: an intravenous neuraminidase inhibitor. Expert Opin Pharmacother. 2015;16:1889–900.
48. Kubo S, Tomozawa T, Kakuta M, Tokumitsu A, Yamashita M. Laninamivir prodrug CS-8958, a long-acting neuraminidase inhibitor, shows superior anti-influenza virus activity after a single administration. Antimicrob Agents Chemother. 2010;54:1256–64.
49. Yamashita M, Tomozawa T, Kakuta M, Tokumitsu A, Nasu H, Kubo S. CS-8958, a prodrug of the new neuraminidase inhibitor R-125489, shows long-acting anti-influenza virus activity. Antimicrob Agents Chemother. 2009;53:186–92.
50. Barnett JM, Cadman A, Burrell FM, Madar SH, Lewis AP, Tisdale M, Bethell R. In vitro selection and characterisation of influenza B/Beijing/1/87 isolates with altered susceptibility to zanamivir. Virology. 1999;265:286–95.
51. Blick TJ, Tiong T, Sahasrabudhe A, Varghese JN, Colman PM, Hart GJ, Bethell RC, McKimm-Breschkin JL. Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. Virology. 1995;214:475–84.
52. Gubareva LV, Bethell R, Hart GJ, Murti KG, Penn CR, Webster RG. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. J Virol. 1996;70:1818–27.
53. Gubareva LV, Robinson MJ, Bethell RC, Webster RG. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. J Virol. 1997;71:3385–90.
54. McKimm-Breschkin JL. Resistance of influenza viruses to neuraminidase inhibitors—a review. Antiviral Res. 2000;47:1–17.
55. McKimm-Breschkin JL, Blick TJ, Sahasrabudhe A, Tiong T, Marshall D, Hart GJ, Bethell RC, Penn CR. Generation and characterization of variants of NWS/G70C influenza virus after in vitro passage in 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en. Antimicrob Agents Chemother. 1996;40:40–6.
56. Staschke KA, Colacino JM, Baxter AJ, Air GM, Bansal A, Hornback WJ, Munroe JE, Laver WG. Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en. Virology. 1995;214:642–6.
57. Tai CY, Escarpe PA, Sidwell RW, Williams MA, Lew W, Wu H, Kim CU, Mendel DB. Characterization of human influenza virus variants selected in vitro in the presence of the neuraminidase inhibitor GS 4071. Antimicrob Agents Chemother. 1998;42:3234–41.
58. McKimm-Breschkin JL, Sahasrabudhe A, Blick TJ, McDonald M, Colman PM, Hart GJ, Bethell RC, Varghese JN. Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. J Virol. 1998;72:2456–62.
59. Herlocher ML, Truscon R, Fenton R, Klimov A, Elias S, Ohmit SE, Monto AS. Assessment of development of resistance to antivirals in the ferret model of influenza virus infection. J Infect Dis. 2003;188:1355–61.
60. Blick TJ, Sahasrabudhe A, McDonald M, Owens IJ, Morley PJ, Fenton RJ, McKimm-Breschkin JL. The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. Virology. 1998;246:95–103.
61. Ginting TE, Shinya K, Kyan Y, Makino A, Matsumoto N, Kaneda S, Kawaoka Y. Amino acid changes in hemagglutinin contribute to the replication of oseltamivir-resistant H1N1 influenza viruses. J Virol. 2012;86:121–7.
62. Gubareva LV. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res. 2004;103:199–203.
63. Hayden F. Developing new antiviral agents for influenza treatment: what does the future hold? Clin Infect Dis. 2009;48 Suppl 1:S3–13.
64. McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, Hayden F, Zambon M. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob Agents Chemother. 2003;47:2264–72.
65. Meijer A, Rebelo-de-Andrade H, Correia V, Besselaar T, Drager-Dayal R, Fry A, Gregory V, Gubareva L, Kageyama T, Lackenby A, Lo J, Odagiri T, Pereyaslov D, Siqueira MM, Takashita E, Tashiro M, Wang D, Wong S, Zhang W, Daniels RS, Hurt AC. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2012–2013. Antiviral Res. 2014;110:31–41.
66. Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, Butler EN, Wallis TR, Klimov AI, Gubareva LV. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob Agents Chemother. 2008;52:3284–92.
67. Okomo-Adhiambo M, Sheu TG, Gubareva LV. Assays for monitoring susceptibility of influenza viruses to neuraminidase inhibitors. Influenza Other Respi Viruses. 2013;7 Suppl 1:44–9.
68. WHO. Meetings of the WHo working group on surveillance of influenza antiviral susceptibility—Geneva, November 2011 and June 2012. MMWR Morb Mortal Wkly Rep. 2012;39:369–80.
69. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet. 2004;364:759–65.
70. Roberts NA. Treatment of influenza with neuraminidase inhibitors: virological implications. Philos Trans R Soc Lond B Biol Sci. 2001;356:1895–7.
71. Whitley RJ, Boucher CA, Lina B, Nguyen-Van-Tam JS, Osterhaus A, Schutten M, Monto AS. Global assessment of resistance to neuraminidase inhibitors, 2008–2011: the Influenza Resistance Information Study (IRIS). Clin Infect Dis. 2013;56:1197–205.
72. Whitley RJ, Hayden FG, Reisinger KS, Young N, Dutkowski R, Ipe D, Mills RG, Ward P. Oral oseltamivir treatment of influenza in children. Pediatr Infect Dis J. 2001;20:127–33.
73. Baz M, Abed Y, McDonald J, Boivin G. Characterization of multidrug-resistant influenza A/H3N2 viruses shed during 1 year by an immunocompromised child. Clin Infect Dis. 2006;43:1555–61.
74. Escuret V, Collins PJ, Casalegno JS, Vachieri SG, Cattle N, Ferraris O, Sabatier M, Frobert E, Caro V, Skehel JJ, Gamblin S, Valla F, Valette M, Ottmann M, McCauley JW, Daniels RS, Lina B. A novel I221L substitution in neuraminidase confers high-level resistance to oseltamivir in influenza B viruses. J Infect Dis. 2014;210:1260–9.
75. Eshaghi A, Shalhoub S, Rosenfeld P, Li A, Higgins RR, Stogios PJ, Savchenko A, Bastien N, Li Y, Rotstein C, Gubbay JB. Multiple influenza A (H3N2) mutations conferring resistance to neuraminidase inhibitors in a bone marrow transplant recipient. Antimicrob Agents Chemother. 2014;58:7188–97.
76. Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J Infect Dis. 1998;178:1257–62.
77. Memoli MJ, Athota R, Reed S, Czajkowski L, Bristol T, Proudfoot K, Hagey R, Voell J, Fiorentino C, Ademposi A, Shoham S, Taubenberger JK. The natural history of influenza infection in the severely immunocompromised vs nonimmunocompromised hosts. Clin Infect Dis. 2014;58:214–24.

78. Piralla A, Gozalo-Marguello M, Fiorina L, Rovida F, Muzzi A, Colombo AA, Alessandrino PE, Baldanti F. Different drug-resistant influenza A(H3N2) variants in two immunocompromised patients treated with oseltamivir during the 2011–2012 influenza season in Italy. J Clin Virol. 2013;58:132–7.
79. Weinstock DM, Gubareva LV, Zuccotti G. Prolonged shedding of multidrug-resistant influenza A virus in an immunocompromised patient. N Engl J Med. 2003;348:867–8.
80. Ison MG, Gubareva LV, Atmar RL, Treanor J, Hayden FG. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. J Infect Dis. 2006;193:760–4.
81. Tamura D, DeBiasi RL, Okomo-Adhiambo M, Mishin VP, Campbell AP, Loechelt B, Wiedermann BL, Fry AM, Gubareva LV. Emergence of multidrug-resistant influenza A(H1N1)pdm09 virus variants in an immunocompromised child treated with oseltamivir and zanamivir. J. Infect. Dis. 2015;212:1209–13.
82. Wang MZ, Tai CY, Mendel DB. Mechanism by which mutations at his274 alter sensitivity of influenza a virus n1 neuraminidase to oseltamivir carboxylate and zanamivir. Antimicrob Agents Chemother. 2002;46:3809–16.
83. Gubareva LV, Kaiser L, Matrosovich MN, Soo-Hoo Y, Hayden FG. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. J Infect Dis. 2001;183:523–31.
84. de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JS, Tran TH, Farrar J. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med. 2005;353:2667–72.
85. Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. Avian flu: isolation of drug-resistant H5N1 virus. Nature. 2005;437:1108.
86. Earhart KC, Elsayed NM, Saad MD, Gubareva LV, Nayel A, Deyde VM, Abdelsattar A, Abdelghani AS, Boynton BR, Mansour MM, Essmat HM, Klimov A, Shuck-Lee D, Monteville MR, Tjaden JA. Oseltamivir resistance mutation N294S in human influenza A(H5N1) virus in Egypt. J Infect Public Health. 2009;2:74–80.
87. Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science. 2010;328:1272–5.
88. Duan S, Govorkova EA, Bahl J, Zaraket H, Baranovich T, Seiler P, Prevost K, Webster RG, Webby RJ. Epistatic interactions between neuraminidase mutations facilitated the emergence of the oseltamivir-resistant H1N1 influenza viruses. Nat Commun. 2014;5:5029.
89. Abed Y, Pizzorno A, Bouhy X, Boivin G. Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses. PLoS Pathog. 2011;7:e1002431.
90. Rameix-Welti MA, Munier S, Le Gal S, Cuvelier F, Agou F, Enouf V, Naffakh N, van der Werf S. Neuraminidase of 2007–2008 influenza A(H1N1) viruses shows increased affinity for sialic acids due to the D344N substitution. Antivir Ther. 2011;16:597–603.
91. Baz M, Abed Y, Papenburg J, Bouhy X, Hamelin ME, Boivin G. Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. N Engl J Med. 2009;361:2296–7.
92. Memoli MJ, Hrabal RJ, Hassantoufighi A, Eichelberger MC, Taubenberger JK. Rapid selection of oseltamivir- and peramivir-resistant pandemic H1N1 virus during therapy in 2 immunocompromised hosts. Clin Infect Dis. 2010;50:1252–5.
93. Hurt AC, Hardie K, Wilson NJ, Deng YM, Osbourn M, Leang SK, Lee RT, Iannello P, Gehrig N, Shaw R, Wark P, Caldwell N, Givney RC, Xue L, Maurer-Stroh S, Dwyer DE, Wang B, Smith DW, Levy A, Booy R, Dixit R, Merritt T, Kelso A, Dalton C, Durrheim D, Barr IG. Characteristics of a widespread community cluster of H275Y oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. J Infect Dis. 2012;206:148–57.
94. Takashita E, Kiso M, Fujisaki S, Yokoyama M, Nakamura K, Shirakura M, Sato H, Odagiri T, Kawaoka Y, Tashiro M. Characterization of a large cluster of influenza A(H1N1) pdm09 viruses cross-resistant to oseltamivir and peramivir during the 2013–2014 influenza season in Japan. Antimicrob Agents Chemother. 2015;59:2607–17.
95. Okomo-Adhiambo M, Fry AM, Su S, Nguyen HT, Elal AA, Negron E, Hand J, Garten RJ, Barnes J, Xiyan X, Villanueva JM, Gubareva LV, Group USIAW. Oseltamivir-resistant influenza A(H1N1)pdm09 viruses, United States, 2013–14. Emerg Infect Dis. 2015;21:136–41.
96. Butler J, Hooper KA, Petrie S, Lee R, Maurer-Stroh S, Reh L, Guarnaccia T, Baas C, Xue L, Vitesnik S, Leang SK, McVernon J, Kelso A, Barr IG, McCaw JM, Bloom JD, Hurt AC. Estimating the fitness advantage conferred by permissive neuraminidase mutations in recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. PLoS Pathog. 2014;10:e1004065.
97. Hurt AC, Lee RT, Leang SK, Cui L, Deng YM, Phuah SP, Caldwell N, Freeman K, Komadina N, Smith D, Speers D, Kelso A, Lin RT, Maurer-Stroh S, Barr IG. Increased detection in Australia and Singapore of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and zanamivir sensitivity due to a S247N neuraminidase mutation. Euro. Surveill. 2011;16(23). pii: 19884.
98. Nguyen HT, Fry AM, Loveless PA, Klimov AI, Gubareva LV. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir. Clin Infect Dis. 2010;51:983–4.
99. Nguyen HT, Trujillo AA, Sheu TG, Levine M, Mishin VP, Shaw M, Ades EW, Klimov AI, Fry AM, Gubareva LV. Analysis of influenza viruses from patients clinically suspected of infection with an oseltamivir resistant virus during the 2009 pandemic in the United States. Antiviral Res. 2012;93:381–6.
100. Centers for Disease C, Prevention. Oseltamivir-resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients—Seattle, Washington, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:893–6.
101. Eshaghi A, Patel SN, Sarabia A, Higgins RR, Savchenko A, Stojios PJ, Li Y, Bastien N, Alexander DC, Low DE, Gubbay JB. Multidrug-resistant pandemic (H1N1) 2009 infection in immunocompetent child. Emerg Infect Dis. 2011;17:1472–4.
102. van der Vries E, Stelma FF, Boucher CA. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. N Engl J Med. 2010;363:1381–2.
103. Takashita E, Meijer A, Lackenby A, Gubareva L, Rebelo-de-Andrade H, Besselaar T, Fry A, Gregory V, Leang SK, Huang W, Lo J, Pereyaslov D, Siqueira MM, Wang D, Mak GC, Zhang W, Daniels RS, Hurt AC, Tashiro M. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013–2014. Antiviral Res. 2015;117:27–38.
104. Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. J Virol. 2009;83:10366–73.
105. Yates PJ, Mehta N, Horton J, Tisdale M. Virus susceptibility analyses from a phase IV clinical trial of inhaled zanamivir treatment in children infected with influenza. Antimicrob Agents Chemother. 2013;57:1677–84.
106. Kaminski MM, Ohnemus A, Staeheli P, Rubbenstroth D. Pandemic 2009 H1N1 influenza A virus carrying a Q136K mutation in the neuraminidase gene is resistant to zanamivir but exhibits reduced fitness in the guinea pig transmission model. J Virol. 2013;87:1912–5.
107. Yen HL, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, Govorkova EA. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother. 2005;49:4075–84.

108. Mishin VP, Hayden FG, Gubareva LV. Susceptibilities of antiviral-resistant influenza viruses to novel neuraminidase inhibitors. Antimicrob Agents Chemother. 2005;49:4515–20.
109. Hurt AC, Leang SK, Tiedemann K, Butler J, Mechinaud F, Kelso A, Downie P, Barr IG. Progressive emergence of an oseltamivir-resistant A(H3N2) virus over two courses of oseltamivir treatment in an immunocompromised paediatric patient. Influenza Other Respi Viruses. 2013;7:904–8.
110. Abed Y, Baz M, Boivin G. A novel neuraminidase deletion mutation conferring resistance to oseltamivir in clinical influenza A/H3N2 virus. J Infect Dis. 2009;199:180–3.
111. Dapat C, Suzuki Y, Saito R, Kyaw Y, Myint YY, Lin N, Oo HN, Oo KY, Win N, Naito M, Hasegawa G, Dapat IC, Zaraket H, Baranovich T, Nishikawa M, Saito T, Suzuki H. Rare influenza A (H3N2) variants with reduced sensitivity to antiviral drugs. Emerg Infect Dis. 2010;16:493–6.
112. Lin YP, Gregory V, Collins P, Kloess J, Wharton S, Cattle N, Lackenby A, Daniels R, Hay A. Neuraminidase receptor binding variants of human influenza A(H3N2) viruses resulting from substitution of aspartic acid 151 in the catalytic site: a role in virus attachment? J Virol. 2010;84:6769–81.
113. Zhu X, McBride R, Nycholat CM, Yu W, Paulson JC, Wilson IA. Influenza virus neuraminidases with reduced enzymatic activity that avidly bind sialic acid receptors. J Virol. 2012;86:13371–83.
114. Tamura D, Nguyen HT, Sleeman K, Levine M, Mishin VP, Yang H, Guo Z, Okomo-Adhiambo M, Xu X, Stevens J, Gubareva LV. Cell culture-selected substitutions in influenza A(H3N2) neuraminidase affect drug susceptibility assessment. Antimicrob Agents Chemother. 2013;57:6141–6.
115. Hu Y, Lu S, Song Z, Wang W, Hao P, Li J, Zhang X, Yen HL, Shi B, Li T, Guan W, Xu L, Liu Y, Wang S, Zhang X, Tian D, Zhu Z, He J, Huang K, Chen H, Zheng L, Li X, Ping J, Kang B, Xi X, Zha L, Li Y, Zhang Z, Peiris M, Yuan Z. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. Lancet. 2013;381:2273–9.
116. Yen HL, McKimm-Breschkin JL, Choy KT, Wong DD, Cheung PP, Zhou J, Ng IH, Zhu H, Webby RJ, Guan Y, Webster RG, Peiris JS. Resistance to neuraminidase inhibitors conferred by an R292K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population. mBio. 2013;4:e00396–13.
117. Marjuki H, Mishin VP, Chesnokov AP, Jones J, De La Cruz JA, Sleeman K, Tamura D, Nguyen HT, Wu HS, Chang FY, Liu MT, Fry AM, Cox NJ, Villanueva JM, Davis CT, Gubareva LV. Characterization of drug-resistant influenza A(H7N9) variants isolated from an oseltamivir-treated patient in Taiwan. J Infect Dis. 2015;211:249–57.
118. Hatakeyama S, Sugaya N, Ito M, Yamazaki M, Ichikawa M, Kimura K, Kiso M, Shimizu H, Kawakami C, Koike K, Mitamura K, Kawaoka Y. Emergence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. JAMA. 2007;297:1435–42.
119. Hurt AC, Iannello P, Jachno K, Komadina N, Hampson AW, Barr IG, McKimm-Breschkin JL. Neuraminidase inhibitor-resistant and -sensitive influenza B viruses isolated from an untreated human patient. Antimicrob Agents Chemother. 2006;50:1872–4.
120. Okomo-Adhiambo M, Sleeman K, Ballenger K, Nguyen HT, Mishin VP, Sheu TG, Smagala J, Li Y, Klimov AI, Gubareva LV. Neuraminidase inhibitor susceptibility testing in human influenza viruses: a laboratory surveillance perspective. Viruses. 2010;2:2269–89.
121. Sheu TG, Deyde VM, Garten RJ, Klimov AI, Gubareva LV. Detection of antiviral resistance and genetic lineage markers in influenza B virus neuraminidase using pyrosequencing. Antiviral Res. 2010;85:354–60.
122. Escuret V, Frobert E, Bouscambert-Duchamp M, Sabatier M, Grog I, Valette M, Lina B, Morfin F, Ferraris O. Detection of human influenza A (H1N1) and B strains with reduced sensitivity to neuraminidase inhibitors. J Clin Virol. 2008;41:25–8.
123. Carr S, Ilyushina NA, Franks J, Adderson EE, Caniza M, Govorkova EA, Webster RG. Oseltamivir-resistant influenza A and B viruses pre- and postantiviral therapy in children and young adults with cancer. Pediatr Infect Dis J. 2011;30:284–8.
124. Higgins RR, Beniprashad M, Chong-King E, Li Y, Bastien N, Low DE, Gubbay JB. Recovery of influenza B virus with the H273Y point mutation in the neuraminidase active site from a human patient. J Clin Microbiol. 2012;50:2500–2.
125. Fujisaki S, Takashita E, Yokoyama M, Taniwaki T, Xu H, Kishida N, Sato H, Tashiro M, Imai M, Odagiri T. A single E105K mutation far from the active site of influenza B virus neuraminidase contributes to reduced susceptibility to multiple neuraminidase-inhibitor drugs. Biochem Biophys Res Commun. 2012;429:51–6.
126. Kim JH, Resende R, Wennekes T, Chen HM, Bance N, Buchini S, Watts AG, Pilling P, Streltsov VA, Petric M, Liggins R, Barrett S, McKimm-Breschkin JL, Niikura M, Withers SG. Mechanism-based covalent neuraminidase inhibitors with broad-spectrum influenza antiviral activity. Science. 2013;340:71–5.

Resistance Mechanisms to HIV-1 Nucleoside Reverse Transcriptase Inhibitors

33

Brian D. Herman, Robert A. Domaoal, Maryam Ehteshami, and Raymond F. Schinazi

1 Introduction

HIV-1 reverse transcriptase (RT) is essential for viral replication and is a major target for antiretroviral therapy. There are 26 FDA-approved drugs for the treatment of HIV, 12 are designed to target RT and some are the most widely prescribed agents especially as fixed-dose combinations. By inhibiting the enzyme required for copying the viral genome, viral replication can be stopped. RT is responsible for synthesizing double-stranded DNA from the viral single-stranded RNA genome during the process of reverse transcription. It is a DNA polymerase that can use RNA or DNA as a template and it also has RNase H activity, which cleaves RNA annealed to DNA [1–3].

RT is a heterodimer consisting of two subunits, a 66 kDa subunit called p66, and a 51 kDa subunit called p51. The p66 subunit contains the N-terminal polymerase domain and C-terminal RNase H domain linked by a connection domain. The p51 subunit is generated by proteolytic cleavage of p66 by the viral protease to remove the C-terminal RNase H domain and provides structural support for p66. The polymerase domain resembles the shape of a right hand with fingers, palm, thumb, and connection subdomains. The polymerase active site is in the palm subdomain. The RNase H domain contains the RNase H active site [4, 5]. RT does not have 3′–5′ exonuclease activity [6]. This can contribute to errors during polymerization that lead to mutagenesis.

There are many factors that contribute to the high genetic variability of HIV-1. Due to a very rapid replication rate, HIV-1 produces 10^7–10^9 new viral particles per day and has a high viral turnover resulting from an in vivo half-life of approximately 2 days [7, 8]. The HIV genome is comprised of two single RNA strands (ssRNA) and RT can transfer from one strand to the other during reverse transcription generating recombinant viral DNA sequences [9]. Reverse transcription is a primary source for genetic variation because RT DNA synthesis is error-prone with a rate of one misincorporation per 10^4 nucleotide incorporations at the enzyme level [10, 11]. The host RNA polymerase can also introduce mutations when transcribing the viral positive-sense DNA into mRNA. Suboptimal drug concentrations in certain compartments can also promote resistance selection [12]. These errors affect the translated viral proteins and future viruses that are produced. Each of these mechanisms generates diversity that can also lead to the development of drug resistance.

As a primary target for antiretroviral therapy, RT has two classes of drugs aimed at inhibiting its enzyme activity. Nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) target the polymerization activity of RT function by different mechanisms. We will focus on the NRTIs and the different mechanisms of resistance that develop. Mutations can develop that cause NRTI resistance by discriminating an NRTI-TP from the natural dNTP substrate. RT also has the ability to excise incorporated NRTIs from the DNA primer by reversing the polymerization reaction. C-terminal mutations also develop in response to drug selection and cause resistance by affecting RNase H activity. Understanding the unique mechanisms of resistance and the relationships among the mutations can help develop new and more effective inhibitors against HIV-1 that can suppress the selection of drug-resistant virus.

2 Nucleoside Reverse Transcriptase Inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs) are analogs of the natural 2′-deoxynucleoside and nucleotide substrates of DNA polymerases. The parent compounds require phosphorylation by host intracellular kinases and phosphotransferases to

B.D. Herman, Ph.D. • R.A. Domaoal, Ph.D.
M. Ehteshami, Ph.D. • R.F. Schinazi, Ph.D., D.Sc. (✉)
Department of Pediatrics, Laboratory of Biochemical Pharmacology, Center for AIDS Research, Emory University School of Medicine, Atlanta, GA, USA
e-mail: rschina@emory.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_33

form 2′-deoxynucleoside triphosphate (dNTP) analogs that can be incorporated by RT (reviewed in [13]). The efficient phosphorylation of NRTIs to their nucleoside triphosphate (NTP) form is essential to their efficacy. NRTI-TP compete with natural dNTPs for binding and incorporation onto the elongating DNA chain. Once incorporated, they act as obligate chain terminators because they lack the 3′-hydroxyl group on the ribose ring, and prevent further DNA synthesis. The active NRTI-TP forms have long intracellular half-lives compared to the parent compounds and have low protein binding [14]. Since there are four possible bases that can be incorporated, the RT is ideal since different nucleoside analogs can be combined effectively so long as they do not share the same first phosphorylation enzyme. Thus RT is considered a target within a target. In addition, NTPs do not diffuse out of the cells and hence usually have long intracellular half-lives.

The current FDA-approved NRTIs used in therapy represent analogs to all four of the natural dNTPs; two thymidine analogs: zidovudine (AZT) and stavudine (d4T); two cytosine analogs: lamivudine (3TC) and emtricitabine ((-)-FTC); the adenosine 2',3'-analog didanosine (ddI); and the guanosine analog abacavir (ABC). Tenofovir disoproxil fumarate (TDF) is an adenosine analog and a prodrug for the oral delivery of the nucleotide analog tenofovir (TFV) (Fig. 33.1). NRTIs remain the cornerstone of current antiretroviral treatment regimens. These regimens effectively suppress viral replication in HIV-infected persons, but can fail due to poor adherence, delayed toxicities, and emergence of drug-resistant virus. Under drug pressure, viral variants containing mutations that confer decreased susceptibility will have a selective advantage over drug-sensitive variants.

3 Mechanisms of NRTI Resistance

Mutations that cause resistance to NRTIs are located in the palm, fingers, and connection subdomains of RT and they use unique mechanisms to be effective (Table 33.1). Some mutations allow RT to discriminate between NRTI-TPs and the natural dNTPs by affecting binding and the rate of incorporation of the nucleotide analog. This involves amino acid residues in direct contact with the incoming NRTI that are located along the dNTP-binding track extending from the β3–β4 finger loop region to YMDD residue M184 (Fig. 33.2). Another set of mutations cause an increased rate of excision of incorporated NRTI-MP and are located close to the dNTP-binding site. These mutations are most often selected by thymidine analog NRTI and are thus termed thymidine analog mutations (TAMs). A recently identified group of mutations has long-range effects on NRTI incorporation by repositioning the primer/template. The effect of each mutation on NRTI and RT activity is unique and the relationship that develops with other mutations can be synergistic or antagonistic. This chapter explores mechanisms of NRTI resistance in more detail and includes information on approved as well as experimental nucleoside analogs.

4 Nucleotide Discrimination

The nucleotide discrimination mechanism of NRTI resistance involves the preferential incorporation of natural dNTP substrates over NRTI-TP substrates (Fig. 33.3). HIV-1 RT containing NRTI discrimination mutations maintains the ability to complete reverse transcription in the presence of drug by excluding NRTI-TP substrates while retaining the ability to incorporate natural dNTP substrates with reasonable efficiency [15, 16]. Discrimination mutations decrease NRTI-TP incorporation efficiency compared to wild-type RT through diminished binding and/or decreased incorporation rate [17]. Pre-steady-state kinetic analyses describe the catalytic efficiency of nucleotide incorporation using the ratio k_{pol}/K_d, where k_{pol} is the turnover rate for phosphodiester bond formation and K_d is the dissociation constant of the nucleotide for RT [18]. Mutations conferring enhanced NRTI-TP discrimination selectively increase K_d for NRTI-TP binding to the RT active site or decrease the k_{pol} value for nucleotide addition. Therefore, impaired NRTI-TP incorporation is a result of decreased binding affinity, slower incorporation rate, or a combination of both effects. The altered incorporation kinetics of NRTI-TP compared to the analogous dNTP results in increased selectivity for incorporation of natural dNTP substrates.

NRTI discrimination mutations occur at residues in the fingers or palm of the polymerase active site including K65R, L74V, Q151M (in complex with other mutations), and M184V/I, some of which form the dNTP-binding pocket [19–24] (Fig. 33.2). These mutations affect NRTI binding and incorporation through direct interaction with the nucleotide or via conformational distortions of the active site. These mutations and their discrimination phenotypes and mechanism toward specific NRTI are discussed below.

4.1 Didanosine

Didanosine (ddI, 2′,3′-dideoxyinosine) is a prodrug that is converted to 2′,3′-dideoxyadenosine triphosphate (ddATP), the active metabolite recognized by RT. After metabolism and activation, ddATP can be incorporated by HIV-1 RT into the nascent viral DNA, thus causing chain termination. In cell culture assays, the concentrations of ddI required to inhibit HIV-1 replication by 50 % (EC_{50}) were between 0.1 and 4 μM depending on the assay system and cell type [25]. ddATP binds RT with similar affinity as dATP; however, the rate of incorporation is >5-fold slower for ddATP compared to dATP [26].

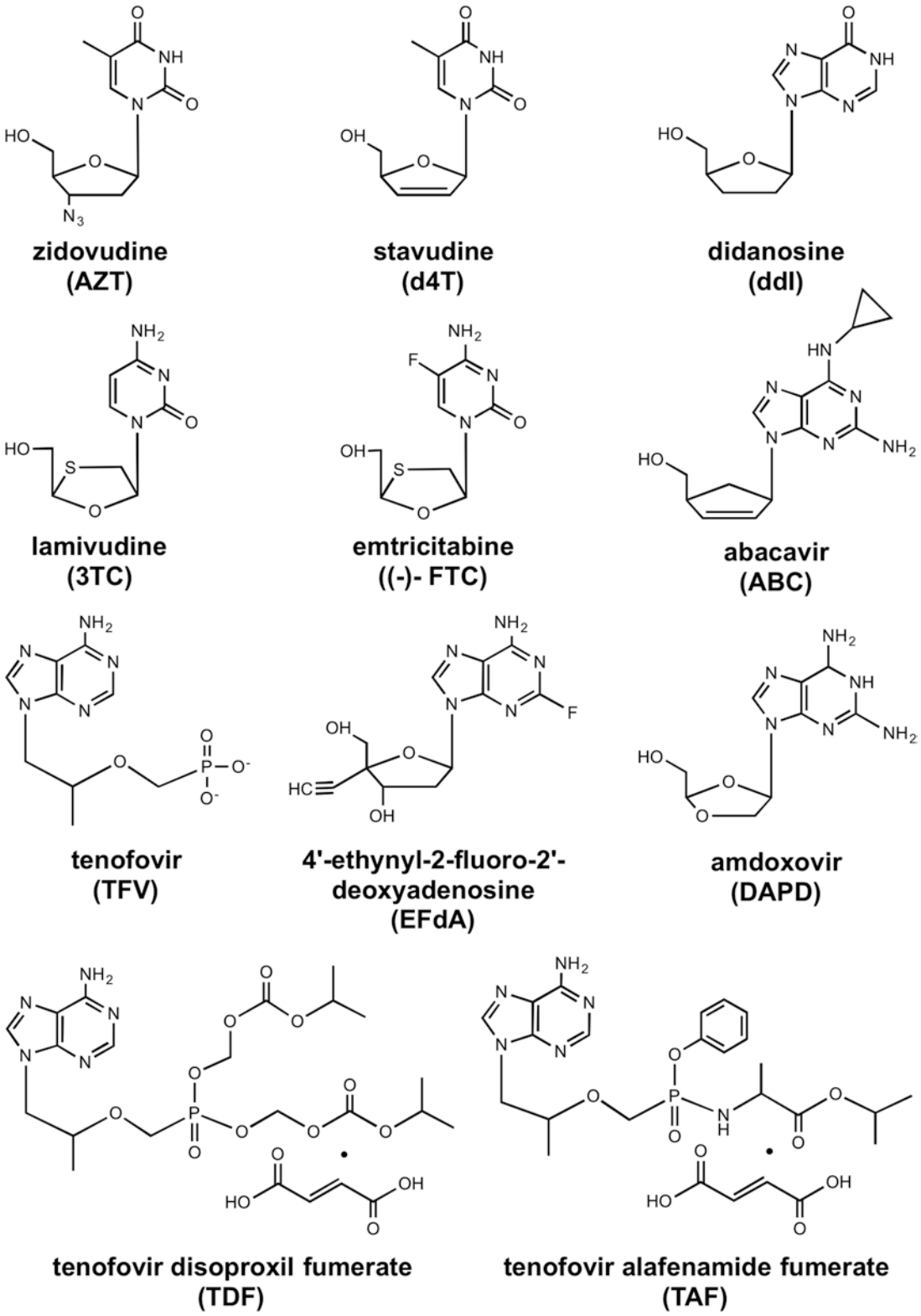


Fig. 33.1 Structures of FDA-approved and investigational NRTI

Clinical isolates from subjects who received ddI monotherapy for 1–2 years harbored the mutations L74V, K65R, M184V, as well as some TAMs, with L74V being most prevalent [27]. The L74V mutation confers at least fivefold resistance to ddI compared to wild-type (WT) HIV-1 and cross-resistance to ddC and ABC [27, 28]. Pre-steady-state incorporation assays with L74V RT indicate that the incorporation rate (k_{pol}) for ddATP is severely decreased (tenfold) compared to WT RT,

Table 33.1 Nucleoside RT inhibitor (NRTI) resistance mutations

Mutations	RT inhibitors
Discrimination	
M184V/I	3TC, FTC, ABC
K65R	3TC, FTC, ABC, TDF
K70E	3TC, FTC, ABC, TDF
L74V/I	ABC
Y115F	ABC, TDF
Q151M complex	All NRTIs
Excision	
M41L	ABC, TDF, AZT
D67N	AZT
K70R	AZT
L210W	ABC, TDF, AZT
T251Y/F	ABC, TDF, AZT
K219Q/E	AZT
T69 insertion	All NRTIs
C-terminal	
G333D	AZT, 3TC
N348I	AZT, d4T, NNRTIs
V371A	AZT, d4T
Q509L	AZT, d4T

indicating a discrimination phenotype [26]. The L74V mutation also causes reduced viral fitness due to decreased ability to incorporate natural dNTPs [29].

Additionally, AZT + ddI combination treatment can select a cluster of mutations with Q151M appearing first followed by A62V, V75I, F77L, and F116Y [30]. Together these mutations are referred to as the Q151M complex (Q151Mc). This complex results in high level of resistance to most NRTI (tenofovir is the exception) by the discrimination mechanism [24, 31]. The rate of incorporation of each NRTI was significantly decreased compared to that of its counterpart dNTP. It is believed that a loss of electrostatic interaction between Q151 and the incoming dNTP or NRTI is responsible for this mechanism [31]. RT harboring the Q151M complex of mutations displayed similar ability as WT RT to unblock NRTI-MP chain-terminated primers.

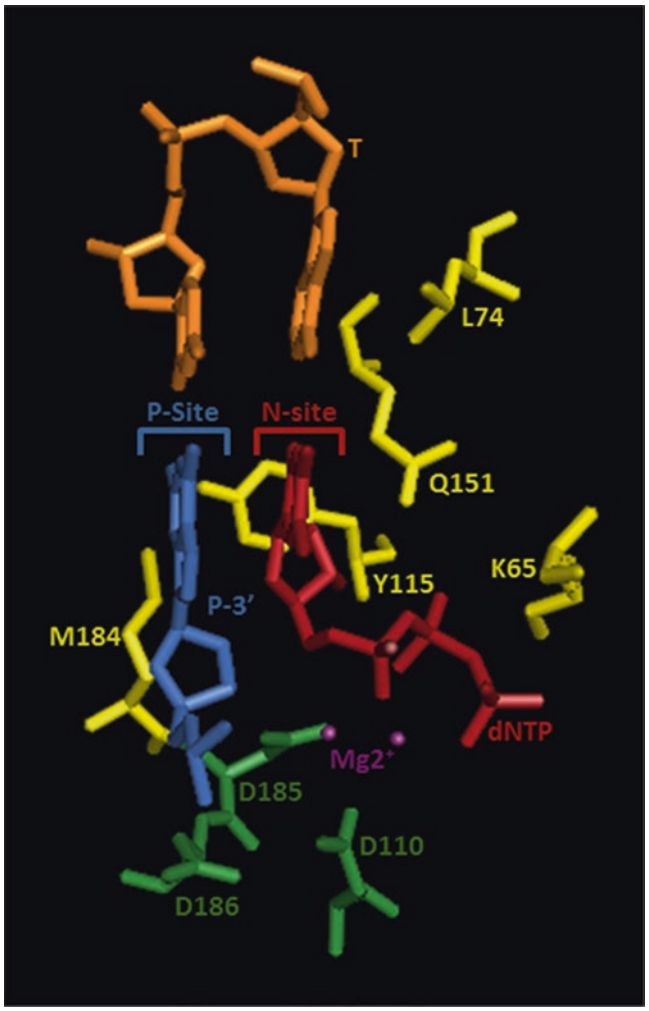


Fig. 33.2 Molecular model of the RT active site showing locations of residues mutated to confer resistance through nucleotide discrimination. The positions of the priming site (P-site) with the primer 3′-residue (P-3′) and the nucleotide-binding site (N-site) with a bound dNTP are indicated. The catalytic triad residues D110, D185, and D186 coordinate the two magnesium ions. Residues K65, L74, Y115, Q151, and M184 contact the incoming dNTP and are often mutated to confer resistance through the discrimination mechanism. The template nucleotides (T) are base paired with the primer 3′-residue and the incoming dNTP. The structure was drawn using PyMol based on the PDB coordinates 1RTD from Huang et al. [166]

4.2 Abacavir

Abacavir(ABC)[(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-en-1-yl-methanol] is a prodrug of carbovir (2-amino-1,9-dihydro-9-[(1*R*,4*S*)-4-(hydroxymethyl)-2-cyclopenten-1-yl]-6*H*-purin-6-one), a 2′-deoxyguanosine analog [32, 33]. Abacavir demonstrated equivalent activity to AZT against HIV-1 clinical isolates in peripheral blood lymphocytes with EC_{50} values of 0.26 and 0.23 μM, respectively. CBV-TP potently and selectively inhibited HIV-1 RT with an inhibitory constant (K_i) value of 21 nM [32].

Both cell culture selection experiments and clinical isolates from persons treated with ABC identified similar resistance mutations as those selected by ddI [34]. The most frequent mutations selected by ABC were M184V and the combination of L74V + M184V. Both of these mutations alone conferred low-level resistance (two- to fourfold over WT) to ABC. In combination, L74V + M184V conferred 11-fold ABC resistance over WT. Like L74V, M184V confers resistance to ABC and other NRTI through the discrimination mechanism [35–37]. Discrimination by M184V has been more thoroughly characterized for 3TC and (-)-FTC, so the molecular mechanism by which M184V discriminates NRTI is discussed further below.

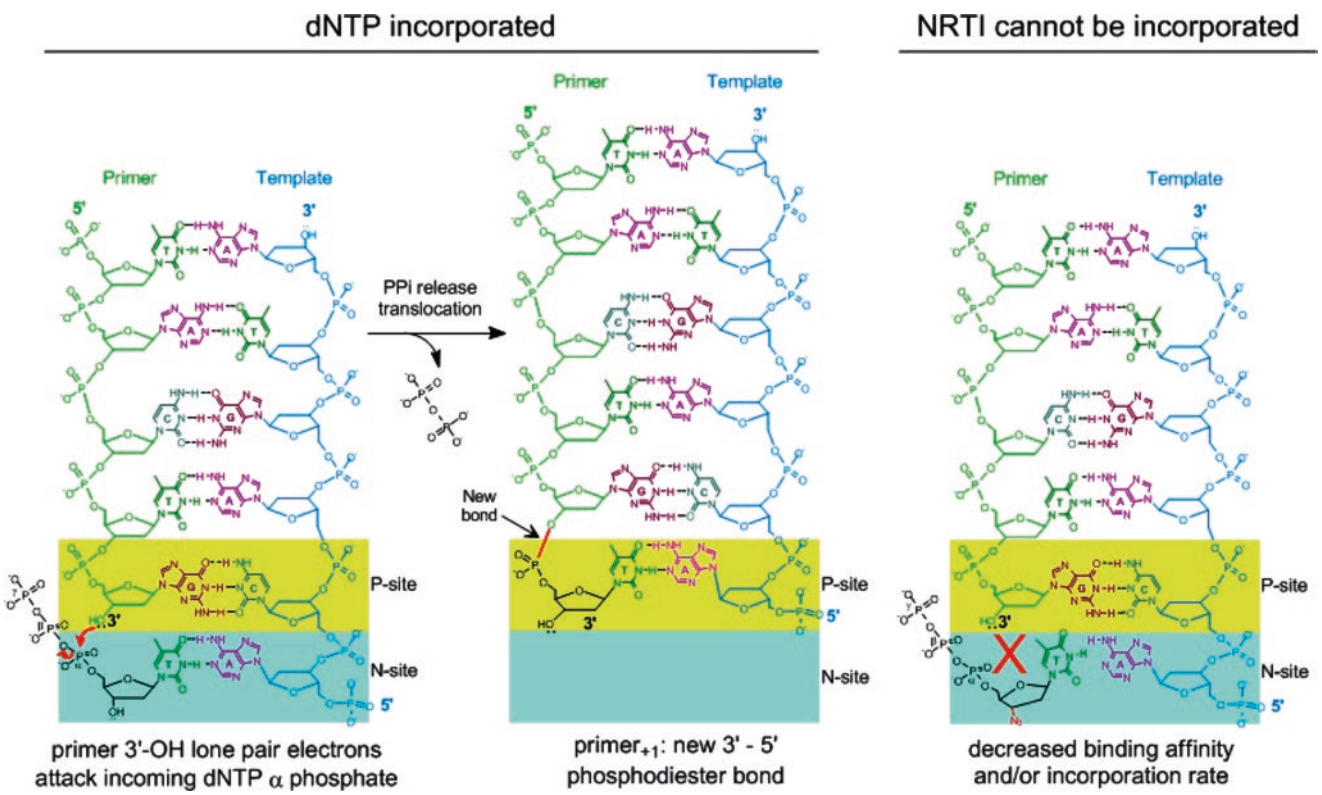


Fig. 33.3 Molecular mechanism of nucleotide incorporation and NRTI discrimination by HIV-1 RT. Mutations that confer resistance to NRTI by the discrimination mechanism maintain the ability to incorporate natural dNTP while selectively excluding NRTI-TP. NRTI-TP exclusion occurs by decreased NRTI-TP binding affinity (K_d) for the RT active site or decreased rate of NRTI incorporation (k_{pol})

4.3 Zalcitabine (ddC), Lamivudine (3TC), and Emtricitabine ((-)-FTC)

Zalcitabine (2′,3′-dideoxycytidine; ddC), lamivudine ((-)-3′-thia-2′,3′-dideoxycytidine; 3TC), and emtricitabine [(-)-3′-thia-5-flouro-2′,3′-dideoxycytidine], [(-)-FTC]; are the three cytidine analog NRTI and were FDA approved in 1992, 1994, and 1995, respectively [38]. Zalcitabine never saw widespread application due to delayed peripheral neuropathy that progressed with cumulative dose and did not resolve with stoppage of use [39]. Neuropathic cytotoxicity was later associated with potent inhibition of mitochondrial polymerase γ [40]. ddC was subsequently removed from the market in 2006 [41].

3TC and (-)-FTC are cytidine analogs that contain the unnatural L-enantiomer ribose with a sulfur atom replacing the C3′ position. (-)-FTC has an additional 5-fluoro modification to the cytosine ring. The modification on the sugar ring results in decreased affinity of 3TC-TP and (-)-FTC-TP as compared to ddCTP for polymerase γ [42].

3TC and (-)-FTC demonstrate antiviral activity against HIV-1 in cell culture and their nucleoside 5′-triphosphate forms are potent chain terminators of DNA synthesis upon incorporation by HIV-1 RT [43–45]. Cell culture selection as well as sequencing of patient isolates identified the mutations M184V or M184I to confer decreased susceptibility to 3TC and (-)-FTC [46–49]. M184I is selected within 2 weeks in persons receiving 3TC and is replaced with M184V within 2 months [49]. The M184V mutation conferred greater than 1400-fold resistance to 3TC and (-)-FTC and cross-resistance to ddI and ABC [47].

The mechanism of M184V resistance has been extensively characterized with structural and kinetic studies. Multiple kinetic studies have demonstrated a severely reduced catalytic efficiency of incorporation of 3TC-TP and (-)-FTC-TP by HIV-1 RT containing the M184V mutation [35–37]. 3TC-TP and (-)-FTC-TP demonstrated drastically impaired binding to M184V RT (K_d values increased 76- and 19-fold, respectively) compared to WT enzyme with relatively unchanged incorporation rates. The catalytic efficiencies of dCTP binding and incorporation for M184V and WT RT were similar, indicating that M184V can effectively discriminate between dCTP and 3TC-TP or (-)-FTC-TP [35–37]. A crystal structure of RT containing the M184I mutation revealed that branched side chains at position 184 (i.e., I and V) cause a steric clash with the L-enantiomer ribose of 3TC-TP or (-)-FTC-TP, but not with the natural D-ribose of dCTP

[19]. This explains why 3TC-TP and (-)-FTC-TP binding is more markedly diminished as compared to dCTP binding. Together, these findings describe the molecular mechanism of discrimination by which the M184V/I mutations confer resistance to 3TC and (-)-FTC.

3TC is also used for the treatment of hepatitis B virus (HBV) and has been shown to select for the M204V mutation in HBV which is structurally and functionally equivalent to M184V in HIV-1 [50]. The 2′-deoxynucleoside analog entecavir (ETV), approved for the treatment of HBV, also demonstrates activity against HIV-1 and is, therefore, not recommended to be used in HBV/HIV-1-coinfected individuals [51]. ETV remains potent against 3TC-resistant HBV; however, it has been shown to select for M184V HIV-1 in coinfected patients [52, 53]. The M184V mutation in RT results in decreased efficiency of ETV incorporation by both diminished binding and slower incorporation rate [54]. ETV, unlike other NRTI, contains a 3′-OH group allowing additional nucleotide incorporation. However, during extension of ETV-MP containing primers, increasing steric hindrance results in delayed chain termination primarily at position +3, thus protecting ETV-MP from nucleotide excision [55, 56].

4.4 Tenofovir, Tenofovir Disoproxil Fumarate, and Tenofovir Alafenamide

The acyclic nucleoside phosphonate tenofovir (*R*-9-(2-phosphonylmethoxypropyl)adenine) has no sugar ring structure, but contains an acyclic methoxypropyl linker between the base N9 atom and a non-hydrolyzable C-P phosphonate bond [57]. Tenofovir is poorly absorbed by the oral route and is, therefore, administered as a lipophilic orally bioavailable prodrug, tenofovir disoproxil fumarate (TDF), a fumaric acid salt of the bis-isopropoxycarbonyloxymethyl ester of tenofovir [57]. Degradation of TDF to its monoester and, subsequently, to tenofovir occurs readily in the intestinal mucosa by the action of carboxylesterases and phosphodiesterases, respectively [58]. Tenofovir is rapidly converted intracellularly to tenofovir-monophosphate (TFV-MP) and the active tenofovir-diphosphate (TFV-DP) forms by adenylate monophosphate kinase and 5′-nucleoside diphosphate kinase, respectively [59].

Cell culture selection experiments identified K65R as the predominant resistance mutation which conferred three- to fourfold resistance to TFV compared to WT HIV-1 [60]. TFV resistance in TDF-experienced patients is associated with the K65R mutation, often in combination with the S68N/K/ or G substitutions [61, 62]. It has been reported that the K70E mutation is also selected in HIV-1 patients receiving tenofovir in combination with other NRTI [63]. However, the K65R and K70E mutations are antagonistic to one another and are not believed to exist on the same genome [64]. The K65R mutation provides some level of cross-resistance to all approved NRTI except AZT [65]. In fact, giving AZT to persons treated with TDF could prevent the development of the K65R mutation commonly seen in persons infected with clade C HIV in Africa [66]. Both K65R and K70E mutations confer resistance to tenofovir via the discrimination mechanism [65, 67–70]. Unlike the discrimination of 3TC-TP and (-)-FTC-TP by M184V, the discrimination phenotype conferred by K65R and K70E toward TFV-DP is driven by decreased k_{pol}, and not by significantly altered binding [65, 67–70].

Tenofovir alafenamide (TAF) is a second-generation prodrug that delivers higher intracellular concentrations of TFV diphosphate (TFV-DP) in peripheral blood mononuclear cells, resulting in increased antiviral potency compared to TDF [71, 72]. TAF is currently in phase III clinical trials in combination with other antiretroviral agents for the treatment of HIV-1- and HBV-infected patients [73]. TAF is more potent than TFV and TDF against WT HIV-1 in cell culture experiments; however their resistance profiles against a panel of NRTI-resistant mutant viruses were highly correlated ($r^2 = 0.97$). Cell culture selection experiments using both TAF and TFV resulted in the emergence of the K65R mutation conferring 6.5-fold reduced susceptibility to TAF [72]. TAF remained potent against HIV-1 isolates resistant to non-nucleoside reverse transcriptase inhibitors, as well as HIV-1 protease and integrase inhibitors.

4.5 EFdA

4′-Ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) is an analog of 2′-deoxyadenosine that contains a 3′-OH that inhibits both HIV and HBV in culture [74]. EFdA inhibits WT and drug-resistant HIV-1 at sub-*nano*molar concentrations making it one of the most potent anti-HIV-1 agents reported to date [75, 76]. EFdA is approximately 500-fold more potent against HIV-1 compared to HBV. Molecular modeling studies by Takamatsu and colleagues indicate that a deeper hydrophobic binding pocket in the HIV-1 RT active site, compared to HBV RT, better accommodated the 4′-ethynyl moiety of bound EFdA-TP [74].

EFdA inhibits HIV-1 RT through multiple mechanisms in a sequence-dependent context [77]. Despite the presence of a 3′-OH, EFdA can block further nucleotide addition at the site of incorporation by preventing RT translocation. In the pre-translocation state RT can excise EFdA-monophosphate (MP), but it is readily converted back to the EFdA-MP-terminated form. EFdA can act as a delayed chain terminator by the incorporation of one additional nucleotide following EFdA-MP incorporation, thus preventing EFdA-MP primer unblocking. Lastly, although EFdA is efficiently misincorporated by RT, the resulting mismatched primer is inefficiently extended, therefore providing additional opportunities for chain termination.

The varied mechanisms of EFdA RT inhibition have been implicated as rational for its potent inhibition of WT HIV-1 and

its favorable drug resistance profile. EFdA potently inhibited clinical isolates from subjects heavily treated with NRTI including multidrug-resistant HIV-1 [75]. Interestingly, the tenofovir resistance mutation K65R confers hypersusceptibility to EFdA through a decreased ability to unblock EFdA-MP-terminated primers [78]. Cell culture selection experiments using WT_{LAI} HIV-1 identified the combination of mutations I142V, T165R, and M184V, which conferred moderate reduced susceptibility to EFdA, but were also associated with significantly reduced viral fitness. The M184V/I mutations were also present in isolates from SIV-infected macaques though viral replication remained suppressed through drug treatment [79]. An in vitro selection experiment using a mix of 11 highly drug-resistant HIV-1 isolates found a delayed emergence of resistance toward EFdA compared to TDF, 3TC, and (-)-FTC [80]. Furthermore, EFdA remained highly potent against variants selected against EFdA and TDF despite the appearance of M184V in all isolates. EFdA's unique inhibitory mechanisms and excellent resistance profile warrant further investigation as a clinical candidate for the treatment of NRTI-naive and -experienced patients.

4.6 Amdoxovir (DAPD) and Dioxolane Guanosine

The purine nucleoside analog 1-β-D-dioxolane guanosine (DXG) has potent activity against HIV and hepatitis B virus [81]. However, it demonstrates poor solubility and limited oral bioavailability in monkeys [82]. The analog 1-β-D-2,6-diaminopurine dioxolane (amdoxovir, DAPD) also exhibits antiviral activity and is more water soluble and orally bioavailable [81, 82]. DAPD serves as a prodrug for DXG by deamination at the six-position by adenosine deaminase [83, 84]. Only DXG-TP was detected in primary lymphocytes and CEM cells following exposure to DXG or DAPD.

DAPD has potent activity against NRTI-resistant HIV-1 containing TAMs, M184V, and the NNRTI resistance mutation K103N. The mutations L74V and K65R resulted in 3.5- and 5.6-fold resistance to HIV-1 in cell culture assays [85, 86]. Pre-steady-state kinetic analyses reveal that both K65R and L74V mutations confer resistance to DAPD through a combination of decreased nucleotide affinity and slower incorporation rate [87].

5 Excision

Another mechanism of NRTI resistance is by the process of excision, in which RT uses a phosphate donor to remove incorporated NRTIs by reversing the catalytic reaction of polymerization [88, 89] (Fig. 33.4). A set of mutations is primarily selected by the thymidine analogs AZT and d4T. The classical mutations are M41L, D67N, K70R, L210W, T215Y/F, and K219Q and are known as thymidine analog mutations (TAMs) [90–92]. They were initially observed in subjects receiving AZT monotherapy, the first approved AIDS drug [90–93].

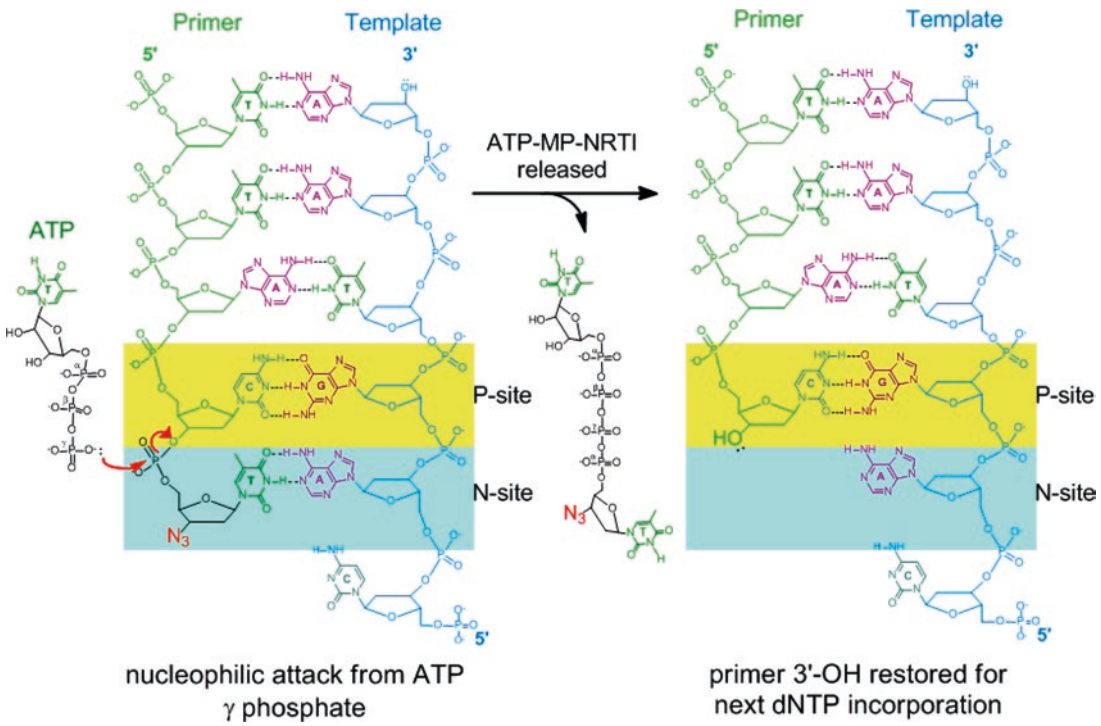


Fig. 33.4 Molecular mechanism of HIV-1 RT primer unblocking by ATP-mediated NRTI-MP excision. Nucleophilic attack of the chain-terminating NRTI-MP from an ATP pyrophosphate donor results in the release of an adenosine-NRTI dinucleoside tetraphosphate and restores the primer 3′-OH for subsequent nucleotide incorporation

AZT was identified in 1985 at the National Cancer Institute with collaborators at the Burroughs-Wellcome Company (now GlaxoSmithKline) to be the first nucleoside inhibitor with in vitro and in vivo activity against HIV [94]. It was shown to inhibit RT as a triphosphate and became the first NRTI [95]. Structurally, AZT replaces the 3′-hydroxyl with a 3′-azido group and is dependent on thymidine kinase 1 (TK1) for activation [95, 96]. AZT is no longer recommended for initial combination regimens for antiretroviral naive persons, but is still recommended for preexposure prophylaxis and postexposure treatment of mother-to-child transmission of HIV during pregnancy, labor, and delivery [97, 98]. AZT is one of the most effective NRTIs for central nervous system (CNS) penetration and has shown to improve cognitive performance in a study of HIV dementia [99].

TAMs are also selected in response to the thymidine analog d4T. Its structure has an unsaturation in the ribose ring to form a 2′,3′-dideoxy-2′,3′-dehydroribose ring analog that is also dependent on TK1 for activation. d4T has been shown to cause peripheral neuropathy that can be corrected by using a lower dosage [100]. This NRTI is rarely used in the USA, but is still used in resource-poor settings [101].

The mutations that develop to thymidine analogs were biochemically shown to excise the NRTI after it was incorporated into the DNA [88, 89]. Excision is the primary mechanism of AZT resistance. RT can excise normal nucleotides or NRTIs by phosphorolysis, the reverse of the polymerization reaction, using pyrophosphate or ATP as a phosphate donor. The RT/primer template complex binds ATP, the phosphate donor, and the gamma phosphate attacks the monophosphate group linking the last two nucleotides of the primer strand generating an AZT/2′-deoxyadenosine dinucleotide tetraphosphate product. The primer, shortened by one nucleotide, contains a free 3′-OH competent for additional nucleotide incorporation (Fig. 33.4). The reaction was initially discovered using DNA templates, but can also use RNA less efficiently [102, 103]. An RT that has TAMs has an increased rate of excision using ATP as the phosphate donor [104, 105]. The mutations help create a pocket for ATP to bind that is adjacent to the dNTP-binding cleft and excise the NRTI or nucleotide [106]. Using ATP for excision generates an AZTp4A product that cannot be reincorporated and is not a substrate for RT. Structural information revealed that the AZT resistance mutations K70R and T215Y are important to ATP binding. The T215Y mutation has a mutated aromatic side chain that stacks with the base and the K70R-mutated side chain forms polar interactions with the alpha phosphate and 3′-OH of ATP [106]. The β- and γ-phosphates of ATP chelate the two active site Mg^{2+} ions to allow phosphorolysis to occur to remove AZT-MP.

The primary mechanism for excision is ATP-dependent phosphorolysis for several reasons. There is a relatively low concentration or pyrophosphate in a cell. Also, a dNTP would favor binding at the N (nucleotide binding) site of the polymerase active site to form a polymerase catalytic complex rather than an excision complex [107]. An NTP with a purine base would have more extensive stacking with the T215Y mutant than a pyrimidine base [106]. GTP has also been shown to be an efficient excision substrate; however ATP has a much higher concentration in cells than GTP [88, 108, 109].

ATP-mediated excision can be inhibited by the formation of a dead-end complex (DEC) [110, 111]. When an NRTI is incorporated onto the end of the primer it is located in the N site of the polymerase active site. After incorporation, it translocates to the P (priming) site and the N site becomes empty. The next complementary dNTP can then bind in the N site. The end of the primer is blocked by the NRTI missing the necessary 3′-OH group for phosphodiester bond formation and the next complementary dNTP cannot be incorporated. The dNTP binding stabilizes the complex by decreasing the dissociation rate of RT and trapping RT and the primer/template in the DEC. The finger domain also closes after dNTP binding and makes the complex more stable. Excision of an NRTI by ATP-dependent pyrophosphorolysis can only be conducted when the 3′ end of the primer is located in the N site [105, 112, 113]. The identity of the incorporated chain terminator, the primer/template sequence, and the mutational background of RT all contribute to the stability of the DEC. Its formation inhibits excision and is beneficial for drug activity.

Modifications of the NRTI that increase the terminated primer fraction in the N site favor the excision mechanism. AZT remains resistant to DEC formation due to its 3′-azido group. The bulkiness of the group prevents translocation to the P site after binding at the N site. This leaves AZT more susceptible to excision and prevents the next complementary dNTP from binding to the N site and forming the DEC [105]. The other NRTIs do not have bulky modifications, such as an azido group at the 3′ position, and will shift toward DEC formation rather than the excision reaction. Unlike d4T, AZT does not form DEC at physiological dNTP concentrations, explaining why TAMs confer higher resistance to AZT than d4T [114].

The contribution of each of the mutations that make up the TAMs is complex with many developing in supportive roles to enhance excision and to increase viral fitness [106, 115, 116]. TAMs develop in two distinct but overlapping patterns. The K70R background gives rise to the D67N, T215F, and K219Q/E mutations, and the other includes M41L, and L210W associated with T215Y [91, 93, 117–121]. The T215Y and K219Q mutations are located in the palm domain and increase the processivity of DNA synthesis by decreasing RT dissociation from the primer/template. They are also known to contribute to the prevention of DEC formation. The mutations D67N and K70R are located in the finger

domain and do not affect processivity but increase excision. The D67N mutation changes the aspartic acid into an asparagine and removes the negative charge. The K70R is often the first mutation selected during AZT therapy and repositions the positive charge allowing for a better interaction with the phosphate donor of ATP or PPi [106]. The T215Y/F mutation also enhances binding to ATP or PPi [112, 122]. A combination of TAMs and a T69 insertion in the β-3–β-4 finger loop allows RT to excise a broader range of NRTIs [112, 123–125]. AZT excision can be enhanced by the addition of the Δ67 deletion with a TAM background [126, 127].

6 C-Terminal Mutations

Mutations that lead to either discrimination-based or excision-based mechanisms of resistance are generally selected at the site of NRTI binding, namely in the polymerase active site located at the N-terminus of RT. It was therefore a somewhat unexpected discovery when mutations in the distal connection domain and RNase H domain of RT were found to be associated with NRTI treatment (reviewed in [128, 129]). Specifically, it was found that several C-terminal mutations are frequently selected in association with TAMs, and cause resistance to predominantly AZT and d4T. Resistance has also been reported to 3TC, ddI, d4T, ABC, and TFV to varying degrees, with various C-terminal mutations [130, 131]. Finally, some C-terminal mutations can also confer resistance to non-nucleoside analog inhibitors (NNRTIs). A complete review of NNRTI-associated resistance can be found elsewhere [132]. This section reviews the prevalence of C-terminal mutations, their mechanism of drug resistance, and their impact on clinical outcome.

6.1 Prevalence of C-Terminal Mutations

The N348I mutation selected in the connection domain of RT has a prevalence of ~12 % in treatment-experienced patients. This mutation is associated with AZT and nevirapine (NNRTI) treatment and appears early during therapy [130, 131, 133–135]. Mutations A360V/I and A371V also show a 10 % and 20 % prevalence in patients treated with AZT, respectively [136]. Mutation G333D is associated with a 12 % increase in prevalence after treatment with AZT and 3TC [135, 137–139]. Other mutations in connection domain that are associated with TAM selection include amino acid changes at residues 322, 356, 359, 360, 369, and 371 [133, 140]. Similar to N348I, amino acid changes at positions 359 and 371 have also been associated with other NNRTI-associated mutations such as K103N and Y181C [130, 131]. In the RNase H domain, the Q509L mutation has been selected in AZT-treated cell culture but rarely appears in vivo [141]. Other RNase H mutations identified in vivo include changes at positions 506, 547, 469, 470, 554, and 558. However most of these positions appear to be highly polymorphic [135, 136, 142, 143].

6.2 Mechanism of NRTI Resistance with C-Terminal Mutations

A number of studies have addressed the mechanism through which C-terminus mutations confer resistance to NRTIs (summarized in Fig. 33.5). Nikolenko et al. proposed that C-terminus mutations reduce RNase H activity. This, in turn, allows more time for ATP-mediated removal of the incorporated chain terminator at the polymerase active site [144–147]. To test this

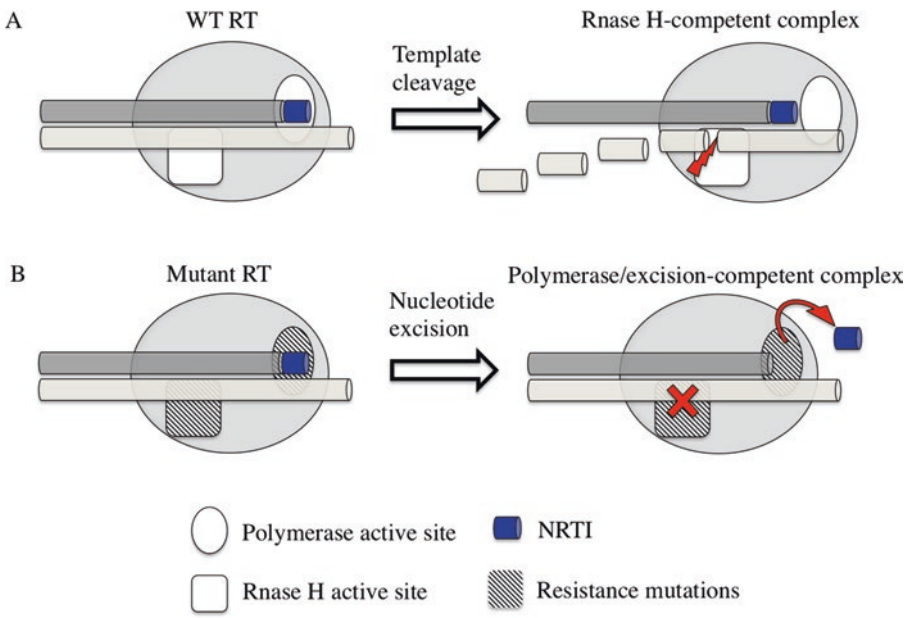


Fig. 33.5 Mechanism of NRTI resistance with C-terminal mutations. (**a**) WT RT engages the RNA/DNA substrate in both polymerase and RNase H active sites. After chain termination with incoming NRTI, the RNA template is degraded in the RNase H-competent complex, leading to complex dissociation and insufficient NRTI excision. (**b**) RT harboring C-terminal mutation has reduced RNase activity. This favors the occurrence of polymerase/excision-competent complexes where the incorporated NRTI occupies the polymerase active site. Increased ATP-dependent excision of incorporated NRTI is in turn facilitated through the selection of resistance-conferring mutations at this site

hypothesis, the authors monitored template switching by RT containing C-terminal mutations as an indication of RNase H activity. They demonstrated that the presence of these mutations led to reduced template switching, which in turn correlates with reduced RNase H activity [146]. This hypothesis was further supported by the observation that C-terminal mutations conferred AZT resistance on RNA/DNA, but not DNA/DNA substrates [131, 141]. Radzio et al. further showed that artificial increases in RNase H activity led to decreased AZT excision, thus highlighting the interplay between RNA cleavage and nucleotide excision [148]. In another study, Ehteshami et al. showed that the presence of A360V and N348I mutations selectively reduced DNA/RNA substrate binding in the RNase H-complex, thus accounting for the observed reduction in RNA cleavage. Interestingly, they observed that when RNase H activity was knocked out through the introduction of E478Q mutant, RT enzymes harboring TAMs/A360V/N348I still showed increased AZT excision as compared to TAMs alone. This suggests that at least some connection domain mutations may increase resistance to AZT in an RNase H-independent manner. Further studies showed that N348I and A360V increase enzyme processivity, which may play a role in increased rates of AZT removal [149].

As described above, the role of C-terminal mutations on antiviral resistance was clearly demonstrated in numerous cell-based, and cell-free studies. Importantly, current standard protocols for HIV genetic testing are based on sequencing the N-terminal region of RT while omitting the C-terminal region. Considering the relatively high frequency of C-terminal mutation selection in the clinic, it was unclear whether the genetic testing of RT should be expanded to include the C-terminal region of this enzyme. Since the discovery of this class of mutations, several independent studies have examined whether C-terminal mutations play an important role in treatment failure in the clinical setting. Considering the strong selection association between C-terminal mutations and other NRTI-associated mutations, it appears that current genetic testing standards are sufficient and additional screening for C-terminal mutations will likely not have a significant impact on clinical outcome [134, 150]. Based on this, current guidelines do not recommend routine genetic testing of the C-terminal region of HIV RT [151, 152].

7 NRTI Synergy and Antagonism Between Resistance Mechanisms

The use of two NRTI together with an antiretroviral drug from another class is currently recommended for combination antiretroviral therapy because several studies have shown synergy between NRTI combinations, such as AZT/ddI and AZT/3TC (combivir) [28, 153–157]. The observed antiviral synergy of these combinations has been attributed to antagonism between the discrimination mutations K65R, L74V, and M184V and TAMs [69, 158–165]. K65R, K70E, L74V, or M184V when present in combination with TAMs reverse the AZT resistance phenotype observed with TAMs alone. The rate of primer unblocking by ATP-mediated excision is decreased by the addition of K65R, K70E, L74V, and M184V in the background of TAMs [69, 70, 162, 164]. Multiple hypotheses have been proposed to explain how these residues diminish excision activity. K65R and M184V may reposition or limit the flexibility of the chain-terminated primer in the active site [158, 161]. L74V may alter the base pairing interaction of the NRTI with the template [158, 166]. Additionally, TAMs also negatively affect the ability of K65R to discriminate between NRTI-TP and dNTP by partially restoring the rate of incorporation [69].

The poor excision and discrimination phenotypes observed with viruses carrying both TAMs and discrimination mutations can be partially restored by the accumulation of additional mutations [131, 138, 139, 157, 167, 168]. For example, the G333D mutation appears to suppress the antagonistic relationship between M184V and TAMs, thus allowing for dual-AZT and -3TC resistance [139]. Another study has shown that TFV resistance becomes significant when N348I is present with TAMs [169]. The N348I mutation is also selected in association with M184V and TAMs. However, it was shown that the presence of this mutation does not result in dual-3TC/AZT resistance [131, 146]. The N348I mutation is thought to neutralize the antagonizing effect of M184V on TAM-mediated AZT resistance, likely through restoring deficits in RT processivity and viral fitness [134, 170, 171]. Other mutations that can mediate dual-discrimination-based and excision-based resistance include amino acid changes E44A/D, T69D, V75M/T, V118I, H208Y, R211K, and K219R.

8 Combination Therapy

Nucleoside analog inhibitors are an important component of antiretroviral combination therapy [172]. Most triple-drug regimens are comprised of two NRTIs and a third antiretroviral agent from a different drug class (such as NNRTIs, integrase inhibitors, or protease inhibitors). The current recommended first-line combination therapy includes (-)-FTC and TDF (sometimes combined in a single pill known as Truvada) in addition to an integrase inhibitor (such as raltegravir and dolutegravir), or a protease inhibitor (such as darunavir/ritonavir) [97]. Other recommended fixed-dose combinations include Atripla [(-)-FTC, TDF and efavirenz], Complera ((-)-FTC, TDF, rilpivirine), Stribild ((-)-FTC, TDF, elvitegravir), Triumeq (ABC, 3TC and dolutegravir), Epzicom (ABC + 3TC), as well as other multi-pill combinations with

protease inhibitors such as atazanavir (boosted with ritonavir) [173, 174]. Combination of TDF with 3TC or (-)-FTC has shown to be particularly effective as the long half-life of these drugs allows for reduced daily pill burden. This, in combination with reduced toxicity, improves adherence to treatment and clinical outcomes [175]. (-)-FTC + TDF combination also has a favorable resistance profile. The M184V mutation selected by (-)-FTC or 3TC treatment results in the generation of a virus with impaired viral fitness. Similarly, the K65R mutation is rarely selected in TDF-treated patients. The antagonistic relationship between K65R and M184V mutations may also play a role in increasing the barrier to drug resistance as dual-TDF/(-)-FTC resistance is not frequently observed in the clinic [175–177].

9 Conclusion

The development of resistance to NRTIs can limit their effectiveness during HIV treatment and it is important to understand the mechanisms and the mutations that are involved. Discrimination, excision, and C-terminal mutations apply unique strategies and specific mutations for overcoming NRTI inhibition and their relationships are complex. Their characteristics can be used to develop new strategies for treatment regimens and drug combinations can be developed that will be more effective due to incompatible resistance mechanisms. On the horizon are more potent antiviral nucleoside analogs for HIV infections as well as long-acting nanoparticle formulations. These novel approaches should increase compliance and provide convenient regimens until a "cure" is found for HIV. In the meantime, all of this knowledge and these advances could contribute to the development of novel anti-HIV compounds that are highly effective against HIV drug resistance mechanisms and reduce transmission of drug-resistant viruses. Let's not forget that "dead viruses don't mutate." Complete viral suppression is the ultimate goal of antiviral therapy [178].

Acknowledgements This work was supported in part by NIH grant 1RO1MH100999-01 and 5P30-AI-50409 (CFAR).

References

1. Arts EJ, Wainberg MA. Mechanisms of nucleoside analog antiviral activity and resistance during human immunodeficiency virus reverse transcription. Antimicrob Agents Chemother. 1996;40(3):527–40.
2. Harrich D, Hooker B. Mechanistic aspects of HIV-1 reverse transcription initiation. Rev Med Virol. 2002;12(1):31–45.
3. Coffin JM, Hughes SH, Varmus HE. Retroviral virions and genomes in retroviruses. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1997.
4. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science. 1992;256(5065):1783–90.
5. Jacobo-Molina A, Ding J, Nanni RG, Clark Jr AD, Lu X, Tantillo C, Williams RL, Kamer G, Ferris AL, Clark P, et al. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proc Natl Acad Sci U S A. 1993;90(13):6320–4.
6. Roberts JD, Bebenek K, Kunkel TA. The accuracy of reverse-transcriptase from HIV-1. Science. 1988;242(4882):1171–3.
7. Coffin JM. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science. 1995;267(5197):483–9.
8. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and Cd4 LYMPHOCYTES in HIV-1 infection. Nature. 1995;373(6510):123–6.
9. Hu WS, Temin HM. Retroviral recombination and reverse transcription. Science. 1990;250(4985):1227–33.
10. Pathak VK, Temin HM. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. Proc Natl Acad Sci U S A. 1990;87(16):6019–23.
11. Mansky LM, Temin HM. Lower in-vivo mutation-rate of human-immunodeficiency-virus type-1 than that predicted from the fidelity of purified reverse-transcriptase. J Virol. 1995;69(8):5087–94.
12. Kepler TB, Perelson AS. Drug concentration heterogeneity facilitates the evolution of drug resistance. Proc Natl Acad Sci U S A. 1998;95(20):11514–9.
13. Herman BD, Sluis-Cremer N. Molecular pharmacology of nucleoside and nucleotide HIV-1 reverse transcriptase inhibitors. In: Gallelli L, editor. Pharmacology. InTech; 2012. DOI: 10.5772/32969. Available from: http://www.intechopen.com/books/pharmacology/molecular-pharmacologyof-nucleoside-and-nucleotide-hiv-1-reverse-transcriptase-inhibitors
14. Bazzoli C, Jullien V, Le Tiec C, Rey E, Mentre F, Taburet AM. Intracellular pharmacokinetics of antiretroviral drugs in HIV-infected patients, and their correlation with drug action. Clin Pharmacokinet. 2010;49(1):17–45.
15. Cihlar T, Ray AS. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. Antiviral Res. 2010;85(1):39–58.
16. Sarafianos SG, Clark Jr AD, Das K, Tuske S, Birktoft JJ, Ilankumaran P, Ramesha AR, Sayer JM, Jerina DM, Boyer PL, Hughes SH, Arnold E. Structures of HIV-1 reverse transcriptase with pre- and post-translocation AZTMP-terminated DNA. EMBO J. 2002;21(23):6614–24.
17. Isel C, Ehresmann C, Walter P, Ehresmann B, Marquet R. The emergence of different resistance mechanisms toward nucleoside inhibitors is explained by the properties of the wild type HIV-1 reverse transcriptase. J Biol Chem. 2001;276(52):48725–32.
18. Reardon JE. Human immunodeficiency virus reverse transcriptase: steady-state and pre-steady-state kinetics of nucleotide incorporation. Biochemistry. 1992;31(18):4473–9.
19. Sarafianos SG, Das K, Clark Jr AD, Ding J, Boyer PL, Hughes SH, Arnold E. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. Proc Natl Acad Sci U S A. 1999;96(18):10027–32.
20. Martin JL, Wilson JE, Haynes RL, Furman PA. Mechanism of resistance of human immunodeficiency virus type 1 to 2′,3′-dideoxyinosine. Proc Natl Acad Sci U S A. 1993;90(13):6135–9.
21. Harrigan PR, Stone C, Griffin P, Najera I, Bloor S, Kemp S, Tisdale M, Larder B. Resistance profile of the human immunodeficiency virus type 1 reverse transcriptase inhibitor abacavir (1592U89) after monotherapy and combination therapy. CNA2001 Investigative Group. J Infect Dis. 2000;181(3):912–20.

22. Margot NA, Lu B, Cheng A, Miller MD, Study T. Resistance development over 144 weeks in treatment-naive patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in Study 903. HIV Med. 2006;7(7):442–50.
23. Ueno T, Shirasaka T, Mitsuya H. Enzymatic characterization of human immunodeficiency virus type 1 reverse transcriptase resistant to multiple 2′,3′-dideoxynucleoside 5′-triphosphates. J Biol Chem. 1995;270(40):23605–11.
24. Menendez-Arias L. Mechanisms of resistance to nucleoside analogue inhibitors of HIV-1 reverse transcriptase. Virus Res. 2008;134(1–2):124–46.
25. Ahluwalia G, Cooney DA, Mitsuya H, Fridland A, Flora KP, Hao Z, Dalal M, Broder S, Johns DG. Initial studies on the cellular pharmacology of 2′,3′-dideoxyinosine, an inhibitor of HIV infectivity. Biochem Pharmacol. 1987;36(22):3797–800.
26. Deval J, Navarro JM, Selmi B, Courcambeck J, Boretto J, Halfon P, Garrido-Urbani S, Sire J, Canard B. A loss of viral replicative capacity correlates with altered DNA polymerization kinetics by the human immunodeficiency virus reverse transcriptase bearing the K65R and L74V dideoxynucleoside resistance substitutions. J Biol Chem. 2004;279(24):25489–96.
27. Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T, Merigan TC. Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy for 1 to 2 years. Antimicrob Agents Chemother. 1997;41(4):757–62.
28. St Clair MH, Martin JL, Tudor-Williams G, Bach MC, Vavro CL, King DM, Kellam P, Kemp SD, Larder BA. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science. 1991;253(5027):1557–9.
29. Sharma PL, Crumpacker CS. Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation. J Virol. 1997;71(11):8846–51.
30. Shirasaka T, Kavlick MF, Ueno T, Gao WY, Kojima E, Alcaide ML, Chokekijchai S, Roy BM, Arnold E, Yarchoan R, et al. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc Natl Acad Sci U S A. 1995;92(6):2398–402.
31. Deval J, Selmi B, Boretto J, Egloff MP, Guerreiro C, Sarfati S, Canard B. The molecular mechanism of multidrug resistance by the Q151M human immunodeficiency virus type 1 reverse transcriptase and its suppression using alpha-boranophosphate nucleotide analogues. J Biol Chem. 2002;277(44):42097–104.
32. Daluge SM, Good SS, Faletto MB, Miller WH, Clair MHS, Boone LR, Tisdale M, Parry NR, Reardon JE, Dornsife RE, Averett DR, Krenitsky TA. 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. Antimicrob Agents Chemother. 1997;41(5):1082–93.
33. Faletto MB, Miller WH, Garvey EP, Clair MHS, Daluge SM, Good SS. Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. Antimicrob Agents Chemother. 1997;41(5):1099–107.
34. Tisdale M, Alnadaf T, Cousens D. Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. Antimicrob Agents Chemother. 1997;41(5):1094–8.
35. Krebs R, Immendorfer U, Thrall SH, Wohrl BM, Goody RS. Single-step kinetics of HIV-1 reverse transcriptase mutants responsible for virus resistance to nucleoside inhibitors zidovudine and 3-TC. Biochemistry. 1997;36(33):10292–300.
36. Feng JY, Anderson KS. Mechanistic studies examining the efficiency and fidelity of DNA synthesis by the 3TC-resistant mutant (184V) of HIV-1 reverse transcriptase. Biochemistry. 1999;38(29):9440–8.
37. Wilson JE, Aulabaugh A, Caligan B, McPherson S, Wakefield JK, Jablonski S, Morrow CD, Reardon JE, Furman PA. Human immunodeficiency virus type-1 reverse transcriptase. Contribution of Met-184 to binding of nucleoside 5′-triphosphate. J Biol Chem. 1996;271(23):13656–62.
38. De Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. Int J Antimicrob Agents. 2009;33(4):307–20.
39. Yarchoan R, Perno CF, Thomas RV, Klecker RW, Allain JP, Wills RJ, McAtee N, Fischl MA, Dubinsky R, McNeely MC, et al. Phase I studies of 2′,3′-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). Lancet. 1988;1(8577):76–81.
40. Chen CH, Cheng YC. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′,3′-dideoxycytidine. J Biol Chem. 1989;264(20):11934–7.
41. Roche-Pharmaceuticals. M.D./Alert. [Letter]. 2006 [cited 2012 June 10]. http://www.fda.gov/downloads/drugs/drug/safety/drug-shortages/ucm086099.pdf.
42. Lee H, Hanes J, Johnson KA. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. Biochemistry. 2003;42(50):14711–9.
43. Schinazi RF, McMillan A, Cannon D, Mathis R, Lloyd RM, Peck A, Sommadossi JP, Clair MS, Wilson J, Furman PA, et al. Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine. Antimicrob Agents Chemother. 1992;36(11):2423–31.
44. Hart GJ, Orr DC, Penn CR, Figueiredo HT, Gray NM, Boehme RE, Cameron JM. Effects of (-)-2′-deoxy-3′-thiacytidine (3TC) 5′-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. Antimicrob Agents Chemother. 1992;36(8):1688–94.
45. Coates JA, Cammack N, Jenkinson HJ, Mutton IM, Pearson BA, Storer R, Cameron JM, Penn CR. The separated enantiomers of 2′-deoxy-3′-thiacytidine (BCH 189) both inhibit human immunodeficiency virus replication in vitro. Antimicrob Agents Chemother. 1992;36(1):202–5.
46. Gao Q, Gu Z, Parniak MA, Cameron J, Cammack N, Boucher C, Wainberg MA. The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine confers high-level resistance to the (-) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. Antimicrob Agents Chemother. 1993;37(6):1390–2.
47. Schinazi RF, Lloyd Jr RM, Nguyen MH, Cannon DL, McMillan A, Ilksoy N, Chu CK, Liotta DC, Bazmi HZ, Mellors JW. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob Agents Chemother. 1993;37(4):875–81.
48. Kavlick MF, Shirasaka T, Kojima E, Pluda JM, Hui Jr F, Yarchoan R, Mitsuya H. Genotypic and phenotypic characterization of HIV-1 isolated from patients receiving (--)-2′,3′-dideoxy-3′-thiacytidine. Antiviral Res. 1995;28(2):133–46.
49. Svicher V, Alteri C, Artese A, Forbici F, Santoro MM, Schols D, Van Laethem K, Alcaro S, Costa G, Tommasi C, Zaccarelli M, Narciso P, Antinori A, Ceccherini-Silberstein F, Balzarini J, Perno CF. Different evolution of genotypic resistance profiles to emtricitabine versus lamivudine in tenofovir-containing regimens. J Acquir Immune Defic Syndr. 2010;55(3):336–44.
50. Gutfreund K, Williams M, George R, Bain V, Ma M, Yoshida E, Villeneuve J, Fischer K, Tyrrel D. Genotypic succession of mutations of the hepatitis B virus polymerase associated with lamivudine resistance. J Hepatol. 2000;33(3):469–75.
51. Lai CL, Shouval D, Lok AS, Chang TT, Cheinquer H, Goodman Z, DeHertogh D, Wilber R, Zink RC, Cross A, Colonno R, Fernandes L, Group BEAS. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N Engl J Med. 2006;354(10):1011–20.
52. Sherman M, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska A, Martin P, Goodman Z, Colonno R, Cross

A, Denisky G, Kreter B, Hindes R, Group AIBS. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. Gastroenterology. 2006;130(7):2039–49.
53. mcMahon M, Jilek B, Brennan T, Shen L, Zhou Y, Wind-Rotolo M, Xing S, Bhat S, Hale B, Hergarty R, Chong C, Liu J, Siliciano R, Thio C. The HBV drug entecavir—effects on HIV-1 replication and resistance. N Engl J Med. 2007;356(25):2614–21.
54. Domaoal RA, McMahon M, Thio CL, Bailey CM, Tirado-Rives J, Obikhod A, Detorio M, Rapp KL, Siliciano RF, Schinazi RF, Anderson KS. Pre-steady-state kinetic studies establish entecavir 5′-triphosphate as a substrate for HIV-1 reverse transcriptase. J Biol Chem. 2008;283(9):5452–9.
55. Tchesnokov EP, Obikhod A, Schinazi RF, Gotte M. Delayed chain termination protects the anti-hepatitis B virus drug entecavir from excision by HIV-1 reverse transcriptase. J Biol Chem. 2008;283(49):34218–28.
56. Langley DR, Walsh AW, Baldick CJ, Eggers BJ, Rose RE, Levine SM, Kapur AJ, Colonno RJ, Tenney DJ. Inhibition of hepatitis B virus polymerase by entecavir. J Virol. 2007;81(8):3992–4001.
57. De Clercq E. The acyclic nucleoside phosphonates from inception to clinical use: historical perspective. Antiviral Res. 2007;75(1):1–13.
58. Naesens L, Bischofberger N, Augustijns P, Annaert P, Van den Mooter G, Arimilli MN, Kim CU, De Clercq E. Antiretroviral efficacy and pharmacokinetics of oral bis(isopropyloxycarbonylo xymethyl)-9-(2-phosphonylmethoxypropyl)adenine in mice. Antimicrob Agents Chemother. 1998;42(7):1568–73.
59. Robbins BL, Srinivas RV, Kim C, Bischofberger N, Fridland A. Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-R-(2-phosphonomethoxypropyl)adenine (PMPA), bis(isopropyloxymethylcarbonyl)PMPA. Antimicrob Agents Chemother. 1998;42(3):612–7.
60. Wainberg MA, Miller MD, Quan Y, Salomon H, Mulato AS, Lamy PD, Margot NA, Anton KE, Cherrington JM. In vitro selection and characterization of HIV-1 with reduced susceptibility to PMPA. Antivir Ther. 1999;4(2):87–94.
61. Margot NA, Waters JM, Miller MD. In vitro human immunodeficiency virus type 1 resistance selections with combinations of tenofovir and emtricitabine or abacavir and lamivudine. Antimicrob Agents Chemother. 2006;50(12):4087–95.
62. McColl D, Margot N, Wulfsohn M, Coakley D, Cheng A, Miller MD. Patterns of resistance emerging in HIV-1 from antiretroviral-experienced patients undergoing intensification therapy with tenofovir disoproxil fumarate. J Acquir Immune Defic Syndr. 2004;37(3):1340–50.
63. Ross L, Gerondelis P, Liao Q, Wine B, Lim M, Shaefer M, Rodriguez A, Limoli K, Huang W, Parkin NT, Gallant J, Lanier R. Selection of the HIV-1 reverse transcriptase mutation K70E in antiretroviral-naive subjects treated with tenofovir/abacavir/lamivudine therapy (abstract no. 92). In: 14th international HIV drug resistance workshop. 2005. Québec City, Canada.
64. Kagan RM, Lee TS, Ross L, Lloyd Jr RM, Lewinski MA, Potts SJ. Molecular basis of antagonism between K70E and K65R tenofovir-associated mutations in HIV-1 reverse transcriptase. Antiviral Res. 2007;75(3):210–8.
65. Parikh UM, Koontz DL, Chu CK, Schinazi RF, Mellors JW. In vitro activity of structurally diverse nucleoside analogs against human immunodeficiency virus type 1 with the K65R mutation in reverse transcriptase. Antimicrob Agents Chemother. 2005;49(3):1139–44.
66. Skhosana L, Steegen K, Bronze M, Lukhwareni A, Letsoalo E, Papathanasopoulos MA, Carmona SC, Stevens WS. High prevalence of the K65R mutation in HIV-1 subtype C infected patients failing tenofovir-based first-line regimens in South Africa. PLoS One. 2015;10(2):e0118145.
67. Sluis-Cremer N, Arion D, Kaushik N, Lim H, Parniak MA. Mutational analysis of Lys65 of HIV-1 reverse transcriptase. Biochem J. 2000;348(Pt 1):77–82.
68. Parikh UM, Zelina S, Sluis-Cremer N, Mellors JW. Molecular mechanisms of bidirectional antagonism between K65R and thymidine analog mutations in HIV-1 reverse transcriptase. AIDS. 2007;21(11):1405–14.
69. Parikh UM, Bacheler L, Koontz D, Mellors JW. The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. J Virol. 2006;80(10):4971–7.
70. Sluis-Cremer N, Sheen CW, Zelina S, Torres PS, Parikh UM, Mellors JW. Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. Antimicrob Agents Chemother. 2007;51(1):48–53.
71. Ruane P, DeJesus E, Berger D, Markowitz M, Bredeek U, Callebaut C, Zhong L, Ramanathan S, Rhee M, Fordyce M, Yale K. Antiviral activity, safety, and pharmacokinetics/pharmacodynamics of tenofovir alafenamide as 10-day monotherapy in HIV-1-positive adults. J Acquir Immune Defic Syndr. 2013;63(4):449–55.
72. Margot NA, Johnson A, Miller MD, Callebaut C. Characterization of HIV-1 resistance to tenofovir alafenamide in vitro. Antimicrob Agents Chemother. 2015;59(10):5917–24.
73. Sax P, Wohl D, Yin M, Post F, DeJesus E, Saag M, Pozniak A, Thompson M, Podzamczer D, Molina J, Oka S, Koenig E, Trottier B, Andrade-Villanueva J, Crofoot G, Custodio J, Plummer A, Zhong L, Cao H, Martin H, Callebaut C, Cheng A, Fordyce M, McCallister S, GS-US Team. Tenofovir alafenamide versus tenofovir disoproxil fumarate, coformulated with elvitegravir, cobicistat, and emtricitabine, for initial treatment of HIV-1 infection: two randomised, double-blind, phase 3, non-inferiority trials. Lancet 2015;385(9987): 2606–15.
74. Takamatsu Y, Tanaka Y, Kohgo S, Murakami S, Singh K, Das D, Venzon DJ, Amano M, Higashi-Kuwata N, Aoki M, Delino NS, Hayashi S, Takahashi S, Sukenaga Y, Haraguchi K, Sarafianos SG, Maeda K, Mitsuya H. 4′-Modified nucleoside analogs: potent inhibitors active against entecavir-resistant hepatitis B virus. Hepatology. 2015;62(4):1024–36.
75. Kawamoto A, Kodama E, Sarafianos SG, Sakagami Y, Kohgo S, Kitano K, Ashida N, Iwai Y, Hayakawa H, Nakata H, Mitsuya H, Arnold E, Matsuoka M. 2′-Deoxy-4′-C-ethynyl-2-halo-adenosines active against drug-resistant human immunodeficiency virus type 1 variants. Int J Biochem Cell Biol. 2008;40(11):2410–20.
76. Kodama EI, Kohgo S, Kitano K, Machida H, Gatanaga H, Shigeta S, Matsuoka M, Ohrui H, Mitsuya H. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob Agents Chemother. 2001;45(5):1539–46.
77. Michailidis E, Huber AD, Ryan EM, Ong YT, Leslie MD, Matzek KB, Singh K, Marchand B, Hagedorn AN, Kirby KA, Rohan LC, Kodama EN, Mitsuya H, Parniak MA, Sarafianos SG. 4′-Ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) inhibits HIV-1 reverse transcriptase with multiple mechanisms. J Biol Chem. 2014;289(35):24533–48.
78. Michailidis E, Ryan EM, Hachiya A, Kirby KA, Marchand B, Leslie MD, Huber AD, Ong YT, Jackson JC, Singh K, Kodama EN, Mitsuya H, Parniak MA, Sarafianos SG. Hypersusceptibility mechanism of tenofovir-resistant HIV to EFdA. Retrovirology. 2013;10:65.
79. Murphey-Corb M, Rajakumar P, Michael H, Nyaundi J, Didier PJ, Reeve AB, Mitsuya H, Sarafianos SG, Parniak MA. Response of simian immunodeficiency virus to the novel nucleoside reverse transcriptase inhibitor 4′-ethynyl-2-fluoro-2′-deoxyadenosine in vitro and in vivo. Antimicrob Agents Chemother. 2012;56(9):4707–12.

80. Maeda K, Desai DV, Aoki M, Nakata H, Kodama EN, Mitsuya H. Delayed emergence of HIV-1 variants resistant to 4′-ethynyl-2-fluoro-2′-deoxyadenosine: comparative sequential passage study with lamivudine, tenofovir, emtricitabine and BMS-986001. Antivir Ther. 2014;19(2):179–89.
81. Kim HO, Schinazi RF, Shanmuganathan K, Jeong LS, Beach JW, Nampalli S, Cannon DL, Chu CK. L-beta-(2S,4S)- and L-alpha-(2S,4R)-dioxolanyl nucleosides as potential anti-HIV agents: asymmetric synthesis and structure-activity relationships. J Med Chem. 1993;36(5):519–28.
82. Chen H, Boudinot F, Chu C, Mcclure H, Schinazi R. Pharmacokinetics of (-)-beta-D-2-aminopurine dioxolane and (-)-beta-D-2-amino-6-chloropurine dioxolane and their antiviral metabolite (-)-beta-D-dioxolane guanine in rhesus monkeys. Antimicrob Agents Chemother. 1996;40(10):2332–6.
83. Gu Z, Wainberg M, Nguyen-Ba N, L'Heureux L, de Muys J, Bowlin T, Rando R. Mechanism of action and in vitro activity of 1′,3′-dioxolanylpurine nucleoside analogues against sensitive and drug-resistant human immunodeficiency virus type 1 variants. Antimicrob Agents Chemother. 1999;43(10):2376–82.
84. Gu Z, Wainberg M, Nguyen-Ba P, L'Heureux L, de Muys J, Rando R. Anti-HIV-1 activities of 1,3-dioxolane guanine and 2,6-diaminopurine dioxolane. Nucleosides Nucleotides Nucleic Acids. 1999;18(4–5):891–2.
85. Mewshaw JP, Myrick FT, Wakefield DA, Hooper BJ, Harris JL, McCreedy B, Borroto-Esoda K. Dioxolane guanosine, the active form of the prodrug diaminopurine dioxolane, is a potent inhibitor of drug-resistant HIV-1 isolates from patients for whom standard nucleoside therapy fails. J Acquir Immune Defic Syndr. 2002;29(1):11–20.
86. Bazmi HZ, Hammond JL, Cavalcanti SC, Chu CK, Schinazi RF, Mellors JW. In vitro selection of mutations in the human immunodeficiency virus type 1 reverse transcriptase that decrease susceptibility to (-)-beta-D-dioxolane-guanosine and suppress resistance to 3′-azido-3′-deoxythymidine. Antimicrob Agents Chemother. 2000;44(7):1783–8.
87. Jeffrey JL, Feng JY, Qi CC, Anderson KS, Furman PA. Dioxolane guanosine 5′-triphosphate, an alternative substrate inhibitor of wild-type and mutant HIV-1 reverse transcriptase. Steady state and pre-steady state kinetic analyses. J Biol Chem. 2003;278(21):18971–9.
88. Meyer PR, Matsuura SE, So AG, Scott WA. Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proc Natl Acad Sci U S A. 1998;95(23):13471–6.
89. Arion D, Kaushik N, McCormick S, Borkow G, Parniak MA. Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry. 1998;37(45):15908–17.
90. Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science. 1989;243(4899):1731–4.
91. Hooker DJ, Tachedjian G, Solomon AE, Gurusinghe AD, Land S, Birch C, Anderson JL, Roy BM, Arnold E, Deacon NJ. An in vivo mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type 1 reverse transcriptase contributes to high-level resistance to 3′-azido-3′-deoxythymidine. J Virol. 1996;70(11):8010–8.
92. Kellam P, Boucher CA, Larder BA. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. Proc Natl Acad Sci U S A. 1992;89(5):1934–8.
93. Harrigan PR, Kinghorn I, Bloor S, Kemp SD, Najera I, Kohli A, Larder BA. Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. J Virol. 1996;70(9):5930–4.
94. Mitsuya H, Weinhold KJ, Furman PA, Clair MHS, Lehrman SN, Gallo RC, Bolognesi D, Barry DW, Broder S. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc Natl Acad Sci U S A. 1985;82(20):7096–100.
95. Furman PA, Fyfe JA, Clair MHS, Weinhold K, Rideout JL, Freeman GA, Lehrman SN, Bolognesi DP, Broder S, Mitsuya H, et al. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci U S A. 1986;83(21):8333–7.
96. Anderson PL, Kakuda TN, Kawle S, Fletcher CV. Antiviral dynamics and sex differences of zidovudine and lamivudine triphosphate concentrations in HIV infected individuals. AIDS. 2003;17(15):2159–68.
97. Department of Health and Human Services. Panel on antiretroviral guidelines for adults and adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. 2015. http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf.
98. Fletcher CV, Anderson PL, Kakuda TN, Schacker TW, Henry K, Gross CR, Brundage RC. Concentration-controlled compared with conventional antiretroviral therapy for HIV infection. AIDS. 2002;16(4):551–60.
99. Sidtis JJ, Gatsonis C, Price RW, Singer EJ, Collier AC, Richman DD, Hirsch MS, Schaerf FW, Fischl MA, Kieburtz K, Simpson D, Koch MA, Feinberg J, Dafni U. Zidovudine treatment of the AIDS dementia complex—results of a placebo-controlled trial. Ann Neurol. 1993;33(4):343–9.
100. Hurwitz SJ, Schinazi RF. In silico study supports the efficacy of a reduced dose regimen for stavudine. Antiviral Res. 2011;92(2):372–7.
101. Broder S. The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. Antiviral Res. 2010;85(1):1–18.
102. Ray AS, Murakami E, Basavapathruni A, Vaccaro JA, Ulrich D, Chu CK, Schinazi RF, Anderson KS. Probing the molecular mechanisms of AZT drug resistance mediated by HIV-1 reverse transcriptase using a transient kinetic analysis. Biochemistry. 2003;42(29):8831–41.
103. Rigourd M, Ehresmann C, Parniak MA, Ehresmann B, Marquet R. Primer unblocking and rescue of DNA synthesis by azidothymidine (AZT)-resistant HIV-1 reverse transcriptase—comparison between initiation and elongation of reverse transcription and between (−) and (+) strand DNA synthesis. J Biol Chem. 2002;277(21):18611–8.
104. Meyer PR, Matsuura SE, Mian AM, So AG, Scott WA. A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. Mol Cell. 1999;4(1):35–43.
105. Boyer PL, Sarafianos SG, Arnold E, Hughes SH. Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. J Virol. 2001;75(10):4832–42.
106. Tu XY, Das K, Han QW, Bauman JD, Clark AD, Hou XR, Frenkel YV, Gaffney BL, Jones RA, Boyer PL, Hughes SH, Sarafianos SG, Arnold E. Structural basis of HIV-1 resistance to AZT by excision. Nat Struct Mol Biol. 2010;17(10):1202–9.
107. Das K, Arnold E. HIV-1 reverse transcriptase and antiviral drug resistance. Part 2. Curr Opin Virol. 2013;3(2):119–28.
108. Jiang LJ, Maret W, Vallee BL. The ATP-metallothionein complex. Proc Natl Acad Sci U S A. 1998;95(16):9146–9.
109. Kennedy EM, Gavegnano C, Nguyen L, Slater R, Lucas A, Fromentin E, Schinazi RF, Kim B. Ribonucleoside triphosphates as substrate of human immunodeficiency virus type 1 reverse transcriptase in human macrophages. J Biol Chem. 2010;285(50):39380–91.
110. Boyer PL, Gao HQ, Clark PK, Sarafianos SG, Arnold E, Hughes SH. YADD mutants of human immunodeficiency virus type 1 and

Moloney murine leukemia virus reverse transcriptase are resistant to lamivudine triphosphate (3TCTP) in vitro. J Virol. 2001;75(14):6321–8.
111. Tong W, Lu CD, Sharma SK, Matsuura S, So AG, Scott WA. Nucleotide-induced stable complex formation by HIV-1 reverse transcriptase. Biochemistry. 1997;36(19):5749–57.
112. Boyer PL, Sarafianos SG, Arnold E, Hughes SH. Nucleoside analog resistance caused by insertions in the fingers of human immunodeficiency virus type 1 reverse transcriptase involves ATP-mediated excision. J Virol. 2002;76(18):9143–51.
113. Marchand B, Gotte M. Site-specific footprinting reveals differences in the translocation status of HIV-1 reverse transcriptase. Implications for polymerase translocation and drug resistance. J Biol Chem. 2003;278(37):35362–72.
114. Naeger LK, Margot NA, Miller MD. ATP-dependent removal of nucleoside reverse transcriptase inhibitors by human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother. 2002;46(7):2179–84.
115. Harrigan PR, Bloor S, Larder BA. Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates in vitro. J Virol. 1998;72(5):3773–8.
116. Hu Z, Giguel F, Hatano H, Reid P, Lu J, Kuritzkes DR. Fitness comparison of thymidine analog resistance pathways in human immunodeficiency virus type 1. J Virol. 2006;80(14):7020–7.
117. Miller MD, Margot N, Lu B, Zhong LJ, Chen SS, Cheng A, Wulfsohn M. Genotypic and phenotypic predictors of the magnitude of response to tenofovir disoproxil fumarate treatment in antiretroviral-experienced patients. J Infect Dis. 2004;189(5):837–46.
118. Cozzi-Lepri A, Ruiz L, Loveday C, Phillips AN, Clotet B, Reiss P, Ledergerber B, Holkmann C, Staszewski S, Lundgren JD. Thymidine analogue mutation profiles: factors associated with acquiring specific profiles and their impact on the virological response to therapy. Antivir Ther. 2005;10(7):791–802.
119. De Luca A, Di Giambenedetto S, Romano L, Gonnelli A, Corsi P, Baldari M, Di Pietro M, Menzo S, Francisci D, Almi P, Zazzi M, Cohort AR. Frequency and treatment-related predictors of thymidine-analogue mutation patterns in HIV-1 isolates after unsuccessful antiretroviral therapy. J Infect Dis. 2006;193(9):1219–22.
120. Yahi N, Tamalet C, Tourres C, Tivoli N, Fantini J. Mutation L210W of HIV-1 reverse transcriptase in patients receiving combination therapy—Incidence, association with other mutations, and effects on the structure of mutated reverse transcriptase. J Biomed Sci. 2000;7(6):507–13.
121. Rhee SY, Liu T, Ravela J, Gonzales MJ, Shafer RW. Distribution of human immunodeficiency virus type 1 protease and reverse transcriptase mutation patterns in 4,183 persons undergoing genotypic resistance testing. Antimicrob Agents Chemother. 2004;48(8):3122–6.
122. Matamoros T, Franco S, Vazquez-Alvarez BM, Mas A, Martinez MA, Menendez-Arias L. Molecular determinants of multinucleoside analogue resistance in HIV-1 reverse transcriptases containing a dipeptide insertion in the fingers subdomain: effect of mutations D67N and T215Y on removal of thymidine nucleotide analogues from blocked DNA primers. J Biol Chem. 2004;279(23):24569–77.
123. Mas A, Parera M, Briones C, Soriano V, Martinez MA, Domingo E, Menendez-Arias L. Role of a dipeptide insertion between codons 69 and 70 of HIV-1 reverse transcriptase in the mechanism of AZT resistance. EMBO J. 2000;19(21):5752–61.
124. Meyer PR, Lennerstrand J, Matsuura SE, Larder BA, Scott WA. Effects of dipeptide insertions between codons 69 and 70 of human immunodeficiency virus type 1 reverse transcriptase on primer unblocking, deoxynucleoside triphosphate inhibition, and DNA chain elongation. J Virol. 2003;77(6):3871–7.
125. White KL, Chen JM, Margot NA, Wrin T, Petropoulos CJ, Naeger LK, Swaminathan S, Miller MD. Molecular mechanisms of tenofovir resistance conferred by human immunodeficiency virus type 1 reverse transcriptase containing a diserine insertion after residue 69 and multiple thymidine analog-associated mutations. Antimicrob Agents Chemother. 2004;48(3):992–1003.
126. Imamichi T, Berg SC, Imamichi H, Lopez JC, Metcalf JA, Falloon J, Lane HC. Relative replication fitness of a high-level 3′-azido-3′-deoxythymidine-resistant variant of human immunodeficiency virus type 1 possessing an amino acid deletion at codon 67 and a novel substitution (Thr → Gly) at codon 69. J Virol. 2000;74(23):10958–64.
127. Boyer PL, Imamichi T, Sarafianos SG, Arnold E, Hughes SH. Effects of the Delta67 complex of mutations in human immunodeficiency virus type 1 reverse transcriptase on nucleoside analog excision. J Virol. 2004;78(18):9987–97.
128. Ehteshami M, Gotte M. Effects of mutations in the connection and RNase H domains of HIV-1 reverse transcriptase on drug susceptibility. AIDS Rev. 2008;10(4):224–35.
129. Delviks-Frankenberry KA, Nikolenko GN, Pathak VK. The "connection" between HIV drug resistance and RNase H. Viruses. 2010;2(7):1476–503.
130. Hachiya A, Kodama EN, Sarafianos SG, Schuckmann MM, Sakagami Y, Matsuoka M, Takiguchi M, Gatanaga H, Oka S. Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. J Virol. 2008;82(7):3261–70.
131. Yap SH, Sheen CW, Fahey J, Zanin M, Tyssen D, Lima VD, Wynhoven B, Kuiper M, Sluis-Cremer N, Harrigan PR, Tachedjian G. N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. PLoS Med. 2007;4(12):e335.
132. Menendez-Arias L, Betancor G, Matamoros T. HIV-1 reverse transcriptase connection subdomain mutations involved in resistance to approved non-nucleoside inhibitors. Antiviral Res. 2011;92(2):139–49.
133. Cane PA, Green H, Fearnhill E, Dunn D. Identification of accessory mutations associated with high-level resistance in HIV-1 reverse transcriptase. AIDS. 2007;21(4):447–55.
134. von Wyl V, Ehteshami M, Demeter LM, Burgisser P, Nijhuis M, Symons J, Yerly S, Boni J, Klimkait T, Schuurman R, Ledergerber B, Gotte M, Gunthard HF. HIV-1 reverse transcriptase connection domain mutations: dynamics of emergence and implications for success of combination antiretroviral therapy. Clin Infect Dis. 2010;51(5):620–8.
135. Waters JM, O'Neal W, White KL, Wakeford C, Lansdon EB, Harris J, Svarovskaia ES, Miller MD, Borroto-Esoda K. Mutations in the thumb-connection and RNase H domain of HIV type-1 reverse transcriptase of antiretroviral treatment-experienced patients. Antivir Ther. 2009;14(2):231–9.
136. Santos AF, Lengruber RB, Soares EA, Jere A, Sprinz E, Martinez AM, Silveira J, Sion FS, Pathak VK, Soares MA. Conservation patterns of HIV-1 RT connection and RNase H domains: identification of new mutations in NRTI-treated patients. PLoS One. 2008;3(3):e1781.
137. Gallego O, Corral A, de Mendoza C, Rodes B, Soriano V. Prevalence of G333D/E in naive and pretreated HIV-infected patients. AIDS Res Hum Retroviruses. 2002;18(12):857–60.
138. Kemp SD, Shi C, Bloor S, Harrigan PR, Mellors JW, Larder BA. A novel polymorphism at codon 333 of human immunodeficiency virus type 1 reverse transcriptase can facilitate dual resistance to zidovudine and L-2′,3′-dideoxy-3′-thiacytidine. J Virol. 1998;72(6):5093–8.
139. Zelina S, Sheen CW, Radzio J, Mellors JW, Sluis-Cremer N. Mechanisms by which the G333D mutation in human

immunodeficiency virus type 1 reverse transcriptase facilitates dual resistance to zidovudine and lamivudine. Antimicrob Agents Chemother. 2008;52(1):157–63.
140. Lengruber RB, Delviks-Frankenberry KA, Nikolenko GN, Baumann J, Santos AF, Pathak VK, Soares MA. Phenotypic characterization of drug resistance-associated mutations in HIV-1 RT connection and RNase H domains and their correlation with thymidine analogue mutations. J Antimicrob Chemother. 2011;66(4):702–8.
141. Brehm JH, Koontz D, Meteer JD, Pathak V, Sluis-Cremer N, Mellors JW. Selection of mutations in the connection and RNase H domains of human immunodeficiency virus type 1 reverse transcriptase that increase resistance to 3′-azido-3′-dideoxythymidine. J Virol. 2007;81(15):7852–9.
142. Ntemgwa M, Wainberg MA, Oliveira M, Moisi D, Lalonde R, Micheli V, Brenner BG. Variations in reverse transcriptase and RNase H domain mutations in human immunodeficiency virus type 1 clinical isolates are associated with divergent phenotypic resistance to zidovudine. Antimicrob Agents Chemother. 2007;51(11):3861–9.
143. Roquebert B, Wirden M, Simon A, Deval J, Katlama C, Calvez V, Marcelin AG. Relationship between mutations in HIV-1 RNase H domain and nucleoside reverse transcriptase inhibitors resistance mutations in naive and pre-treated HIV infected patients. J Med Virol. 2007;79(3):207–11.
144. Delviks-Frankenberry KA, Nikolenko GN, Barr R, Pathak VK. Mutations in human immunodeficiency virus type 1 RNase H primer grip enhance 3′-azido-3′-deoxythymidine resistance. J Virol. 2007;81(13):6837–45.
145. Delviks-Frankenberry KA, Nikolenko GN, Boyer PL, Hughes SH, Coffin JM, Jere A, Pathak VK. HIV-1 reverse transcriptase connection subdomain mutations reduce template RNA degradation and enhance AZT excision. Proc Natl Acad Sci U S A. 2008;105(31):10943–8.
146. Nikolenko GN, Delviks-Frankenberry KA, Palmer S, Maldarelli F, Fivash Jr MJ, Coffin JM, Pathak VK. Mutations in the connection domain of HIV-1 reverse transcriptase increase 3′-azido-3′-deoxythymidine resistance. Proc Natl Acad Sci U S A. 2007;104(1):317–22.
147. Nikolenko GN, Palmer S, Maldarelli F, Mellors JW, Coffin JM, Pathak VK. Mechanism for nucleoside analog-mediated abrogation of HIV-1 replication: balance between RNase H activity and nucleotide excision. Proc Natl Acad Sci U S A. 2005;102(6):2093–8.
148. Radzio J, Sluis-Cremer N. Efavirenz accelerates HIV-1 reverse transcriptase ribonuclease H cleavage, leading to diminished zidovudine excision. Mol Pharmacol. 2008;73(2):601–6.
149. Ehteshami M, Beilhartz GL, Scarth BJ, Tchesnokov EP, McCormick S, Wynhoven B, Harrigan PR, Gotte M. Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3′-azido-3′-deoxythymidine through both RNase H-dependent and -independent mechanisms. J Biol Chem. 2008;283(32):22222–32.
150. Brehm JH, Lalama CM, Hughes MD, Haubrich R, Riddler SA, Sluis-Cremer N, Mellors JW, A.C.T.G.S.A.P. Team. Failure of initial therapy with two nucleosides and efavirenz is not associated with early emergence of mutations in the C-terminus of HIV-1 reverse transcriptase. J Acquir Immune Defic Syndr. 2011;56(4):344–8.
151. Gupta S, Vingerhoets J, Fransen S, Tambuyzer L, Azijn H, Frantzell A, Paredes R, Coakley E, Nijs S, Clotet B, Petropoulos CJ, Schapiro J, Huang W, Picchio G. Connection domain mutations in HIV-1 reverse transcriptase do not impact etravirine susceptibility and virologic responses to etravirine-containing regimens. Antimicrob Agents Chemother. 2011;55(6):2872–9.
152. Wensing AM, Calvez V, Gunthard HF, Johnson VA, Paredes R, Pillay D, Shafer RW, Richman DD. 2014 Update of the drug resistance mutations in HIV-1. Top Antivir Med. 2014;22(3):642–50.
153. Dornsife RE, Clair MHS, Huang AT, Panella TJ, Koszalka GW, Burns CL, Averett DR. Anti-human immunodeficiency virus synergism by zidovudine (3′-azidothymidine) and didanosine (dideoxyinosine) contrasts with their additive inhibition of normal human marrow progenitor cells. Antimicrob Agents Chemother. 1991;35(2):322–8.
154. Yarchoan R, Lietzau JA, Nguyen BY, Brawley OW, Pluda JM, Saville MW, Wyvill KM, Steinberg SM, Agbaria R, Mitsuya H, et al. A randomized pilot study of alternating or simultaneous zidovudine and didanosine therapy in patients with symptomatic human immunodeficiency virus infection. J Infect Dis. 1994;169(1):9–17.
155. Bridges EG, Dutschman GE, Gullen EA, Cheng YC. Favorable interaction of beta-L(-) nucleoside analogues with clinically approved anti-HIV nucleoside analogues for the treatment of human immunodeficiency virus. Biochem Pharmacol. 1996;51(6):731–6.
156. Villahermosa ML, Martinez-Irujo JJ, Cabodevilla F, Santiago E. Synergistic inhibition of HIV-1 reverse transcriptase by combinations of chain-terminating nucleotides. Biochemistry. 1997;36(43):13223–31.
157. Nijhuis M, Schuurman R, de Jong D, van Leeuwen R, Lange J, Danner S, Keulen W, de Groot T, Boucher CA. Lamivudine-resistant human immunodeficiency virus type 1 variants (184V) require multiple amino acid changes to become co-resistant to zidovudine in vivo. J Infect Dis. 1997;176(2):398–405.
158. Das K, Bandwar RP, White KL, Feng JY, Sarafianos SG, Tuske S, Tu X, Clark Jr AD, Boyer PL, Hou X, Gaffney BL, Jones RA, Miller MD, Hughes SH, Arnold E. Structural basis for the role of the K65R mutation in HIV-1 reverse transcriptase polymerization, excision antagonism, and tenofovir resistance. J Biol Chem. 2009;284(50):35092–100.
159. White KL, Chen JM, Feng JY, Margot NA, Ly JK, Ray AS, Macarthur HL, McDermott MJ, Swaminathan S, Miller MD. The K65R reverse transcriptase mutation in HIV-1 reverses the excision phenotype of zidovudine resistance mutations. Antivir Ther. 2006;11(2):155–63.
160. Gotte M, Arion D, Parniak MA, Wainberg MA. The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. J Virol. 2000;74(8):3579–85.
161. Boyer PL, Sarafianos SG, Arnold E, Hughes SH. The M184V mutation reduces the selective excision of zidovudine 5′-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. J Virol. 2002;76(7):3248–56.
162. Naeger LK, Margot NA, Miller MD. Increased drug susceptibility of HIV-1 reverse transcriptase mutants containing M184V and zidovudine-associated mutations: analysis of enzyme processivity, chain-terminator removal and viral replication. Antivir Ther. 2001;6(2):115–26.
163. Miranda LR, Gotte M, Liang F, Kuritzkes DR. The L74V mutation in human immunodeficiency virus type 1 reverse transcriptase counteracts enhanced excision of zidovudine monophosphate associated with thymidine analog resistance mutations. Antimicrob Agents Chemother. 2005;49(7):2648–56.
164. Frankel FA, Marchand B, Turner D, Gotte M, Wainberg MA. Impaired rescue of chain-terminated DNA synthesis associated with the L74V mutation in human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother. 2005;49(7):2657–64.
165. Parikh UM, Barnas DC, Faruki H, Mellors JW. Antagonism between the HIV-1 reverse-transcriptase mutation K65R and

thymidine-analogue mutations at the genomic level. J Infect Dis. 2006;194(5):651–60.
166. Huang H, Chopra R, Verdine GL, Harrison SC. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science. 1998;282(5394):1669–75.
167. Romano L, Venturi G, Bloor S, Harrigan R, Larder BA, Major JC, Zazzi M. Broad nucleoside-analogue resistance implications for human immunodeficiency virus type 1 reverse-transcriptase mutations at codons 44 and 118. J Infect Dis. 2002;185(7):898–904.
168. Stoeckli TC, MaWhinney S, Uy J, Duan C, Lu J, Shugarts D, Kuritzkes DR. Phenotypic and genotypic analysis of biologically cloned human immunodeficiency virus type 1 isolates from patients treated with zidovudine and lamivudine. Antimicrob Agents Chemother. 2002;46(12):4000–3.
169. Sluis-Cremer N, Moore K, Radzio J, Sonza S, Tachedjian G. N348I in HIV-1 reverse transcriptase decreases susceptibility to tenofovir and etravirine in combination with other resistance mutations. AIDS. 2010;24(2):317–9.
170. Radzio J, Yap SH, Tachedjian G, Sluis-Cremer N. N348I in reverse transcriptase provides a genetic pathway for HIV-1 to select thymidine analogue mutations and mutations antagonistic to thymidine analogue mutations. AIDS. 2010;24(5):659–67.
171. von Wyl V, Ehteshami M, Symons J, Burgisser P, Nijhuis M, Demeter LM, Yerly S, Boni J, Klimkait T, Schuurman R, Ledergerber B, Gotte M, Gunthard HF. Epidemiological and biological evidence for a compensatory effect of connection domain mutation N348I on M184V in HIV-1 reverse transcriptase. J Infect Dis. 2010;201(7):1054–62.
172. Schinazi RF. Combined therapeutic modalities for viral infections—rationale and clinical potential. In: Chou T-C, Rideout DC, editors. Synergism and antagonism in chemotherapy. Orlando, Fl: Academic; 1991. p. 110–81.
173. Wong A. The HIV pipeline. Nat Rev Drug Discov. 2014;13(9):649–50.
174. Services., D.o.H.a.H. Panel on antiretroviral guidelines for adults and adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. 2015. http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf.
175. Masho SW, Wang CL, Nixon DE. Review of tenofovir-emtricitabine. Ther Clin Risk Manag. 2007;3(6):1097–104.
176. Miller MD, Haddad M, Su C, Gibbs C, McColl DJ, Guyer B. Trends in HIV-1 reverse transcriptase resistance-associated mutations and antiretroviral prescription data from 2003–2010. Antivir Ther. 2012;17(6):993–9.
177. Munoz de Benito RM, Arribas Lopez JR. Tenofovir disoproxil fumarate-emtricitabine coformulation for once-daily dual NRTI backbone. Expert Rev Anti Infect Ther. 2006;4(4):523–35.
178. Schinazi RF. Dead viruses don't mutate. Int Antiviral News. 1997;5(8):122.

HIV-1 Resistance to the Nonnucleoside Reverse Transcriptase Inhibitors

34

Nicolas Sluis-Cremer

1 Introduction

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are widely used to treat and prevent HIV-1 infection. Most first-line antiretroviral therapies typically include two nucleoside reverse transcriptase inhibitors with one NNRTI (nevirapine (NVP), efavirenz or rilpivirine (RPV)). Etravrine has been approved for the treatment of HIV-infected antiretroviral therapy-experienced individuals, including those with prior NNRTI exposure. In the HIV-1 prevention arena, single-dose NVP is used to prevent mother-to-child transmission ((MTCT)); the ASPIRE and Ring studies are evaluating whether a vaginal ring containing dapivirine can prevent HIV-1 infection in women; a microbicide gel formulation containing the urea-PETT derivative MIV-150 is in a phase I study to evaluate safety, pharmacokinetics, pharmacodynamics, and acceptability; and a long-acting RPV formulation is under development for preexposure prophylaxis (PrEP). Given their widespread use, particularly in resource-limited settings, there is concern in regard to overlapping resistance between the different NNRTIs. In this chapter we comprehensively review the mechanisms of action and resistance to the NNRTIs that are used clinically. A better understanding of NNRTI resistance—including the mechanisms involved—is important for (1) predicting response to treatment; (2) surveillance of transmitted drug resistance; and (3) development of new classes of NNRTIs with higher genetic barriers to resistance.

N. Sluis-Cremer, Ph.D. (✉)
Department of Medicine, Division of Infectious Diseases, University of Pittsburgh School of Medicine, S817 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261, USA
e-mail: nps2@pitt.edu

2 HIV-1 Reverse Transcriptase

Reverse transcription of the human immunodeficiency virus type 1 (HIV-1) single-stranded (+) RNA genome into double-stranded DNA is an essential step in the virus life cycle. Although several viral and host cell proteins may contribute toward the regulation and/or efficiency of HIV-1 reverse transcription [1,2], retroviral DNA synthesis is entirely catalyzed by the RNA- and DNA-dependent DNA polymerase and ribonuclease H (RNase H) activities of the virally encoded multifunctional enzyme, reverse transcriptase (RT).

Multiple structures of HIV-1 RT have been solved by X-ray crystallography. These include structures of (1) the unliganded form of the wild-type (WT) enzyme, or RT containing key drug resistance mutations [3–5]; (2) the RT–template/primer (T/P) binary complex [6–10]; (3) the RT–T/P–dNTP ternary complex [10,11]; and (4) different therapeutic classes of drugs bound to the WT or mutant unliganded RT, or the RT–T/P complex [8–10,12–16]. This wealth of data has provided considerable insight into structure–function relationships, including the overall architecture of the enzyme; key amino acid residues involved in substrate binding and catalysis; the precise location of inhibitor-binding sites; conformational changes associated with inhibitor binding; and the mechanisms by which mutations facilitate drug resistance. Indeed, this chapter utilizes this valuable resource to help explain the mechanism of action, and resistance to, the nonnucleoside RT inhibitors (NNRTIs), as described below.

HIV-1 RT is an asymmetric heterodimer composed of 66 kDa (p66) and 51 kDa (p51) subunits [17]. The p66 subunit, which contains both the DNA polymerase and RNase H active sites, is composed of DNA polymerase (residues 1–318), connection (residues 319–426), and RNase H (residues 427–560) domains. The polymerase domain has a similar architecture as the Klenow fragment of *Escherichia coli* DNA polymerase, and consists of the finger (residues 1–85 and 118–155), palm (residues 86–117 and 156–237), and thumb (residues 238–318) subdomains [12]. The polymerase

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_34

active site, as defined by the three aspartic acid residues D110, D185, and D186, resides in the palm subdomain. The connection domain acts as a tether between the polymerase and RNase H domains, but is also involved in nucleic acid substrate interactions and RT inter-subunit interactions. The p51 subunit is derived by HIV-1 protease-mediated cleavage of the RNase H domain of p66 [17]. However, the spatial arrangement of the polymerase and connection domains differs markedly from that of p66: the larger subunit adopts an "open" catalytically competent conformation that can accommodate a nucleic acid template strand, whereas the p51 subunit is in a "closed" conformation and is considered to play a largely structural role [18].

3 The Nonnucleoside RT Inhibitors

The NNRTIs constitute a group of small (<600 Da), chemically diverse compounds which interact with HIV-1 RT by binding to a single site in p66, termed the NNRTI-binding pocket (NNRTI-BP). NNRTIs are highly specific inhibitors of HIV-1 replication, and are not active against HIV-2 or other retroviruses. 1-[(2-Hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) and tetrahydroimidazobenzodiazepinone (TIBO) were the first NNRTIs identified more than 24 years ago [19,20]. Since then a large number of NNRTIs have been identified that can be classified into >50 different chemical classes [21]. To date, five NNRTIs have been approved by the United States Food and Drug Administration (FDA) for use in HIV-1-infected individuals. These include nevirapine (NVP, Viramune, approved in 1996), efavirenz (EFV, Sustiva, approved in 2006), etravirine (ETR, Intelence, approved in 2008), and rilpivirine (RPV, Edurant, approved 2011) (Fig. 34.1). Delavirdine (Rescriptor) was also approved by the FDA in 1997; however, its efficacy is lower than that of the other NNRTIs, especially EFV, and it also has an inconvenient dosing schedule. These factors have led the US Department of Health and Human Services (DHHS) Antiretroviral Guidelines Panel to recommend that it not be used as part of initial therapy. Due to cross-resistance between delavirdine and the other approved NNRTIs, it is also never in second-line or salvage therapy. Consequently, we have excluded this NNRTI from further discussion in this chapter. In addition to the approved NNRTIs, there are at least three other NNRTIs currently being investigated in clinical trials. These include dapivirine (DAP) and MIV-150 for HIV-1 prevention (Fig. 34.1) and doravirine for therapy.

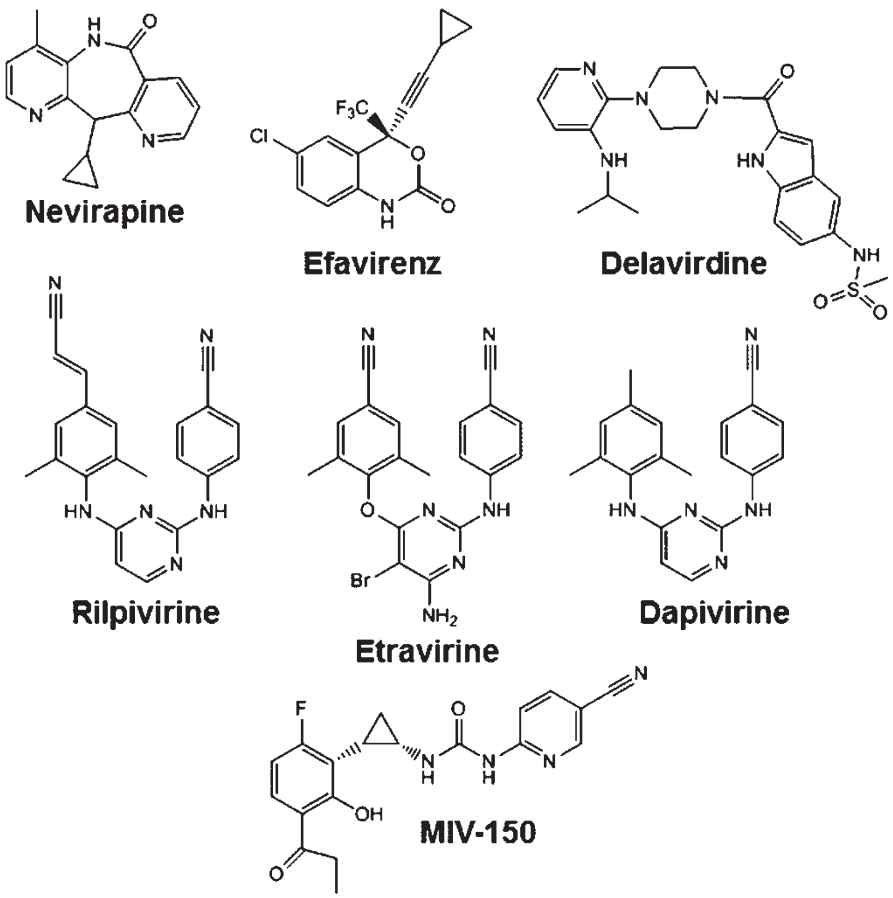


Fig. 34.1 Chemical structures of the NNRTIs used for the prevention and treatment of HIV-1 inefction

4 Clinical Use of NNRTIs

NNRTIs are used for both the treatment and prevention of HIV-1 infection, as described below.

4.1 First-Line Therapy

For antiretroviral therapy (ART)-naïve individuals, a first-line regimen typically consists of two nucleoside RT inhibitors (NRTIs) plus an NNRTI. Alternatively, a ritonavir-boosted protease inhibitor, or an integrase inhibitor, can be used in lieu of the NNRTI. NVP, EFV, and RPV have all been approved for first-line therapy.

4.2 Salvage Therapy

ETR is the only approved NNRTI which demonstrates in vitro and in vivo antiviral activity against a broad range of HIV-1 groups and subtypes, including viral strains that exhibit resistance to NVP and EFV [22–24]. Accordingly, ETR has been approved by the FDA for use in treatment-experienced adults who are experiencing virologic failure with HIV-1 strains resistant to an NNRTI and/or other antiretroviral agents.

4.3 Maintenance Therapy

The development of once-daily ART represented a major advance because it significantly improved adherence to therapy. As such, less frequent dosing options (e.g., once a month or even once a quarter) could become an even more convenient option. Long-acting parenteral formulations of RPV (RPV-LA), and the integrase inhibitor GSK1265744 (744), have been developed to address this niche [25–27]. Currently, the long-acting antiretroviral treatment enabling (LATTE) study is assessing whether a combination of these two antiviral drugs can maintain virologic suppression in HIV-1-infected individuals. In a proof-of-concept study using daily dosing, subjects initially received two NRTIs + 744 or two NRTIs + EFV during an induction phase. Those who demonstrated virologic suppression (viral RNA < 50 copies/mL) after 24 weeks became eligible for the maintenance phase in which the NRTIs were discontinued in one arm of the study, and replaced with RPV + 744. An interim analysis at week 48 highlighted that RPV + 744 maintained virologic suppression at a rate similar to two NRTIs + EFV [28].

4.4 Prevention of Mother-to-Child Transmission

The HIVNET 012 Trial demonstrated that a single dose of NVP (sdNVP) given to an infected mother during labor, and to HIV-1-infected mothers of breastfeeding infants after birth, reduced MTCT by 50 % at 6 weeks of age, and by 38 % at 18 months of age [29]. Unfortunately, sdNVP selects resistant viruses at high rates in both mothers and infected infants and is no longer recommended [30–33]. Instead, the World Health Organization currently recommends two different options. These include (A) twice-daily zidovudine for the mother and infant prophylaxis with either zidovudine or NVP for 6 weeks after birth if the infant is not breast-feeding. If the infant is breast-feeding, daily NVP infant prophylaxis should be continued for 1 week after the end of the breast-feeding period; or (B) a three-drug prophylactic regimen (that can include NVP) for the mother taken during pregnancy and throughout the breast-feeding period, as well as infant prophylaxis for 6 weeks after birth, whether or not the infant is breast-feeding.

4.5 Microbicides

An intravaginal ring (IVR) that releases DAP to cells inside the vagina throughout the 1-month period that it's worn is currently being tested in two separate phase III clinical studies (ASPIRE and the Ring study) [34–36]. Additionally, a gel formulation of MIV-150 in combination with carrageenan and zinc acetate is being evaluated in a phase 1, double-blind, parallel, placebo-controlled, randomized study to evaluate safety, pharmacokinetics, pharmacodynamics, and acceptability of the microbicide gel formulation [37].

4.6 Preexposure Prophylaxis

A multiple-compartment pharmacokinetic study of RPV-LA revealed that all doses (300, 600, or 1200 mg) gave prolonged plasma and genital-tract RPV exposure for up to 84 days [38]. Additionally, all RPV-LA doses were well tolerated. Collectively, these data advocate for its use as a potential PrEP intervention.

5 Mechanism of Action

The NNRTI-BP, which is located in the p66 palm subdomain, ~10 Å from the DNA polymerase active site (Fig. 34.2a) [12], is formed upon inhibitor binding as a result of rotational movements of key amino acids delineating the pocket (mainly Y188 and Y181), and by relocalization of a short three-strand β-sheet containing F227 and W229 [3,4]. These conformational changes alter the positioning of (1) the β4–β7–β8 sheet that contains the catalytic aspartic acid residues, and (2) the structural elements that constitute the "primer grip" (amino acids 227–235), which positions the DNA primer in the active site (Figs. 34.2b and 34.3b). Another major change observed upon NNRTI binding to unliganded RT (Fig. 34.2a), but not the RT–T/P complex

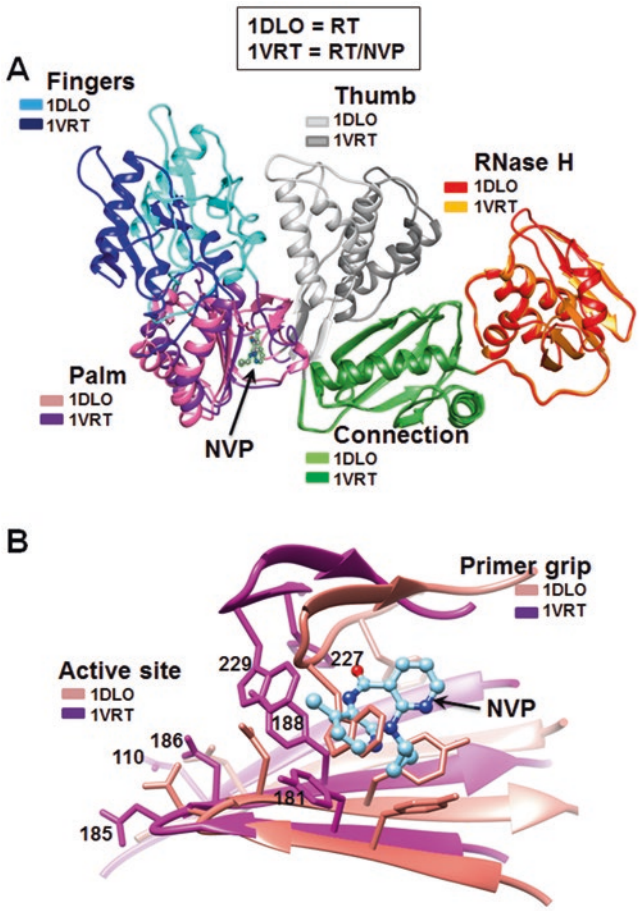


Fig. 34.2 Overlay of the crystal structures of unliganded RT (pdb: 1DLO) and an RT–NVP complex (pdb: 1VRT). (**a**) Overaly of the p66 subunits illustrating the location of the NNRTI-BP, and the conformational changes in the finger and thumb subdomains associated with NNRTI binding. (**b**) Overlay of the DNA polymerase active site illustrating how NNRTIs distort the catalytic triad (residues D110, D185, and D186) and the "primer grip" region

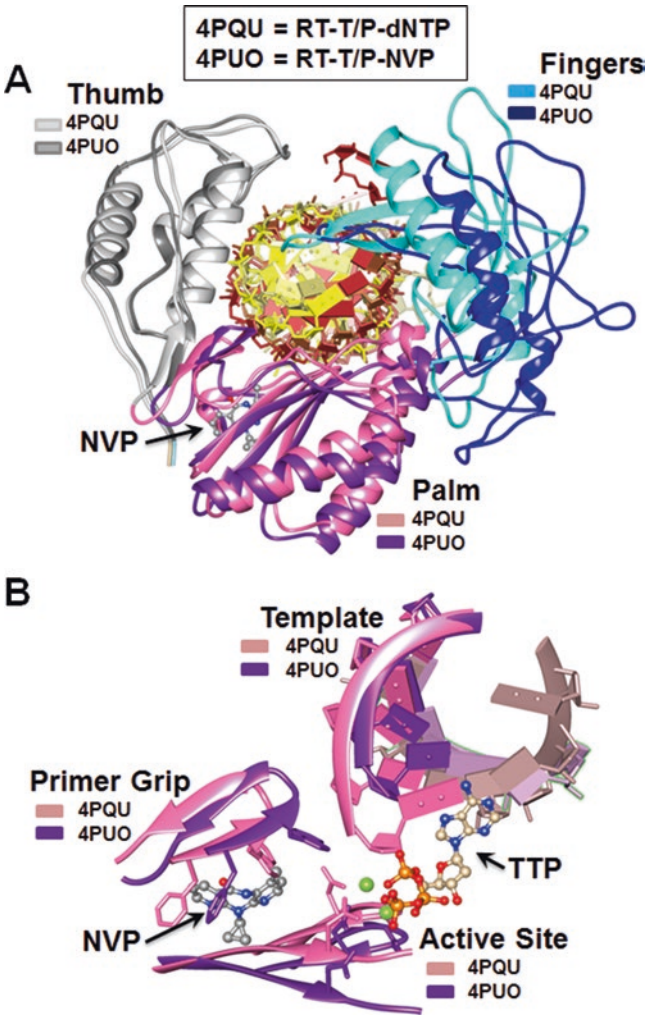


Fig. 34.3 Overlay of the crystal structures of an RT–T/P–dNTP complex (pdb: 4PQU) and an RT–T/P–NVP complex (pdb: 4PUO). (**a**) Overaly of the p66 subunits illustrating the location of the NNRTI-BP, and the conformational changes in the finger subdomain associated with NNRTI binding. (**b**) Overlay of the DNA polymerase active site illustrating how NNRTIs distort the catalytic triad (residues D110, D185, and D186) and the "primer grip" region

(Fig. 34.3a), is the hyperextended conformation of the p66 thumb subdomain. Based on the conformational changes described above, and several different kinetic or thermodynamic studies, at least three mechanisms by which NNRTIs inhibit HIV-1 reverse transcription have been proposed, as described below.

5.1 DNA Polymerase Active Site Distortion

In 1995, Spence et al. reported that NNRTIs did not interfere with dNTP binding, or the dNTP-induced conformational change required for chemical catalysis [39]. Instead in the presence of saturating concentrations of NNRTIs, the next correct dNTP bound tightly to the RT–T/P complex ($K_d \sim 100$ nM vs. 1–5 μM in the absence of inhibitor), but nonproductively. Accordingly, it was suggested that the observed slow rate of nucleotide incorporation (k_{pol}) in the presence of NNRTIs was due to a block at the chemical step of the reaction [39]. However, Xia et al. reported that the large increase in dNTP-binding affinity (K_d) was metal dependent, whereas the decrease in the maximum rate of dNTP incorporation (k_{pol}) was metal independent [40]. Furthermore, no phosphorothioate elemental effects were evident irrespective of the metal ion used in the assay [40]. (Phosphorothioate elemental effects, derived from experiments which compare the rates of incorporation of the natural dNTP substrate versus dNTPαS, are frequently used as a diagnostic for determining whether the chemical step of polymerization reactions is rate limiting.) Collectively, these data suggested that the slow rate of dNTP incorporation observed for NNRTI–RT–T/P complexes might not be due to a direct effect of the chemistry step, but rather an indirect effect through alteration/perturbation of the constella-

tion of amino acids involved in positioning the active site for efficient catalysis. Irrespective of the interpretation of these transient kinetic analyses, they both support a model whereby NNRTIs inhibit the DNA polymerization by active site distortion [39,40]. This active site distortion model is entirely consistent with the observed conformational changes in the catalytic triad and primer grip regions of the NNRTI–RT or NNRTI–RT–T/P–dNTP complexes (see Figs. 34.2 and 34.3).

5.2 Disruption of the Dynamic Intermolecular Interactions of the RT–T/P and RT–T/P–dNTP Complexes

Single-molecule studies have demonstrated that NNRTI binding to RT increases its dynamic sliding motion on the T/P [41,42], which prevents dNTP binding and the formation of a stable RT–T/P–dNTP complex [8–10,42,43]. This increase in dynamic sliding of RT on the T/P appears to be directly related to the magnitude of the NNRTI-induced finger-thumb opening [42].

5.3 Disruption of the Balance Between DNA Polymerase and RNase H Activity

The efficiency of HIV-1 reverse transcription is dependent on a delicate balance between the DNA polymerase and RNase H activities of RT. Consequently, the activity and specificity of RNase H must be finely tuned during reverse transcription. Kinetic studies have revealed that NNRTIs show greater inhibitory potential in reactions that require both DNA polymerase and RNase H activity, such as strand-transfer reactions or the initiation of (+)strand DNA synthesis [44]. In this regard, NNRTIs enhance the enzyme's RNase H activity [45–48]. The mechanisms by which NNRTIs enhance RNase H cleavage are T/P dependent. For example, on duplexes that contain the unique polypurine RNA primer used for the initiation of (+)strand DNA synthesis, RT can rapidly switch between two orientations that support either DNA synthesis or RNA hydrolysis [49]. NNRTI binding to RT pushes the enzyme to adopt a binding orientation that favors RNase H cleavage [49]. On other RNA/DNA T/P substrates, NNRTIs have been shown to cause RT to slide nonuniformly over the T/P complex such that the RNA/DNA hybrid has ready access to the RNase H active site [9,10].

6 HIV-1 Resistance to NNRTIs

HIV-1 resistance to NNRTIs has been documented in HIV-1 individuals failing first-line or salvage therapies containing NVP, EFV, RPV, or ETR, and in prevention strategies that use sdNVP (see [50] for recent review). Typically, resistance is associated with the acquisition of one or more mutations in the NNRTI-BP, although mutations in the connection domain, specifically N348I, have also been associated with NNRTI resistance [51–53]. Table 34.1 lists mutations in RT associated with decreased HIV-1 susceptibility to NVP, EFV, RPV, and ETR. Additionally, Table 34.2 documents the fold change in susceptibility of HIV-1 strains containing frequently selected NNRTI resistance mutations to the FDA-approved NNRTIs.

6.1 Cross-Resistance Between the NNRTIs

In general, there is a high level of cross-resistance within the NNRTI class as a result of two mechanisms:

Table 34.1 Mutations associated with decreased HIV-1 susceptibility to NVP, EFV, RPV, and ETR

	V90	L100	K101	K103	V106	V108	E138	V179	Y181	Y188	G190	H221	P225	F227	M230	N348
NVP	I	I	**EP**	**NS**	**AIM**	I		DEL	**CIV**	**LCH**	**ASE**		H	LC	**L**	I
EFV	I	**I**[a]	**EP**	**NS**	**AIM**	I		DEL	CIV	**LCH**	**ASE**		H	LC	**L**	I
ETR	I	**I**	**EP**		I		AG**KQ**	DEFITL	**CIV**	L	ASE			C	**L**	
RPV	I	**I**	**EP**		I		**AGKQ**	DEFITL	**CIV**	**L**	ASE	Y		C	**L**	

The data have been adapted from the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/)
[a]Mutations in *bold* are associated with the highest levels of reduced susceptibility or virological response to the relevant NNRTI

Table 34.2 Median fold change in the susceptibility of HIV-1 containing single NNRTI resistance mutations to NVP, EFV, ETR, and RPV

	L100I	K101E	K103N	V106M	E138K	Y181C	G190A	M230L	N348I
NVP	7.3	170	>43.0	6.3	0.4	>43.0	>85	20.4	4.2
EFV	20.3	2.1	32.5	2.6	0.5	2.1	8.1	5.9	2.8
RPV	0.9	3.0	0.9	0.9	3.0	2.8	1.1	3.4	N.D.
ETR	1.3	4.9	0.9	0.9	2.8	4.0	1.1	3.6	2.1

Data were adapted from [55, 78]

1. Nearly all of the NNRTI resistance mutations are within or adjacent to the NNRTI-BP. There is no evidence that any one mutation only confers resistance to a single agent; most NNRTI resistance mutations reduce susceptibility to two or more NNRTIs (Table 34.1).
2. The genetic barrier to NNRTI resistance is low. Typically, EFV, NVP, and RPV require only a single mutation to reduce clinical efficacy. ETR requires two mutations, but in certain circumstances (i.e., Y181I/V) a single mutation may be sufficient (Table 34.2) [22–24].

6.2 Subtype Differences in HIV-1 Resistance to NNRTIs

The diversity of HIV-1 has given rise to a large number of variants, including nine subtypes (A–D, F–H, J–K), six sub-subtypes (A1–A4, F1–F2), multiple (>48) circulating recombinant forms, and thousands of unique recombinant forms. The majority of research into HIV-1 drug resistance has focused on subtype B viruses, yet non-subtype B strains are responsible for 90 % of global infections. Importantly, there is increasing evidence of subtype differences in NNRTI resistance. For example (1) subtype C viruses harbor GTG (valine) at codon 106 in RT whereas subtype B harbors GTA (valine). The GTG polymorphism facilitates the emergence of subtype C virus with the V106M mutation (GTG to ATG) that confers resistance to NVP and EFV [54]. (2) Recent studies show that a glutamic acid-to-alanine substitution at codon 138 in RT occurs significantly more frequently in subtype C than B sequences in both treatment-naïve and RT inhibitor-experienced HIV-1-infected individuals [55]. E138A has been clinically associated with virologic failure of regimens that contain RPV or ETR [56,57]. (3) We reported that N348I in the connection domain of RT emerges in 45 % and 12 % of subtype C-infected individuals on failing regimens containing NVP or EFV, respectively [53].

6.3 Transmission of NNRTI-Resistant HIV-1

The widespread use of ART has contributed to the emergence of epidemics of transmitted HIV drug resistance (TDR) [58]. Indeed, recent analyses have shown a high but stable TDR prevalence in high-income countries, and a relatively low but rapidly increasing prevalence in low- and middle-income countries [59]. In view of the fact that they form the foundation of first-line ART and prevention of MTCT regimens, the most frequent TDR mutations are single-amino acid mutations conferring high-level resistance to NNRTIs (typically K103N, Y181C, and G190A). Importantly, these mutations have been associated with treatment failure in cases in which they exist before the initiation of first-line ART [60–63].

6.4 Impact of NNRTI Resistance Mutations on Viral Fitness

Although some NNRTI resistance mutations have been associated with a decline in viral replication fitness [64–66], the most prevalent NNRTI resistance mutations, such as Y181C and K103N, have minimal consequences on viral replication [67,68]. This is supported by clinical data demonstrating that these mutations can persist for several months after cessation of NNRTI therapy, or after transmission to an ART-naïve individual [69,70]. Furthermore, HIV-1 strains containing multiple NNRTI mutations may have improved replication capacity compared to virus harboring only single or double mutants [67], which may provide an explanation for the continued accumulation of NNRTI-associated mutations despite the presence of high-level resistance. There is also evidence of compensatory interactions between NNRTI and NRTI resistance mutations. For example (1) the L74V mutation has been demonstrated to compensate for the reduced replicative capacity of L100I/K103N HIV-1 [71]; and (2) E138K may compensate for the fitness deficits of both M184I and M184V and restore the replicative capacity of viruses containing M184I/V [72,73].

7 Mechanisms by Which Mutations in RT Confer HIV-1 Resistance

Typically, resistance mutations in the NNRTI-BP of RT decrease HIV-1 susceptibility to the inhibitors by directly impacting drug binding [74,75], although more complex mechanisms have been reported [42]. Below, using the available biochemical and structural data, we summarize the mechanism(s) by which several key mutations confer NNRTI resistance. The mutations discussed were selected based on clinical significance (including frequency), and available biochemical and structural data. Of note, the reader is encouraged to keep in mind the fold-change values for each mutation discussed (Table 34.2), as oftentimes it is challenging to correlate these values with the available structural/biochemical explanations for resistance.

7.1 L100I

L100I has been selected in vitro by EFV, ETR, and RPV [22,76–79], and has been detected in HIV-1-infected individuals failing EFV-, ETR-, or RPV-based therapies [80–84]. L100I rarely occurs in isolation, but when it does it reduces NVP and EFV susceptibility about 7- and 20-fold, respectively (Table 34.2). A crystal structure of L100I RT in complex with NVP has been solved [85]. Comparison of this structure with that of the WT RT–NVP complex (Fig. 34.4)

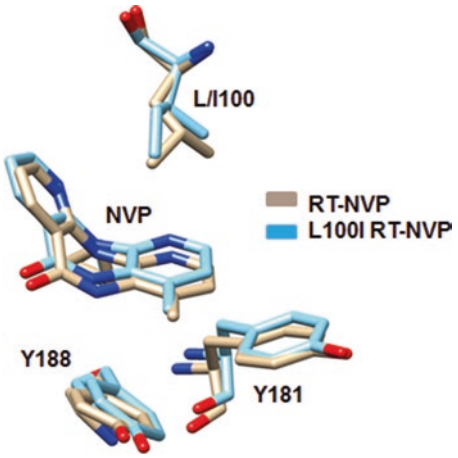


Fig. 34.4 Overlay of the NNRTI-BP of RT–NVP (pdb: 1VRT) and L100I RT–NVP (pdb: 1S1U)

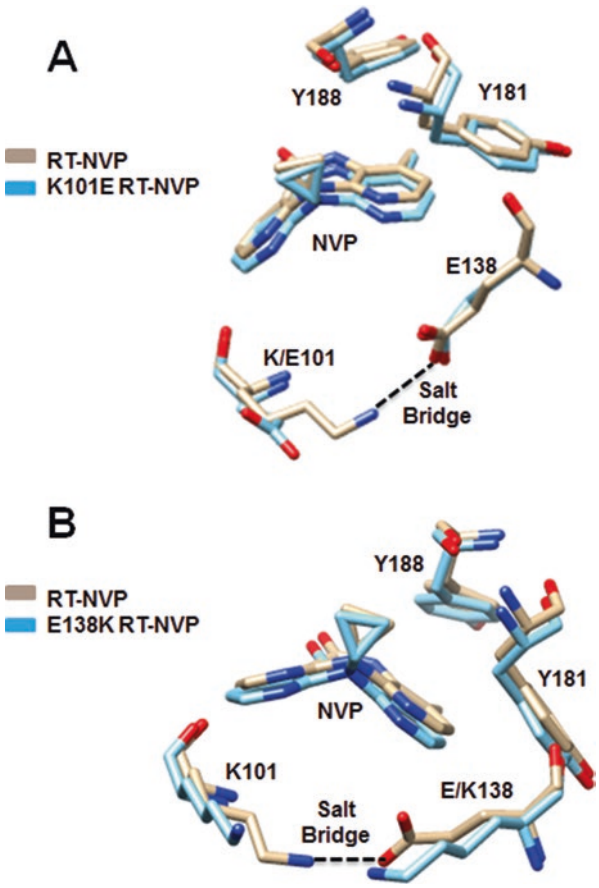


Fig. 34.5 (**a**) Overlay of the NNRTI-BP of RT–NVP (pdb: 1VRT) and K101E RT–NVP (pdb: 2HND). (**b**) Overlay of the NNRTI-BP of RT–NVP (pdb: 1VRT) and E138K RT–NVP (pdb: 2HNY)

reveals that the side chains at residue 100 are significantly overlapped, particularly the CD1 methyl group of L100 and CD methyl group of I100. However, the CG2 methyl of I100 is positioned 0.8 Å from the CD2 atom of L100 leading to a shift in the positioning of NVP. The biggest protein movement seen in the L100I RT-NVP structure is the side chain of Y188, which rotates by 13° and has a 0.6 Å shift of the ring center. There is also a movement of Y181. It is likely that the conformational shifts associated with Y188 and Y181 reduce the ring stacking interactions with NVP, which leads to decreased binding affinity between the inhibitor and the NNRTI-BP [85].

7.2 K101E and E138K

At the solvent-exposed entrance to the NNRTI-BP, the positively charged K101 residue in p66 forms a salt bridge with the negatively charged E138 residue in p51. Mutations at both residues are associated with NNRTI resistance. The K101E mutation has been selected in patients receiving each of the NNRTIs [80,81,84,86]. Alone, it reduces HIV-1 susceptibility to NVP by ~170-fold, to EFV by 2.1-fold, and to RPV and ETR by 3- to 5-fold (Table 34.2). The E138K mutation is selected in patients receiving RPV and ETR, and reduces HIV-1 susceptibility to both NNRTI by ~2-fold [55,84,86]. E138K does not appear to confer significant cross-resistance to NVP and EFV [55]. In the K101E RT-NVP structure [87], there is a significant movement of E101 relative to the position of the WT K101 residue, such that the side-chain carboxyl group points away from the NNRTI-BP (Fig. 34.5a). Accompanying the side-chain movement due to K101E, there is a small shift in the position of NVP, and also a small displacement of the E138 side chain with a shift of ~0.5 Å relative to WT. In the E138K RT-NVP structure [87], K138 moves away from the NNRTI-BP as indicated by the shift in position of the CD atom of 2.8 Å (Fig. 34.5b), while K101 becomes much less ordered than in the WT structure. There is also a shift in the position of NVP outwards from the binding pocket. Collectively, both K101E and E138K cause similar perturbations in the NNRTI-BP, with the most obvious effect being the disruption of the salt bridge between the two residues. Molecular modeling studies of an E138K RT–RPV complex suggested that disruption of the salt bridge causes an increased opening of the NNRTI-BP to solvent, and decreases van der Waals contact between RPV and the protein [88]. This observation is consistent with the finding that other mutations at resiues K101 (P) or E138 (A, G, K, R, or Q) can also disrupt the salt bridge, leading to decrease in HIV-1 susceptibilty to ETR and RPV [55].

7.3 K103N

The K103N mutation is selected in patients receiving NVP- or EFV-based therapies [80,81,89,90]. K103N reduces HIV-1 susceptibility to NVP (~50-fold) and EFV (~30-fold), but does

not confer cross-resistance to ETR or RPV (Table 34.2). Crystal structures of K103N RT in complex with NVP, EFV, RPV, and ETR have been reported [13,14,16,91]. In general, direct contacts between these NNRTI and K103 are not observed, and a stereochemical explanation for the loss of NNRTI binding due to K103N cannot be inferred from these structures. In contrast, in unliganded K103N RT structures the asparagine side chain forms a hydrogen bond with the hydroxyl group of Y188 (Fig. 34.6a), and it has been suggested that this interaction helps stabilize the apo-form of the enzyme which prevents NNRTI binding [5,92,93]. However, K103N does not decrease virus susceptibility to ETR or RPV, which suggests that further factors must be in play to explain resistance to NVP and EFV resistance. Using single-molecule and ensemble biophysical approaches, we recently proposed a mechanism of resistance that relies on modulation of the conformational dynamics of drug-bound RT [42]. Briefly, we found that K103N does not impact the affinity of EFV binding to RT but relieves the drug-induced molecular arthritis in the finger and thumb subdomains of RT, likely through disruption of the salt bridge between K101 and E138 (Fig. 34.6b). This, in turn, inhibits the EFV-bound enzyme from sliding excessively on the T/P substrate and allows it to efficiently bind an incoming nucleotide and form a functional RT–T/P–dNTP complex. In contrast to EFV, K103N decreased the binding affinity of NVP for the RT–T/P complex, a finding which underscores a concept that a single mutation in RT can differentially affect the binding of structurally diverse NNRTI. Of note, in the crystal structure of K103N RT in complex with RPV [92], the salt bridge between K101 and E138 remains intact (Fig. 34.6c), and we found that RPV binds with increased affinity to the RT–T/P complex [42].

7.4 Y181C

The Y181C mutaton is primarily selected by therapies containing NVP, ETR, or RPV [27,81,84]. It causes >40-fold reduced susceptibility to NVP, and 3- to 4-fold reduced susceptibility to RPV and ETR (Table 34.2). Y181C also reduces HIV-1 susceptibility to EFV by ~2-fold, and has been associated with a reduced response to treatment with an EFV-contaning regimen. In the Y181C RT-NVP structure [94], the inhibitor is located in almost exactly the same position that it occupies in the WT RT-NVP structure (Fig. 34.7a). There are however some slight

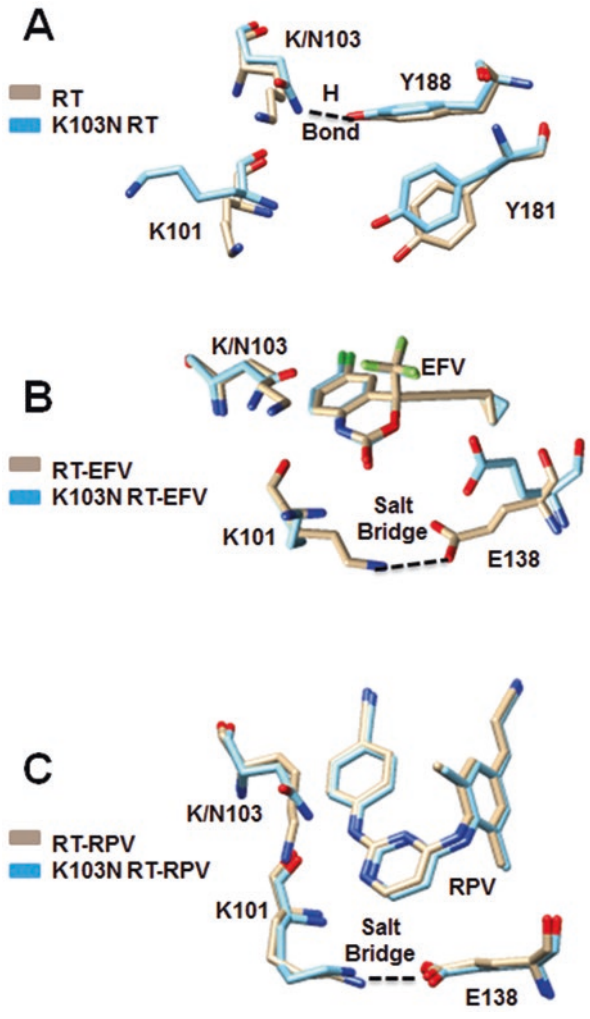


Fig. 34.6 (**a**) Overlay of the NNRTI-BP of HIV-1 RT (pdb: 1DLO) and K103N RT (pdb: 1HQE). (**b**) Overlay of the NNRTI-BP of RT-EFV (pdb: 1FK9) and K103N RT-EFV (pdb: 1FKO). (**c**, **b**) Overlay of the NNRTI-BP of RT–RPV (pdb: 3MEE) and K103N RT–RPV (pdb: 3MEG)

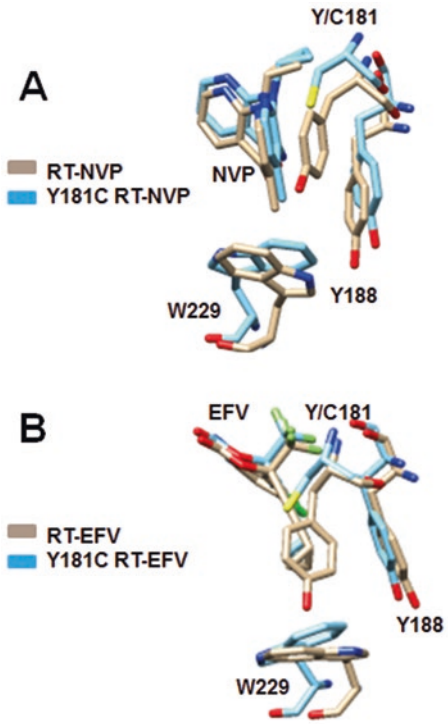


Fig. 34.7 (**a**) Overlay of the NNRTI-BP of RT–NVP (pdb: 1VRT) and Y181C RT–NVP (pdb: 1JLB). (**b**) Overlay of the NNRTI-BP of RT-EFV (pdb: 1FK9) and Y181C RT-EFV (pdb: 1JKH)

perturbations in the NNRTI-BP that could explain the observed resistance. Specifically, the reduced bulk of the Cys side chain allows Y188 to move closer toward it, which causes W229 to reorientate, resulting in a rearrangement of the β-9–β-11 sheet region. In the Y181C RT-EFV structure [94], there is also very little movement of the inhibitor in the NNRTI-BP (Fig. 34.7b). However the cyclopropyl group is less bulky than the corresponding aromatic rings of NVP, which allows W229 to flip, thus providing one explanation for the greater resilience of EFV toward the Y181C mutation.

7.5 N348I

The N348I mutation in the connection domain of HIV-1 RT confers resistance to NVP and EFV [51–53], and possibly also RPV and ETR. Interestingly, residue N348 in both subunits of RT is located distal to the NNRTI-BP, and it is not evident how these mutations decrease HIV-1 susceptibility to the NNRTIs. Several studies have reported that N348I decreases the RNase H cleavage activity of HIV-1 RT [98–101]. In this regard, Nikolenko et al. proposed that this decrease in RNase H activity preserves the RNA template and provides more time for NNRTIs to dissociate from the RT, resulting in the resumption of DNA synthesis and enhanced NNRTI resistance [98]. However, other studies have shown that N348I confers NVP resistance on both RNA/DNA and DNA/DNA T/P substrates [101], suggesting that factors in addition to RNase H cleavage impact nevirapine binding. Interestingly, NNRTI resistance can be attributed to the mutation in either subunit of the enzyme [101].

8 Conclusion

NNRTIs are widely used to treat HIV-1-infected individuals. Most first-line antiretroviral therapies typically include two NRTIs in combination with an NNRTI. ETR can be used to treat HIV-infected antiretroviral therapy-experienced individuals, including those with prior NNRTI exposure. NNRTIs are also increasingly being included in strategies to prevent HIV-1 infection. Given their widespread use, particularly in resource-limited settings, as well as their low genetic barriers to resistance, there are concerns about overlapping resistance between the different NNRTIs. Consequently, a better understanding of all aspects of NNRTI resistance—including the mechanisms involved—is important for (1) predicting response to treatment; (2) surveillance of TDR; and (3) development of new classes of NNRTIs with higher genetic barriers to resistance.

Acknowledgements Research in the Sluis-Cremer laboratory was supported by grants AI081571 and GM068406 from the National Institutes of Health (NIH), USA.

References

1. Hu WS, Hughes SH. HIV-1 reverse transcription. Cold Spring Harb Perspect Med. 2012;2:10.
2. Warrilow D, Tachedjian G, Harrich D. Maturation of the HIV reverse transcription complex: putting the jigsaw together. Rev Med Virol. 2009;19:324–37.
3. Rodgers DW, Gamblin SJ, Harris BA, Ray S, Culp JS, Hellmig B, Woolf DJ, Debouck C, Harrison SC. The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 1995;92:1222–6.
4. Hsiou Y, Ding J, Das K, Clark Jr AD, Hughes SH, Arnold E. Structure of unliganded HIV-1 reverse transcriptase at 2.7 A resolution: implications of conformational changes for polymerization and inhibition mechanisms. Structure. 1996;4:853–60.
5. Hsiou Y, Ding J, Das K, Clark Jr AD, Boyer PL, Lewi P, Janssen PA, Kleim JP, Rösner M, Hughes SH, Arnold E. The Lys103Asn mutation of HIV-1 RT: a novel mechanism of drug resistance. J Mol Biol. 2001;309:437–45.
6. Ding J, Das K, Hsiou Y, Sarafianos SG, Clark Jr AD, Jacobo-Molina A, Tantillo C, Hughes SH, Arnold E. Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 A resolution. J Mol Biol. 1998;284:1095–111.
7. Sarafianos SG, Das K, Tantillo C, Clark Jr AD, Ding J, Whitcomb JM, Boyer PL, Hughes SH, Arnold E. Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA:DNA. EMBO J. 2001;20:1449–61.
8. Das K, Martinez SE, Bauman JD, Arnold E. HIV-1 reverse transcriptase complex with DNA and nevirapine reveals non-nucleoside inhibition mechanism. Nat Struct Mol Biol. 2012;19:253–9.
9. Lapkouski M, Tian L, Miller JT, Le Grice SF, Yang W. Complexes of HIV-1 RT, NNRTI and RNA/DNA hybrid reveal a structure compatible with RNA degradation. Nat Struct Mol Biol. 2013;20:230–6.
10. Das K, Martinez SE, Bandwar RP, Arnold E. Structures of HIV-1 RT-RNA/DNA ternary complexes with dATP and nevirapine reveal conformational flexibility of RNA/DNA: insights into requirements for RNase H cleavage. Nucleic Acids Res. 2014;42:8125–37.
11. Huang H, Chopra R, Verdine GL, Harrison SC. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science. 1998;282:1669–75.
12. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science. 1992;256:1783–90.
13. Ren J, Milton J, Weaver KL, Short SA, Stuart DI, Stammers DK. Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase. Structure. 2000;8:1089–94.
14. Das K, Clark Jr AD, Lewi PJ, Heeres J, De Jonge MR, Koymans LM, Vinkers HM, Daeyaert F, Ludovici DW, Kukla MJ, De Corte B, Kavash RW, Ho CY, Ye H, Lichtenstein MA, Andries K, Pauwels R, De Béthune MP, Boyer PL, Clark P, Hughes SH, Janssen PA, Arnold E. Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. J Med Chem. 2004;47:2550–60.
15. Das K, Bauman JD, Clark Jr AD, Frenkel YV, Lewi PJ, Shatkin AJ, Hughes SH, Arnold E. High-resolution structures of HIV-1 reverse transcriptase/TMC278 complexes: strategic flexibility explains potency against resistance mutations. Proc Natl Acad Sci U S A. 2008;105:1466–71.

16. Lansdon EB, Brendza KM, Hung M, Wang R, Mukund S, Jin D, Birkus G, Kutty N, Liu X. Crystal structures of HIV-1 reverse transcriptase with etravirine (TMC125) and rilpivirine (TMC278): implications for drug design. J Med Chem. 2010;53:4295–9.
17. Le Grice SF. Human immunodeficiency virus reverse transcriptase: 25 years of research, drug discovery, and promise. J Biol Chem. 2012;287:40850–7.
18. Wang J, Smerdon SJ, Jäger J, Kohlstaedt LA, Rice PA, Friedman JM, Steitz TA. Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. Proc Natl Acad Sci U S A. 1994;91:7242–6.
19. Baba M, Tanaka H, De Clercq E, Pauwels R, Balzarini J, Schols D, Nakashima H, Perno CF, Walker RT, Miyasaka T. Highly specific inhibition of human immunodeficiency virus type 1 by a novel 6-substituted acyclouridine derivative. Biochem Biophys Res Commun. 1989;165:1375–81.
20. Debyser Z, Pauwels R, Andries K, Desmyter J, Kukla M, Janssen PA, De Clercq E. An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo-[4,5,1-jk] [1,4]benzodiazepin-2 (1H)-one and -thione derivatives. Proc Natl Acad Sci U S A. 1991 Feb 15;88(4):1451–5.
21. de Béthune MP. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989–2009). Antiviral Res. 2010;85:75–90.
22. Vingerhoets J, Azijn H, Fransen E, De Baere I, Smeulders L, Jochmans D, Andries K, Pauwels R, de Béthune MP. TMC125 displays a high genetic barrier to the development of resistance: evidence from in vitro selection experiments. J Virol. 2005;79:12773–82.
23. Lazzarin A, Campbell T, Clotet B, Johnson M, Katlama C, Moll A, Towner W, Trottier B, Peeters M, Vingerhoets J, de Smedt G, Baeten B, Beets G, Sinha R, Woodfall B, DUET-2 Study Group. Efficacy and safety of TMC125 (etravirine) in treatment-experienced HIV-1-infected patients in DUET-2: 24-week results from a randomised, double-blind, placebo-controlled trial. Lancet. 2007;370:39–48.
24. Madruga JV, Cahn P, Grinsztejn B, Haubrich R, Lalezari J, Mills A, Pialoux G, Wilkin T, Peeters M, Vingerhoets J, de Smedt G, Leopold L, Trefiglio R, Woodfall B, DUET-1 Study Group. Efficacy and safety of TMC125 (etravirine) in treatment-experienced HIV-1-infected patients in DUET-1: 24-week results from a randomised, double-blind, placebo-controlled trial. Lancet. 2007;370:29–38.
25. Baert L, van 't Klooster G, Dries W, François M, Wouters A, Basstanie E, Iterbeke K, Stappers F, Stevens P, Schueller L, Van Remoortere P, Kraus G, Wigerinck P, Rosier J. Development of a long-acting injectable formulation with nanoparticles of rilpivirine (TMC278) for HIV treatment. Eur J Pharm Biopharm. 2009;72:502–8.
26. van 't Klooster G, Hoeben E, Borghys H, Looszova A, Bouche MP, van Velsen F, Baert L. Pharmacokinetics and disposition of rilpivirine (TMC278) nanosuspension as a long-acting injectable antiretroviral formulation. Antimicrob Agents Chemother. 2010;54:2042–50.
27. Yoshinaga T, Kobayashi M, Seki T, Miki S, Wakasa-Morimoto C, Suyama-Kagitani A, Kawauchi-Miki S, Taishi T, Kawasuji T, Johns BA, Underwood MR, Garvey EP, Sato A, Fujiwara T. Antiviral characteristics of GSK1265744, an HIV integrase inhibitor dosed orally or by long-acting injection. Antimicrob Agents Chemother. 2015;59:397–407.
28. Margolis D, Brinson C, Eron J, Richmond G, LeBlanc R, Griffith S, St. Clair M, Stevens M, Williams P, Spreen W. 744 and Rilpivirine as two-drug oral maintenance therapy: LAI116482 (LATTE) week 48 results. In: 21st conference on retroviruses and opportunistic infections (CROI 2014). Boston; 3–6 March 2014. Abstract 91LB.
29. Guay LA, Musoke P, Fleming T, Bagenda D, Allen M, Nakabiito C, Sherman J, Bakaki P, Ducar C, Deseyve M, Emel L, Mirochnick M, Fowler MG, Mofenson L, Miotti P, Dransfield K, Bray D, Mmiro F, Jackson JB. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. Lancet. 1999;354:795–802.
30. Flys T, Nissley DV, Claasen CW, Jones D, Shi C, Guay LA, Musoke P, Mmiro F, Strathern JN, Jackson JB, Eshleman JR, Eshleman SH. Sensitive drug-resistance assays reveal long-term persistence of HIV-1 variants with the K103N nevirapine (NVP) resistance mutation in some women and infants after the administration of single-dose NVP: HIVNET 012. J Infect Dis. 2005;192:24–9.
31. Eshleman SH, Guay LA, Wang J, Mwatha A, Brown ER, Musoke P, Mmiro F, Jackson JB. Distinct patterns of emergence and fading of K103N and Y181C in women with subtype A vs. D after single-dose nevirapine: HIVNET 012. J Acquir Immune Defic Syndr. 2005;40:24–9.
32. Micek MA, Blanco AJ, Beck IA, Dross S, Matunha L, Montoya P, Seidel K, Gantt S, Matediane E, Jamisse L, Gloyd S, Frenkel LM. Nevirapine resistance by timing of HIV type 1 infection in infants treated with single-dose nevirapine. Clin Infect Dis. 2010;50:1405–14.
33. Johnson JA, Li JF, Morris L, Martinson N, Gray G, McIntyre J, Heneine W. Emergence of drug-resistant HIV-1 after intrapartum administration of single-dose nevirapine is substantially underestimated. J Infect Dis. 2005;192:16–23.
34. Woolfson AD, Malcolm RK, Morrow RJ, Toner CF, McCullagh SD. Intravaginal ring delivery of the reverse transcriptase inhibitor TMC 120 as an HIV microbicide. Int J Pharm. 2006;325:82–9.
35. Romano J, Variano B, Coplan P, Van Roey J, Douville K, Rosenberg Z, Temmerman M, Verstraelen H, Van Bortel L, Weyers S, Mitchnick M. Safety and availability of dapivirine (TMC120) delivered from an intravaginal ring. AIDS Res Hum Retroviruses. 2009;25:483–8.
36. Nel A, Smythe S, Young K, Malcolm K, McCoy C, Rosenberg Z, Romano J. Safety and pharmacokinetics of dapivirine delivery from matrix and reservoir intravaginal rings to HIV-negative women. J Acquir Immune Defic Syndr. 2009;51:416–23.
37. Creasy G, Hoesley C, Friedland B, Zhang S, Plagianos M, Kleinbeck K, Levendosky K, Fernández-Romero J, Zydowsky T. First-in-human safety and pharmacokinetics (PK) of a MIV-150/zinc acetate/carrageenan gel (PC-1005). AIDS Res Hum Retroviruses. 2014;30 Suppl 1:A151.
38. Jackson AG, Else LJ, Mesquita PM, Egan D, Back DJ, Karolia Z, Ringner-Nackter L, Higgs CJ, Herold BC, Gazzard BG, Boffito M. A compartmental pharmacokinetic evaluation of long-acting rilpivirine in HIV-negative volunteers for pre-exposure prophylaxis. Clin Pharmacol Ther. 2014;96:314–23.
39. Spence RA, Kati WM, Anderson KS, Johnson KA. Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. Science. 1995;267:988–93.
40. Xia Q, Radzio J, Anderson KS, Sluis-Cremer N. Probing nonnucleoside inhibitor-induced active-site distortion in HIV-1 reverse transcriptase by transient kinetic analyses. Protein Sci. 2007;16:1728–37.
41. Liu S, Abbondanzieri EA, Rausch JW, Le Grice SF, Zhuang X. Slide into action: dynamic shuttling of HIV reverse transcriptase on nucleic acid substrates. Science. 2008;322:1092–7.
42. Schauer GD, Huber KD, Leuba SH, Sluis-Cremer N. Mechanism of allosteric inhibition of HIV-1 reverse transcriptase revealed by single-molecule and ensemble fluorescence. Nucleic Acids Res. 2015;42:11687–96.
43. Bec G, Meyer B, Gerard MA, Steger J, Fauster K, Wolff P, Burnouf D, Micura R, Dumas P, Ennifar E. Thermodynamics of HIV-1

reverse transcriptase in action elucidates the mechanism of action of non-nucleoside inhibitors. J Am Chem Soc. 2013;135:9743–52.
44. Grobler JA, Dornadula G, Rice MR, Simcoe AL, Hazuda DJ, Miller MD. HIV-1 reverse transcriptase plus-strand initiation exhibits preferential sensitivity to non-nucleoside reverse transcriptase inhibitors in vitro. J Biol Chem. 2007;282:8005–10.
45. Shaw-Reid CA, Feuston B, Munshi V, Getty K, Krueger J, Hazuda DJ, Parniak MA, Miller MD, Lewis D. Dissecting the effects of DNA polymerase and ribonuclease H inhibitor combinations on HIV-1 reverse-transcriptase activities. Biochemistry. 2005;44:1595–606.
46. Radzio J, Sluis-Cremer N. Efavirenz accelerates HIV-1 reverse transcriptase ribonuclease H cleavage, leading to diminished zidovudine excision. Mol Pharmacol. 2008;73:601–6.
47. Herman BD, Sluis-Cremer N. Transient kinetic analyses of the ribonuclease H cleavage activity of HIV-1 reverse transcriptase in complex with efavirenz and/or a β-thujaplicinol analogue. Biochem J. 2013;455:179–84.
48. Muchiri JM, Li D, Dykes C, Bambara RA. Efavirenz stimulates HIV-1 reverse transcriptase RNase H activity by a mechanism involving increased substrate binding and secondary cleavage activity. Biochemistry. 2013;52:4981–90.
49. Abbondanzieri EA, Bokinsky G, Rausch JW, Zhang JX, Le Grice SF, Zhuang X. Dynamic binding orientations direct activity of HIV reverse transcriptase. Nature. 2008;453:184–9.
50. Sluis-Cremer N. The emerging profile of cross-resistance among the nonnucleoside HIV-1 reverse transcriptase inhibitors. Viruses. 2014;6:2960–73.
51. Yap SH, Sheen CW, Fahey J, Zanin M, Tyssen D, Lima VD, Wynhoven B, Kuiper M, Sluis-Cremer N, Harrigan PR, Tachedjian G. N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. PLoS Med. 2007;4:e335.
52. Hachiya A, Kodama EN, Sarafianos SG, Schuckmann MM, Sakagami Y, Matsuoka M, Takiguchi M, Gatanaga H, Oka S. Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. J Virol. 2008;82:3261–70.
53. Brehm JH, Koontz DL, Wallis CL, Shutt KA, Sanne I, Wood R, McIntyre JA, Stevens WS, Sluis-Cremer N, Mellors JW, CIPRA-SA Project 1 Study Team. Frequent emergence of N348I in HIV-1 subtype C reverse transcriptase with failure of initial therapy reduces susceptibility to reverse-transcriptase inhibitors. Clin Infect Dis. 2012;55:737–45.
54. Brenner B, Turner D, Oliveira M, Moisi D, Detorio M, Carobene M, Marlink RG, Schapiro J, Roger M, Wainberg MA. A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. AIDS. 2003;17:F1–5.
55. Sluis-Cremer N, Jordan MR, Huber K, Wallis CL, Bertagnolio S, Mellors JW, Parkin NT, Harrigan PR. E138A in HIV-1 reverse transcriptase is more common in subtype C than B: implications for rilpivirine use in resource-limited settings. Antiviral Res. 2014;107:31–4.
56. Siegel MO, Swierzbinski M, Kan VL, Parenti DM. Baseline E138 reverse transcriptase resistance-associated mutations in antiretroviral-naive HIV-infected patients. AIDS. 2012;26:1181–2.
57. Jeulin H, Foissac M, Boyer L, Agrinier N, Perrier P, Kennel A, Velay A, Goehringer F, Henard S, Rabaud C, May T, Schvoerer E. Real-life rilpivirine resistance and potential emergence of an E138A-positive HIV strain in north-eastern France. J Antimicrob Chemother. 2014;69:3095–102.
58. Gupta RK, Jordan MR, Sultan BJ, Hill A, Davis DH, Gregson J, Sawyer AW, Hamers RL, Ndembi N, Pillay D, Bertagnolio S. Global trends in antiretroviral resistance in treatment-naive individuals with HIV after rollout of antiretroviral treatment in resource-limited settings: a global collaborative study and meta-regression analysis. Lancet. 2012;380:1250–8.
59. Pham QD, Wilson DP, Law MG, Kelleher AD, Zhang L. Global burden of transmitted HIV drug resistance and HIV-exposure categories: a systematic review and meta-analysis. AIDS. 2014;28:2751–62.
60. Wittkop L, Gunthard HF, de Wolf F, Dunn D, Cozzi-Lepri A, de Luca A, Kücherer C, Obel N, von Wyl V, Masquelier B, Stephan C, Torti C, Antinori A, García F, Judd A, Porter K, Thiébaut R, Castro H, van Sighem AI, Colin C, Kjaer J, Lundgren JD, Paredes R, Pozniak A, Clotet B, Phillips A, Pillay D, Chêne G, EuroCoord-CHAIN Study Group. Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. Lancet Infect Dis. 2011;11:363–71.
61. Bertagnolio S, Perno CF, Vella S, Pillay D. The impact of HIV drug resistance on the selection of first- and second-line art in resource-limited settings. J Infect Dis. 2013;207:S45–8.
62. Phanuphak P, Sirivichayakul S, Jiamsakul A, Sungkanuparph S, Kumarasamy N, Lee MP, Kumarasamy N, Lee MP, Sirisanthana T, Kantipong P, Lee C, Kamarulzaman A, Mustafa M, Ditangco R, Merati T, Ratanasuwan W, Singtoroj T, Kantor R. Transmitted drug resistance and antiretroviral treatment outcomes in non-subtype B HIV1-infected patients in South East Asia. J Acquir Immune Defic Syndr. 2014;66:74–9.
63. Hamers RL, Schuurman R, Sigaloff KCE, Wallis CL, Kityo C, Siwale M, Mandaliya K, Ive P, Botes ME, Wellington M, Osibogun A, Wit FW, van Vugt M, Stevens WS, de Wit TF, PharmAccess African Studies to Evaluate Resistance (PASER) Investigators. Effect of pretreatment HIV-1 drug resistance on immunological, virological, and drug-resistance outcomes of first-line antiretroviral treatment in sub-Saharan Africa: a multicentre cohort study. Lancet Infect Dis. 2012;12:307–17.
64. Brumme CJ, Huber KD, Dong W, Poon AF, Harrigan PR, Sluis-Cremer N. Replication fitness of multiple nonnucleoside reverse transcriptase-resistant HIV-1 variants in the presence of etravirine measured by 454 deep sequencing. J Virol. 2013;87:8805–7.
65. Armstrong KL, Lee TH, Essex M. Replicative fitness costs of nonnucleoside reverse transcriptase inhibitor drug resistance mutations on HIV subtype C. Antimicrob Agents Chemother. 2011;55:2146–53.
66. Wang J, Bambara RA, Demeter LM, Dykes C. Reduced fitness in cell culture of HIV-1 with nonnucleoside reverse transcriptase inhibitor-resistant mutations correlates with relative levels of reverse transcriptase content and RNase H activity in virions. J Virol. 2010;84:9377–89.
67. Collins JA, Thompson MG, Paintsil E, Ricketts M, Gedizor J, Alexander L. Competitive fitness of Nevirapine-resistant HIV-1 mutants. J Virol. 2004;78:603–11.
68. Iglesias-Ussel MD, Casado C, Yuste E, Olivares I, Lopez-Galindez C. In vitro analysis of HIV-1 resistance to nevirapine and fitness determination of resistant variants. J Gen Virol. 2002;83:93–101.
69. Joly V, Descamps D, Peytavin G, Touati F, Mentre F, Duval X, Delarue S, Yeni P, Brun-Vezinet F. Evolution of HIV-1 resistance mutations in HIV-1-infected patients switched to antiretroviral therapy without NNRTIs. Antimicrob Agents Chemother. 2004;48:172–5.
70. Delaugerre C, Morand-Joubert L, Chaix ML, Picard O, Marcelin AG, Schneider V, Krivine A, Compagnucci A, Katlama C, Girard PM, Calvez V. Persistence of multidrug-resistant HIV-1 without antiretroviral treatment two years after sexual transmission. Antivir Ther. 2004;9:415–21.

71. Korval CE, Dykes C, Wang J, Demeter LM. Relative replicative fitness of efavirenz-resistant mutants of HIV-1: correlation with frequency during clinical therapy and evidence of compensation for the reduced fitness of K103N + L100I by the nucleoside resistance mutation L74V. Virology. 2006;353:184–92.
72. Hu Z, Kuritzkes DR. Interaction of reverse transcriptase (RT) mutations conferring resistance to lamivudine and etravirine: effects on fitness and RT activity of human immunodeficiency virus type 1. J Virol. 2011;85:11309–14.
73. Xu HT, Asahchop EL, Oliveira M, Quashie PK, Quan Y, Brenner BG, Wainberg MA. Compensation by the E138K mutation in HIV-1 reverse transcriptase for deficits in viral replication capacity and enzyme processivity associated with the M184I/V mutations. J Virol. 2011;85:11300–8.
74. Geitmann M, Unge T, Danielson UH. Interaction kinetic characterization of HIV-1 reverse transcriptase non-nucleoside inhibitor resistance. J Med Chem. 2006;49:2375–87.
75. Ren J, Stammers DK. Structural basis for drug resistance mechanisms for non-nucleoside inhibitors of HIV reverse transcriptase. Virus Res. 2008;134:157–70.
76. Winslow DL, Garber S, Reid C, Scarnati H, Baker D, Rayner MM, Anton ED. Selection conditions affect the evolution of specific mutations in the reverse transcriptase gene associated with resistance to DMP 266. AIDS. 1996;10:1205–9.
77. Corbau R, Mori J, Phillips C, Fishburn L, Martin A, Mowbray C, Panton W, Smith-Burchnell C, Thornberry A, Ringrose H, Knochel T, Irving S, Westby M, Wood A, Perros M. Lersivirine, a nonnucleoside reverse transcriptase inhibitor with activity against drug-resistant human immunodeficiency virus type 1. Antimicrob Agents Chemother. 2010;54:4451–63.
78. Azijn H, Tirry I, Vingerhoets J, de Bethune MP, Kraus G, Boven K, Jochmans D, Van Craenenbroeck E, Picchio G, Rimsky LT. TMC278, a next-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), active against wild-type and NNRTI-resistant HIV-1. Antimicrob Agents Chemother. 2010;54:718–27.
79. Asahchop EL, Wainberg MA, Oliveira M, Xu H, Brenner BG, Moisi D, Ibanescu IR, Tremblay C. Distinct resistance patterns to etravirine and rilpivirine in viruses containing NNRTI mutations at baseline. Antimicrob Agents Chemother. 2010;54:718–27.
80. Bacheler LT, Anton ED, Kudish P, Baker D, Bunville J, Krakowski K, Bolling L, Aujay M, Wang XV, Ellis D, Becker MF, Lasut AL, George HJ, Spalding DR, Hollis G, Abremski K. Human immunodeficiency virus type 1 mutations selected in patients failing efavirenz combination therapy. Antimicrob Agents Chemother. 2000;44:2475–84.
81. Reuman EC, Rhee SY, Holmes SP, Shafer RW. Constrained patterns of covariation and clustering of HIV-1 non-nucleoside reverse transcriptase inhibitor resistance mutations. J Antimicrob Chemother. 2010;65:1477–85.
82. Croxtall JD. Etravirine: a review of its use in the management of treatment-experienced patients with HIV-1 infection. Drugs. 2012;72:847–69.
83. Marcelin AG, Descamps D, Tamalet C, Cottalorda J, Izopet J, Delaugerre C, Morand-Joubert L, Trabaud MA, Bettinger D, Rogez S, Ruffault A, Henquell C, Signori-Schmuck A, Bouvier-Alias M, Vallet S, Masquelier B, Flandre P, Calvez V. Emerging mutations and associated factors in patients displaying treatment failure on an etravirine-containing regimen. Antivir Ther. 2012;17:119–23.
84. Rimsky L, Vingerhoets J, Van Eygen V, Eron J, Clotet B, Hoogstoel A, Boven K, Picchio G. Genotypic and phenotypic characterization of HIV-1 isolates obtained from patients on rilpivirine therapy experiencing virologic failure in the phase 3 ECHO and THRIVE studies: 48-week analysis. J Acquir Immune Defic Syndr. 2012;59:39–46.
85. Ren J, Nichols CE, Chamberlain PP, Weaver KL, Short SA, Stammers DK. Crystal structures of HIV-1 reverse transcriptases mutated at codons 100, 106 and 108 and mechanisms of resistance to non-nucleoside inhibitors. J Mol Biol. 2004;336:569–78.
86. Tambuyzer L, Vingerhoets J, Azijn H, Daems B, Nijs S, de Bethune MP, Picchio G. Characterization of genotypic and phenotypic changes in HIV-1-infected patients with virologic failure on an etravirine-containing regimen in the DUET-1 and DUET-2 clinical studies. AIDS Res Hum Retroviruses. 2010;26:1197–205.
87. Ren J, Nichols CE, Stamp A, Chamberlain PP, Ferris R, Weaver KL, Short SA, Stammers DK. Structural insights into mechanisms of non-nucleoside drug resistance for HIV-1 reverse transcriptases mutated at codons 101 or 138. FEBS J. 2006;273:3850–60.
88. Kulkarni R, Babaoglu K, Lansdon EB, Rimsky L, Van Eygen V, Picchio G, Svarovskaia E, Miller MD, White KL. The HIV-1 reverse transcriptase M184I mutation enhances the E138K-associated resistance to rilpivirine and decreases viral fitness. J Acquir Immune Defic Syndr. 2012;59:47–54.
89. Gulick RM, Ribaudo HJ, Shikuma CM, Lustgarten S, Squires KE, Meyer 3rd WA, Acosta EP, Schackman BR, Pilcher CD, Murphy RL, Maher WE, Witt MD, Reichman RC, Snyder S, Klingman KL, Kuritzkes DR. Triple-nucleoside regimens versus efavirenz-containing regimens for the initial treatment of HIV-1 infection. N Engl J Med. 2004;350:1850–61.
90. Margot NA, Lu B, Cheng A, Miller MD. Resistance development over 144 weeks in treatment-naive patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in Study 903. HIV Med. 2006;7:442–50.
91. Lindberg J, Sigurosson S, Lowgren S, Andersson HO, Sahlberg C, Noreen R, Fridborg K, Zhang H, Unge T. Structural basis for the inhibitory efficacy of efavirenz (DMP-266), MSC194 and PNU142721 towards the HIV-1 RT K103N mutant. Eur J Biochem. 2002;269:1670–7.
92. Rodriguez-Barrios F, Gago F. Understanding the basis of resistance in the irksome Lys103Asn HIV-1 reverse transcriptase mutant through targeted molecular dynamics simulations. J Am Chem Soc. 2004;126:15386–7.
93. Das K, Sarafianos SG, Clark Jr AD, Boyer PL, Hughes SH, Arnold E. Crystal structures of clinically relevant Lys103Asn/Tyr181Cys double mutant HIV-1 reverse transcriptase in complexes with ATP and non-nucleoside inhibitor HBY 097. J Mol Biol. 2007;365:77–89.
94. Ren J, Nichols C, Bird L, Chamberlain P, Weaver K, Short S, Stuart DI, Stammers DK. Structural mechanisms of drug resistance for mutations at codons 181 and 188 in HIV-1 reverse transcriptase and the improved resilience of second generation non-nucleoside inhibitors. J Mol Biol. 2001;312:795–805.
95. Sluis-Cremer N, Moore K, Radzio J, Sonza S, Tachedjian G. N348I in HIV-1 reverse transcriptase decreases susceptibility to tenofovir and etravirine in combination with other resistance mutations. AIDS. 2010;24:317–9.
96. Ehteshami M, Beilhartz GL, Scarth BJ, Tchesnokov EP, McCormick S, Wynhoven B, Harrigan PR, Götte M. Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3′-azido-3′-deoxythymidine through both RNase H-dependent and -independent mechanisms. J Biol Chem. 2008;283:22222–32.
97. Delviks-Frankenberry KA, Nikolenko GN, Boyer PL, Hughes SH, Coffin JM, Jere A, Pathak VK. HIV-1 reverse transcriptase connection subdomain mutations reduce template RNA degradation and enhance AZT excision. Proc Natl Acad Sci U S A. 2008;105:10943–8.
98. Nikolenko GN, Delviks-Frankenberry KA, Pathak VK. A novel molecular mechanism of dual resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. J Virol. 2010;84:5238–49.

99. Biondi MJ, Beilhartz GL, McCormick S, Götte M. N348I in HIV-1 reverse transcriptase can counteract the nevirapine-mediated bias toward RNase H cleavage during plus-strand initiation. J Biol Chem. 2010;285:26966–75.
100. Schuckmann MM, Marchand B, Hachiya A, Kodama EN, Kirby KA, Singh K, Sarafianos SG. The N348I mutation at the connection subdomain of HIV-1 reverse transcriptase decreases binding to nevirapine. J Biol Chem. 2010;285:38700–9.
101. Radzio J, Sluis-Cremer N. Subunit-specific mutational analysis of residue N348 in HIV-1 reverse transcriptase. Retrovirology. 2011;8:69.

Drug Resistance to HIV-1 Protease Inhibitors: Molecular Mechanisms and Substrate Coevolution

35

Nese Kurt Yilmaz and Celia A. Schiffer

1 Introduction

HIV-1 protease inhibitors (PIs) are competitive active-site inhibitors that mimic the transition state of the enzyme's substrate and are the most potent antiretroviral drugs against HIV infection. HIV-1 protease processes the viral polyproteins at specific cleavage sites and allows infectious mature virions and hence spread of the virus. Unfortunately rapid viral evolution combined with selective pressure of therapy causes selection of many drug-resistant variants that are no longer efficiently inhibited by the PIs. HIV-1 protease can tolerate extensive mutations, with close to half of the 99-residues making up each of the chains in the homodimeric protease and residues at substrate cleavage sites mutating to escape PI pressure. Structural and biophysical studies of many drug-resistant HIV-1 protease variants revealed insights into how mutations at and outside of the protease active site are able to confer PI resistance while still allowing recognition and processing of substrates, and why substrate mutations coevolve with primary protease mutations. We summarize the main molecular mechanisms underlying PI resistance due to primary, secondary, and substrate coevolved mutations and how this knowledge may guide the design of robust inhibitors to avoid resistance.

N.K. Yilmaz, Ph.D.
Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, USA

C.A. Schiffer (✉)
Institute of Drug Resistance, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA
e-mail: Nese.KurtYilmaz@umassmed.edu; Celia.Schiffer@umassmed.edu

2 HIV-1 Protease as a Drug Target

In the fourth decade after the first reporting of what became the worldwide AIDS epidemic, a cure for HIV-1 still eludes the medical community. According to the recent reports published by UNAIDS, there are ~35 million people living with HIV/AIDS around the globe [1]. Although no permanent cure or vaccine for AIDS exists, there are over 30 direct-acting antiviral (DAA) drugs that belong to seven classes targeting various stages in the life cycle of HIV [2], including protease inhibitors (PIs). With the introduction of DAA combinations as highly active antiretroviral therapy (HAART), overall, the quality and life expectancy of HIV-infected patients have greatly improved [3–5]. However, low drug adherence, toxicity, and high pill burden with some second-line therapies, coupled with the error-prone mechanism of HIV reverse transcriptase, have led to the emergence of drug resistance in HIV-infected patients under therapy.

In the last 25 years, drug discovery efforts aided by structure-based design have led to the development of nine FDA-approved PIs (Fig. 35.1): saquinavir (SQV) [6], indinavir (IDV) [7], ritonavir (RTV) [8], nelfinavir (NFV) [9], amprenavir (APV) [10], lopinavir (LPV) [11], atazanavir (ATV) [12], tipranavir (TPV) [13], and darunavir (DRV) [14–16]. All PIs are competitive inhibitors that bind at the protease active site (Fig. 35.2). The active site of this homodimeric aspartyl protease is formed at the interface of two identical 99-residue monomers and contains the catalytic aspartic acid at residue 25 in both subunits [17, 18]. In unliganded state, the protease is symmetric with highly flexible flaps that open up to allow access to the active site, but close to cover and interact with the bound ligand (substrate or inhibitor). When bound, PIs interact mainly with the hydrophobic S2–S2′ pockets at the active site. The peptidomimetic (except tipranavir) inhibitors were designed to mimic the transition state intermediate of peptide substrate by forming critical interactions with the catalytic Asp25, and contain non-cleavable peptide isosteres as core scaffolds. PIs are the most potent anti-HIV drugs with IC50

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_35

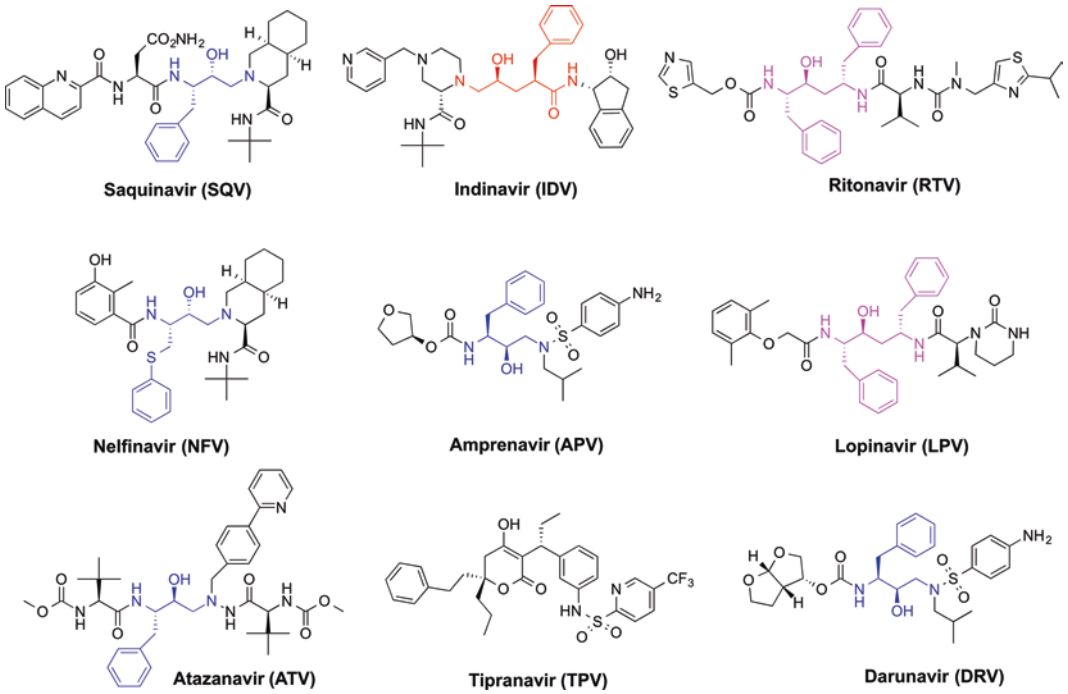


Fig. 35.1 Chemical structures of FDA-approved HIV-1 protease inhibitors. The non-cleavable dipeptide isostere cores mimicking the transition state are hydroxyethylamine (*blue*), hydroxyaminopentane (*red*), and hydroxyethylene (*magenta*)

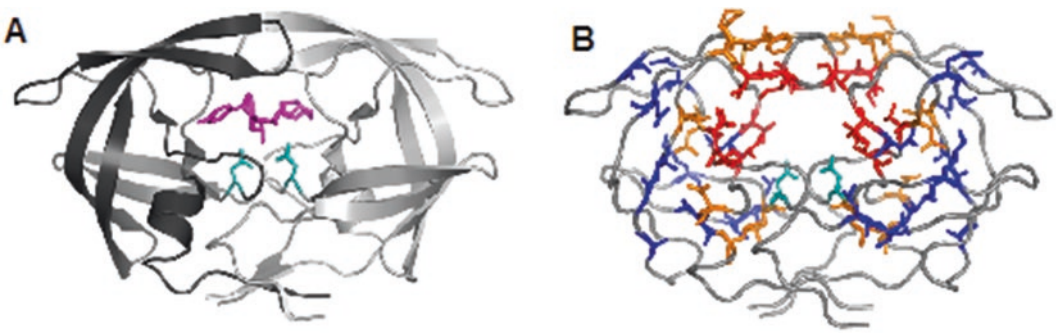


Fig. 35.2 Structure of HIV-1 protease bound to inhibitor DRV (PDB 1T3R). (**a**) The enzyme is a homodimer of two non-covalently assembled 99-residue chains (in *dark and light gray*). Each monomer contributes a catalytic Asp (teal side chain) to the active site where the inhibitor (*magenta*) binds. The flaps close over the bound ligand. (**b**) Residues that mutate to confer resistance to protease inhibitors are depicted by colored side chains. Location of primary resistance mutations at the active site (D30, V32, I47, G48, I50, V82, I84; *red*), primary resistance mutations outside the active site (M46, F53, I54 in flaps and L24, L33, L76, N88, L90; *orange*), and secondary resistance mutations (L10, V11, K20, E35, K43, Q58, V71, G73, T74, N83, L89; *blue*)

values in the low picomolar range. The cooperative dose–response curves with high slopes allow for extraordinarily high level of inhibition at clinical concentrations, which are well above the IC50 [19, 20].

3 HIV-1 Protease in the Viral Life Cycle

HIV infects and replicates in CD4+ immune cells by reverse-transcribing its single-stranded RNA genome. The viral genome includes *gag* and *pol* genes encoding polyprotein precursors Gag and Gag/Pol that need to be processed by HIV protease into individual viral proteins (Fig. 35.3a). Proteolytic cleavage of Gag yields the structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6. Gag/Pol is transcribed as a result of ribosomal frameshifting occurring ~10 % of the time near the end of the *gag* gene [21], and in addition to the Gag structural proteins includes viral enzymes protease (PR), reverse transcriptase (RT), RNase H (RH), and integrase (IN). The newly assembled budding HIV particles are released from the host cell as noninfectious immature virions that contain unprocessed Gag. Processing of Gag by

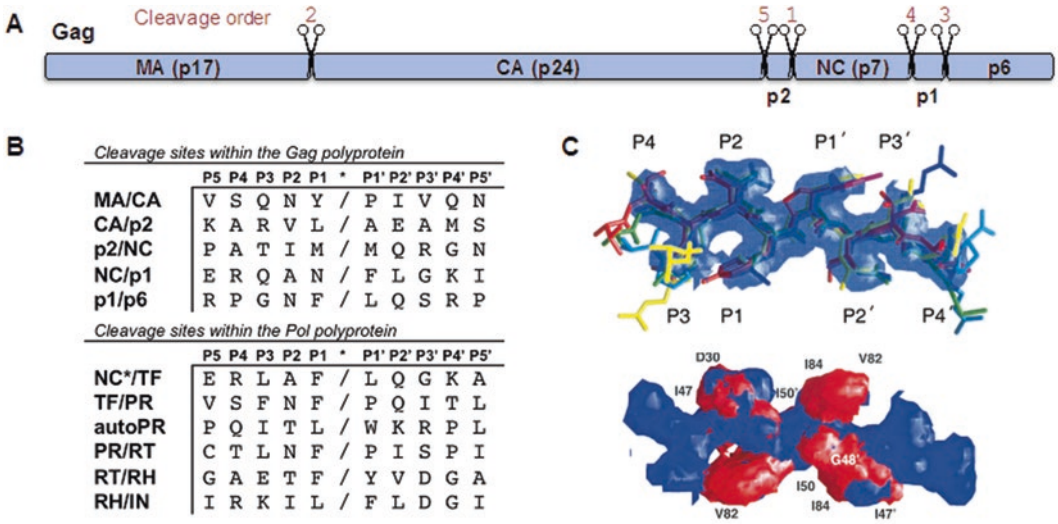


Fig. 35.3 HIV-1 protease substrates and the substrate envelope. (**a**) Processing of Gag to individual viral proteins at five specific sites allows viral maturation. (**b**) The amino acid sequences of cleavage sites within Gag and Pol polyproteins. Notice the lack of any conserved substrate recognition motif at the sequence level. (**c**) The overlay of cleavage site sequences in protease-bound crystal structures reveals the substrate envelope (*blue volume*). The inhibitors (*below*, *red volume*) protrude out the substrate envelope to contact protease residues (*labeled*) that mutate to confer resistance. Panel (**c**) reprinted from King et al. [35], Copyright (2004), with permission from Elsevier

the viral protease induces a major structural rearrangement and triggers the maturation of infectious virus. In total, HIV-1 protease recognizes and cleaves five sites in Gag including those between the viral proteins and spacer peptides p1 and p2 (Fig. 35.3b). The specific, sequential, and ordered processing of Gag by protease is essential for viral maturation and infectivity [22–25]. In addition to viral polyprotein precursors, HIV-1 protease cleaves host cell proteins, including translation initiation factors eIF4 and eIF3d, to inhibit host translation [26, 27].

4 PI Resistance Mutations In and Outside the Protease

The high replication rate of HIV coupled with the error-prone viral reverse transcriptase enables a highly heterogeneous viral population with different mutations. This preexisting diverse pool includes mutations that are expanded to confer resistance under the selective pressure of inhibitor therapy. Combinations of three or more DAAs have high enough selective pressure to minimize the emergence of resistance; however resistance has been observed for each of the HIV DAAs, including the PIs. Highly mutated viral variants can be selected under low plasma concentrations such as due to low patient adherence, or transmitted to newly infected individuals to cause therapy failure.

In viral sequences from patient isolates, up to 60–63 % of the HIV-1 protease sequences can vary, indicating very high tolerance to amino acid substitutions [28, 29] (Fig. 35.2b). Of the 99 positions in each monomer, only 37 are invariant (with mutation frequencies <0.5 %) and 17 are sites of nontreatment-related polymorphisms [28, 29]. The remaining 45 positions have been implicated in drug resistance. Mutations at 26 of these 45 positions can significantly decrease susceptibility to one or more PIs [29, 30], 16 of which are located outside the active site region and the flaps. In most cases, multiple mutations within and outside the protease active site coevolve to confer resistance to a particular inhibitor. Mutations that directly confer resistance—mostly located at the protease active site—are classified as primary mutations, while other mutations selected in the presence of primary mutations but that do not confer resistance by themselves are called secondary mutations. The most common primary resistance mutations include D30N, G48V, I50V/L, V82A/V/T, I84V (within the active site), and L90M (no direct contact with the inhibitor). The resistance pathway and accumulation of mutations depend on the HIV clade (and/or preexisting variants), and inhibitor(s) administered and therefore selected against. The first-generation PIs RTV, SQV, IDV, and NFV lose significant potency against drug-resistant variants and are susceptible to single "signature" active-site mutations. The latest and most potent PI, DRV, is active against most of the multidrug-resistant variants and typically up to 20 mutations need to coexist to confer high levels of DRV resistance.

HIV-1 PI resistance is also associated with coevolution of mutations in the viral genome outside the protease, particularly within the Gag cleavage sites NC/p1 and p1/p6 (reviewed in [31]). While avoiding inhibitor binding, the mutated protease needs to maintain its biological function of

substrate recognition and cleavage. The coevolution of cleavage sites may compensate for lost efficiency due to primary protease resistance mutations. Several Gag substrate mutations have also been classified as primary resistance mutations as they confer PI resistance in the absence of any protease mutations [32–34].

5 Molecular Mechanisms of Resistance

Drug resistance in HIV-1 protease has been extensively studied at the molecular level, particularly by biophysical and structural analysis of various protease mutants, yielding a plethora of information on structural, enzymatic, and dynamic changes associated with inhibitor resistance [34, 36–49]. These data enabled formulating hypotheses on molecular mechanisms of resistance, which led to strategies for designing inhibitors that avoid resistance, and may be applicable to other disease targets where resistance quickly emerges.

5.1 Active-Site Mutations and the Substrate Envelope

The active site of HIV-1 protease is mainly formed by residues 25–32 (including the catalytic Asp25), 47–53, and 80–84 from both monomers. Active-site mutations at residues that directly contact the inhibitor are quickly selected under PI monotherapy (red in Fig. 35.2b). Although chemically different, the three-dimensional shape and electrostatic character of the HIV-1 PIs are fairly similar; therefore a small set of mutations can result in a protease variant with multidrug resistance. Nevertheless, in most cases, specific signature active-site mutations confer resistance to a given inhibitor. Why a specific mutation is selected against an inhibitor, and how the protease is able to maintain its biological function despite an active-site mutation, is effectively explained by the protease *substrate envelope*.

The cleavage site sequences are highly heterogeneous, and amino acid sequence alone cannot explain how protease is able to recognize its substrates with high specificity. High-resolution crystal structures of HIV-1 protease bound to peptides corresponding to these cleavage sites revealed that the substrates adopt a specific, conserved three-dimensional shape when bound at the active site (Fig. 35.3c) [50, 51]. This overlapping volume occupied by bound protease substrates and spanning P4′–P4 sites defines the substrate envelope. The P1–P3 region of the substrates forms a toroid, likely critical in specific recognition of asymmetrical ligands by the homodimeric protease. In addition to describing the *structural* substrate recognition motif of the protease, the substrate envelope serves as a template for contrasting the binding of inhibitors to that of the natural substrates in resistance development, and comparing substrates among themselves in relation to substrate coevolution.

Similar to the substrates, the chemically diverse HIV-1 PIs share a conserved *inhibitor envelope* in protease-bound structures [35, 51, 52] spanning P2′–P2 sites. Superposition of the two envelopes reveals locations where inhibitors protrude out the substrate envelope and contact protease active-site residues. Such protrusions render an inhibitor vulnerable to mutations, as protease contacts at these locations are more important for inhibitor binding compared to substrates. An amino acid substitution could differentially weaken inhibitor contacts without substantially affecting substrate binding. Accordingly, protease residues that contact inhibitors beyond the substrate envelope correspond to locations of major active-site resistance mutations.

Several primary mutations are signature for resistance to a particular inhibitor, such as D30N to NFV, I50V/L to APV/DRV/ATV, G48V to SQV/ATV, and V82A to SQV/RTV. These signature mutations also primarily correspond to locations where individual inhibitors protrude out the substrate envelope. As the protease active site is mostly hydrophobic, side-chain substitutions due to primary mutations mainly affect van der Waals contacts with the ligand. However, analysis of protease–inhibitor complex structures with both wild-type and resistant variants has revealed that structural changes are often more complex than a simple loss of van der Waals contact at the site of mutation [39, 41, 50]. Rather, drug resistance mutations often cause an overall rearrangement of contacts around the inhibitor at the active site.

The substrate envelope broadly defines the evolutionary constraints on the selection of active-site mutations to confer drug resistance from a structural viewpoint. Mutations that abrogate essential contacts with the substrates would be detrimental to biological function, and thus are selected against. Instead, mutations are selected to weaken inhibitor contacts while still maintaining functionally essential substrate interactions. Such mutations tip the competition between inhibitor binding versus substrate recognition/processing in favor of the substrates, thus conferring drug resistance.

In addition to physical contacts with the inhibitor, drug resistance mutations can also alter the conformational dynamics of HIV-1 protease. The protease is a highly flexible enzyme that undergoes major conformational changes involving the flaps and the hydrophobic core during ligand binding and release [53–56]. This concerted change requires extensive side-chain repacking at the hydrophobic core, or *hydrophobic sliding*, as revealed in molecular dynamics (MD) simulations [56]. Reversible cross-linking of core hydrophobic residues carefully chosen based on the MD results elegantly demonstrated that the core dynamics directly modulates the enzyme's activity [57]. Considering drug resistance in the context of the balance between inhibitor binding and substrate processing,

any dynamic change that disfavors the inhibitor over the substrates would contribute to conferring resistance. As the inhibitor needs to stay bound at the active site for efficient inhibition with the flaps closed, while the substrates need to get processed and released for efficient turnover, flap dynamics would differentially affect the two processes. Such changes in flap dynamics have been revealed in MD simulations as well as experimental NMR and EPR dynamics of HIV-1 protease drug-resistant variants [36, 58–60]. This resistance mechanism through changes in the protease conformational dynamics may be common to mutations both at and outside the active site.

5.2 Resistance Mutations Outside the Protease Active Site

In addition to the major mutations at the protease active site, many mutations elsewhere in the protease are selected in resistance to protease inhibitors. Some of these mutations are major resistance mutations, even though they are located outside the active site and do not physically contact the ligand (orange in Fig. 35.2b). Yet others have been classified as *secondary* or *minor* as they do not confer significant levels of resistance when present alone (blue in Fig. 35.2b), but may assist in recovering the enzyme fitness or stability lost due to primary mutations.

While the *substrate envelope* provides an efficient framework to rationalize the selection of active-site mutations, understanding the molecular mechanisms underlying resistance due to changes in a side chain not in physical contact with the inhibitor is more challenging. Recent studies suggest that protease conformational dynamics and changes therein may play a major role in propagating the effect of such mutations to the active site. HIV-1 protease variants with single- or double-secondary resistance mutations bound to DRV were characterized by crystal structures and MD simulations, and displayed changes both in dynamics and subtle but significant rearrangements in the structure around the active site [45]. Interestingly, secondary mutations located at different positions in the protease structure had a common mechanism of propagating their effects to the active site and altering mainly the interactions of residue 47 with DRV. The *network hypothesis* was proposed to explain how distal mutations are able to affect the interactions at the active site through common mechanisms (Fig. 35.4): Residues that undergo secondary resistance mutations and active-site residues affected by secondary mutations are all part of a hydrogen-bonded interaction network in the protease structure. Although much less is known on how the mutations outside the active site contribute to resistance, hydrophobic sliding in relation to conformational dynamics and the more recent network hypothesis have provided inroads that may lead to more detailed and perhaps unified hypothesis to explain the underlying molecular mechanisms by which mutations at these sites directly contribute to resistance—rather than being compensatory.

5.3 Substrate Mutations and Coevolution

In addition to extensive mutations selected in HIV-1 protease under drug pressure to evade inhibition, the viral genome mutates elsewhere as well, especially at polyprotein Gag

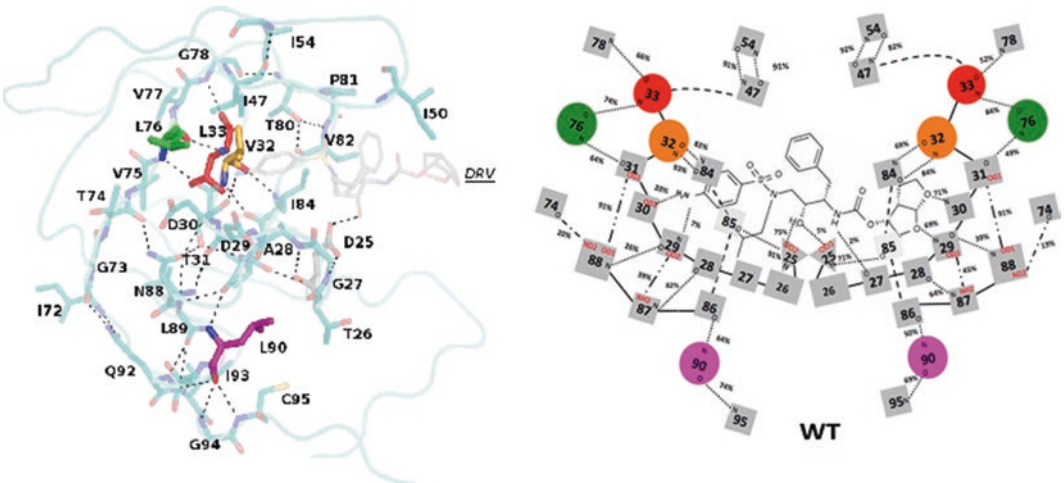


Fig. 35.4 The network hypothesis postulates that the network of hydrogen bonds in the protease structure connects the distal drug resistance mutation sites to the active site. Mutation at residues outside the active site (colored *magenta*, *green*, *red*, and *orange*) are able to affect the interactions with the bound inhibitor and active-site dynamics through common mechanisms, as they are all part of this connected network. Adapted with permission from Ragland et al. [45]. Copyright (2014) American Chemical Society

cleavage sites [31, 33, 61]. Evaluation of coevolution in terms of substrate envelope provided two mechanistic insights [62, 63]: (1) the two most divergent substrates with respect to fit within the envelope are the ones that are the most susceptible to mutations. Nc/p1 and p1/p2 protrude beyond the substrate envelope more than expected based on their size, and mutations therein are more frequent compared to the other substrates. (2) When protease resistance mutations abrogate the fit of a particular substrate within the consensus envelope, substrate mutations may help restore the fit within the substrate envelope. Thus, the substrate envelope is preserved by coevolution of protease and substrate.

Gag mutations have been thought to be *compensatory* mutations that rescue viral fitness lost due to protease mutations. However, some substrate mutations are able to directly confer PI resistance even in the absence of protease mutations and accumulating evidence suggests substrate mutations as an alternative pathway to resistance in patients failing therapy. Some of the most common substrate mutations at the NC/p1 and p1/p6 cleavage sites are classified as primary resistance mutations. A431V mutation at the NC/p1 cleavage site is the most frequent substrate mutation selected under PI pressure, and confers resistance to all PIs except DRV [33, 34]. Both A431V and I437V mutations at the NC/p1 cleavage site have been shown to have little effect on replicative capacity but instead directly confer antiviral resistance [32].

Statistical analysis of viral sequences specifically correlates primary drug resistance mutations in HIV-1 protease to substrate mutations, indicating coevolution [33]. A431V is often observed in combination with major protease mutations I50L, V82A, and I84V, while I437V correlates with I54V and I84V. Under drug pressure, the resistance mutations selected may differentially impair the protease activity on Gag cleavage sites, which would interfere with the ordered processing of Gag. Coevolution of substrates possibly restores proper Gag processing by more efficient cleavage by the protease.

Coevolved mutations of the substrate do not necessarily restore the specific protease–substrate interactions lost due to primary mutations. Structural analysis of coevolution for the Gag A431V and V82A protease mutations revealed the mechanism to be much more complex than a simple switch of A and V side-chain contacts (Fig. 35.5) [64]: V82A protease mutation causes loss of vdW contacts with F433 (not A431), while the A431V substrate mutation optimally fills the P2 pocket and reorients the substrate peptide to a more favorable conformation to stabilize overall interactions with the protease. Similarly, the coevolution mutations at the p1/p6 cleavage site (L449F or S451N) with NFV resistance mutations D30N/N88D do not restore the lost interactions of residue 30 but establish alternate contacts between the protease and substrate [65]. The individual coevolution mutations L449F and S451N enhance protease contacts and fit within the envelope. However, two large side chains together do not further improve contacts with the protease or fit within the substrate envelope, causing protrusions. This structural finding explains why, although frequently selected in correlation with protease NFV resistance mutations, L449F and S451N do not occur simultaneously at the p1/p6 cleavage site in viral sequences [33, 65].

Mutations at the p1/p6 cleavage site also coevolve with protease I50V major resistance mutation. I50V is commonly observed in patients failing therapy with APV and DRV, and also impairs protease catalytic efficiency. I50V often occurs together with the secondary mutation A71V, which compensates for protease efficiency [66, 67]. The substrate Gag L449F mutation rescues the protease activity by 10-fold, whereas P453L, although located distal from the catalytic site, causes a 23-fold enhancement [68]. The WT protease processes the mutated substrates more efficiently compared to the native substrate. This suboptimal cleavage efficiency at the p1/p6 site may be key for temporal regulation of Gag processing preventing premature viral maturation [23, 69]. A recent study

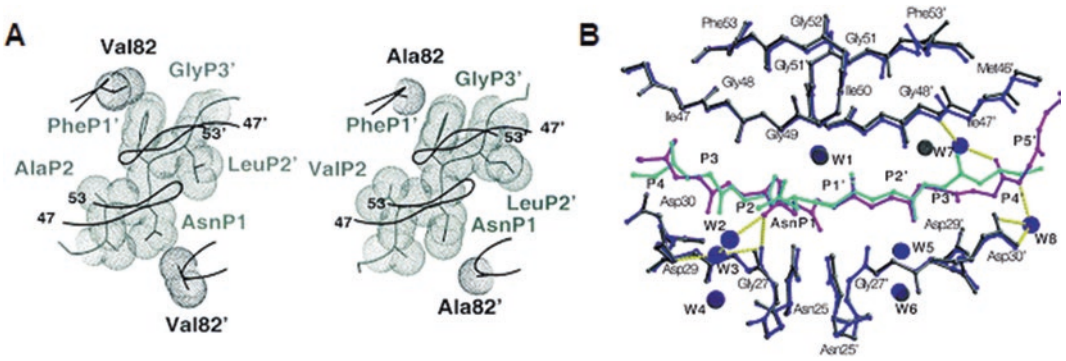


Fig. 35.5 Coevolution of NC-p1 cleavage site with V82A protease mutation. (**a**) Drug resistance mutation V82A causes loss of vdW contacts with Gag F433 (PheP1′), but not with A431 (AlaP2). Coevolution of the substrate to A431V does not enhance intermolecular vdW contacts at the mutation site. Rather, (**b**) the whole substrate peptide reorients (*magenta* versus *cyan*) and new water-mediated hydrogen bonds are formed between the peptide and protease (*yellow dotted lines*). Adapted from Prabu-Jeyabalan et al. [64] with permission. Copyright © 2004, American Society for Microbiology

with a series of crystal structures of I50V/A71V protease bound to p1/p6 substrate variants and MD simulations revealed molecular mechanisms underlying this coevolution [70]. The substrate residue Gag 453 is located away from the protease active site and does not make substantial contacts with the protease. Why P453L coevolution mutation is selected and how it may affect protease binding were not clear. P453L substrate mutation was demonstrated to induce a distal conformational change in one of the protease loops to enhance vdW contacts at residue 449 (Fig. 35.6). Reciprocally, L449F mutation propagates to a conformational change at residue 453, indicating interdependency between the two sites. In general, the coevolution mutations at the substrate do not directly restore interactions lost due to I50V, but instead establish other interactions that are not restricted to the site of mutation. The Gag mutations L449F and P453L enhance vdW interactions between the substrate and mutant protease by distal effects, whereas R452S results in an additional hydrogen bond.

In addition to enhancing substrate–protease interactions, coevolution may restore conformational dynamics at the active site, which is crucial for substrate binding and processing. In the case of I50V protease with p1/p6 substrate coevolution, mutation of the protease or the native substrate alone disturbed the dynamics, which was restored to a wild-type-like state in all coevolved complexes bearing complementary mutations in both the protease and the substrate [70]. Hence, in addition to the specific shape adopted and shared by all substrates when bound to the HIV-1 protease, as defined by the substrate envelope, a conserved dynamic behavior around the active site may be an additional substrate recognition and selection constraint. This dynamic constraint may contribute to the selection of substrate coevolution mutations in response to the disturbed dynamics in mutated drug-resistant protease.

5.4 Thermodynamics of PI Binding to Resistant Variants

Design and development of potent HIV-1 protease inhibitors require maximizing the binding affinity to target, which is dictated by the free energy of binding composed of enthalpy and entropy change between the unbound and bound states. Binding enthalpy mainly depends on the favorable interactions between the ligand and the protease, while the change in degrees of freedom (of the ligand, target, and solvent) determines the binding entropy. The first-generation PIs were entropy-driven binders as the strategy was to design conformational constraints to preposition the compound in a binding-competent state, with additional favorable solvation entropy due to burial of hydrophobic groups and release of structured water molecules. Further optimization yielded more potent PIs with both favorable enthalpy and entropy of binding, such as DRV [71–73]. However, highly potent entropy-driven inhibitors are also possible, such as TPV [74]. The interplay between entropy and enthalpy of binding at the molecular level is not straightforward in drug design, and enhancing one may inadvertently affect the other, resulting in entropy–enthalpy compensation.

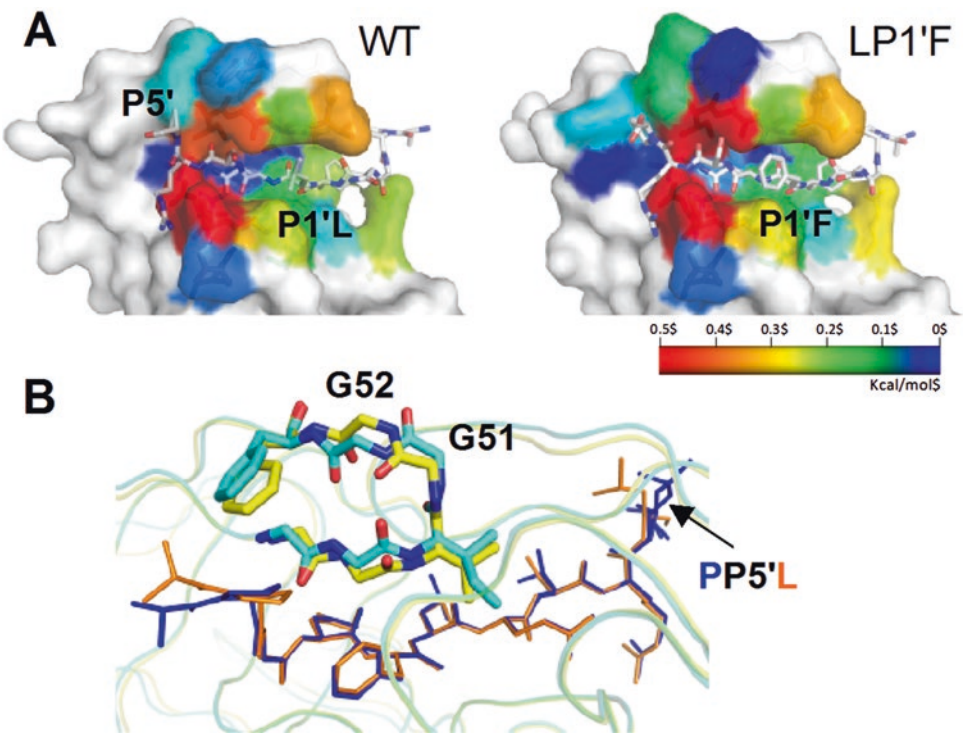


Fig. 35.6 Distal effects of p1–p6 substrate coevolution mutations in binding drug-resistant I50V/A71V protease. (**a**) The vdW contacts of residues in HIV-1 protease–substrate cocrystal structures colored *blue* to *red* for increasing contacts. The substrate mutation at P1′ position (L449F) enhances contacts at P5′ (Gag 453). (**b**) The distal substrate mutation PP5′L (P453L) causes a conformational change in the protease flap and alters substrate–protease interactions. The protease flaps are in *cyan* and *yellow* in complex structures with WT (*navy blue*) and P5′L (*orange*) substrates, respectively. Reprinted from Ozen et al. [70]

The enthalpic and entropic contributions to binding various drug-resistant variants of HIV-1 protease have been determined by isothermal titration calorimetry to understanding how mutations affect the energetics of inhibitor binding [39, 41–43, 71, 74]. In one variant with multiple mutations both within and outside the active site (L10I/G48V/I54V/V82A), the resistance mutations drastically altered the thermodynamics of binding, regardless of the PI tested [39]. Contrary to another variant (V82T/I84V) with similar levels of affinity loss, the first variant displayed extreme entropy–enthalpy compensation on the order of 10–15 kcal/mol. Thus drug resistance mutations in the protease can modulate the thermodynamics and hence affinity of binding. However, when the mutations in this variant are introduced individually or when the I54V mutation is replaced with I54A, this extreme entropy–enthalpy compensation no longer exists [75]. NMR and MD results suggested that alterations in protease conformational dynamics especially at the flap region may be underlying the observed thermodynamic behavior [58, 59], but a better understanding of the molecular mechanisms involved warrants further analysis, in particular of changes in water solvation. This complex and cooperative interdependency in altering thermodynamics of PI binding and conferring resistance presents an additional challenge in the rational design of robust drugs to avoid resistance.

6 Designing Robust Drugs to Avoid Resistance

HIV-1 protease is arguably the most extensively studied drug target to structurally and dynamically characterize how selected mutations confer resistance to inhibitors. We have learned critical insights, which should be transferable to other rapidly evolving disease targets where resistance emerges and impairs treatment options. Perhaps the main message from the HIV-1 protease drug resistance field to the drug design community is the need to shift the current paradigm of regarding resistance only as an afterthought, toward employing strategies to avoid resistance at the very first design and optimization steps of drug development. An effective approach to avoid susceptibility to major active-site mutations is to design inhibitors that stay within the substrate envelope. The highly potent and robust DRV provides a proof of concept for this strategy [14, 16, 71]. Additional libraries designed to stay within the envelope versus paired compound that protrude out provided additional support to validate this strategy [76]. In fact, exploiting the unused regions of the substrate envelope and exploring the chemical space while staying within the substrate envelope was successful in designing compounds even more potent and more robust than DRV [77]. More recently, the substrate envelope hypothesis and the related design strategy have been shown to hold true for HCV NS3/4A protease and its inhibitors as well [78, 79], and should be more generally applicable to other targets.

While we have some valuable insights into how mutations at the protease active site and elsewhere confer resistance, the molecular mechanisms of resistance due to the complex combination of mutations and interdependency in drug resistance are far more complex. To further our understanding of these molecular mechanisms underlying resistance to HIV-1 protease inhibitors, we need more comprehensive approaches unifying the structure, conformational dynamics, and energetics of inhibitor binding. Such an approach may lead to compounds that target and potently inhibit not only the wild-type enzyme but also a wide variety of variants that exist in patient populations. In the absence of a cure and considering the rapid evolution of the virus, the chances of replacing combination therapies with such a compound as single agent may be slim. Regardless, a detailed understanding of the wide variety of mutations and molecular mechanisms underlying resistance to HIV-1 protease inhibitors would provide the opportunity to develop design strategies to avoid drug resistance, by exploiting the biological and functional constraints on the evolution of the drug target.

Acknowledgments This work was supported by the National Institutes of Health Grants specifically from NIGMS P01 GM109767 and R01 GM65347. We acknowledge past and present members of the Schiffer research group, including Akbar Ali, for help with preparing Fig. 35.1 and Madhavi Kolli for useful suggestions.

References

1. UNAIDS. (2013). Global report: UNAIDS report on the global AIDS epidemic.
2. Mehellou Y, De Clercq E. Twenty-six years of anti-HIV drug discovery: where do we stand and where do we go? J Med Chem. 2010;53:521–38.
3. Hogg RS, Heath KV, Yip B, Craib KJP, O'Shaughnessy MV, Schechter MT, Montaner JSG. Improved survival among HIV-infected individuals following initiation of antiretroviral therapy. JAMA. 1998;279:450–4.
4. Hogg RS, OShaughnessy MV, Gataric N, Yip B, Craib K, Schechter MT, Montaner JSG. Decline in deaths from AIDS due to new antiretrovirals. Lancet. 1997;349:1294.
5. Palella FJ, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD, Investigators HOS. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N Engl J Med. 1998;338:853–60.
6. Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Krohn A, et al. Rational design of peptide-based HIV proteinase inhibitors. Science. 1990;248:358–61.
7. Dorsey BD, Levin RB, McDaniel SL, Vacca JP, Guare JP, Darke PL, Zugay JA, Emini EA, Schleif WA, Quintero JC, et al. L-735,524: the design of a potent and orally bioavailable HIV protease inhibitor. J Med Chem. 1994;37:3443–51.

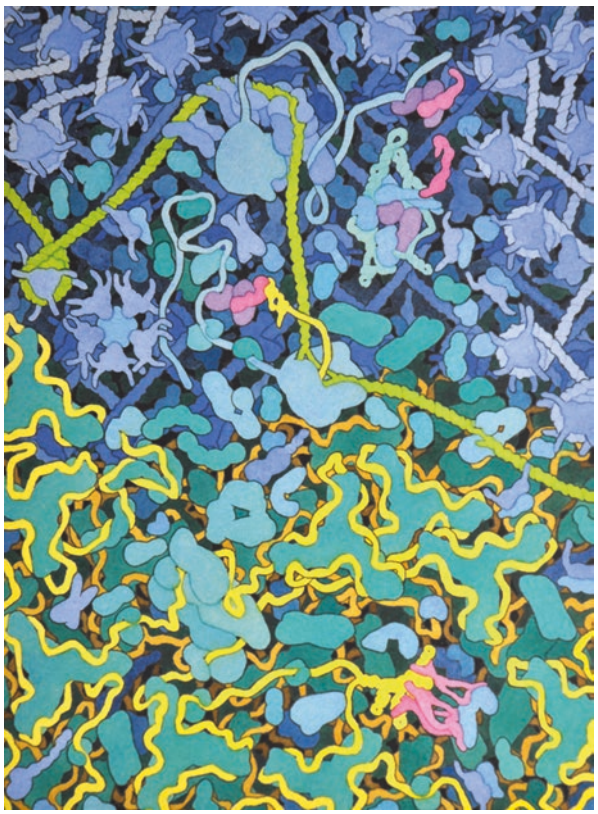
8. Kempf DJ, Marsh KC, Denissen JF, McDonald E, Vasavanonda S, Flentge CA, Green BE, Fino L, Park CH, Kong XP, et al. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. Proc Natl Acad Sci U S A. 1995;92:2484–8.
9. Kaldor SW, Kalish VJ, Davies 2nd JF, Shetty BV, Fritz JE, Appelt K, Burgess JA, Campanale KM, Chirgadze NY, Clawson DK, et al. Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. J Med Chem. 1997;40:3979–85.
10. Kim EE, Baker CT, Dwyer MD, Murcko MA, Rao BG, Tung RD, Navia MA. Crystal-structure of HIV-1 protease in complex with Vx-478, a potent and orally bioavailable inhibitor of the enzyme. J Am Chem Soc. 1995;117:1181–2.
11. Sham HL, Kempf DJ, Molla A, Marsh KC, Kumar GN, Chen CM, Kati W, Stewart K, Lal R, Hsu A, et al. ABT-378, a highly potent inhibitor of the human immunodeficiency virus protease. Antimicrob Agents Chemother. 1998;42:3218–24.
12. Robinson BS, Riccardi KA, Gong YF, Guo Q, Stock DA, Blair WS, Terry BJ, Deminie CA, Djang F, Colonno RJ, et al. BMS-232632, a highly potent human immunodeficiency virus protease inhibitor that can be used in combination with other available antiretroviral agents. Antimicrob Agents Chemother. 2000;44:2093–9.
13. Turner SR, Strohbach JW, Tommasi RA, Aristoff PA, Johnson PD, Skulnick HI, Dolak LA, Seest EP, Tomich PK, Bohanon MJ, et al. Tipranavir (PNU-140690): a potent, orally bioavailable nonpeptidic HIV protease inhibitor of the 5,6-dihydro-4-hydroxy-2-pyrone sulfonamide class. J Med Chem. 1998;41:3467–76.
14. De Meyer S, Azijn H, Surleraux D, Jochmans D, Tahri A, Pauwels R, Wigerinck P, de Bethune MP. TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. Antimicrob Agents Chemother. 2005;49:2314–21.
15. Koh Y, Nakata H, Maeda K, Ogata H, Bilcer G, Devasamudram T, Kincaid JF, Boross P, Wang YF, Tie Y, et al. Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus in vitro. Antimicrob Agents Chemother. 2003;47:3123–9.
16. Surleraux DL, Tahri A, Verschueren WG, Pille GM, de Kock HA, Jonckers TH, Peeters A, De Meyer S, Azijn H, Pauwels R, et al. Discovery and selection of TMC114, a next generation HIV-1 protease inhibitor. J Med Chem. 2005;48:1813–22.
17. Navia MA, Fitzgerald PM, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP. Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. Nature. 1989;337:615–20.
18. Wlodawer A, Miller M, Jaskolski M, Sathyanarayana BK, Baldwin E, Weber IT, Selk LM, Clawson L, Schneider J, Kent SB. Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. Science. 1989;245:616–21.
19. Jilek BL, Zarr M, Sampah ME, Rabi SA, Bullen CK, Lai J, Shen L, Siliciano RF. A quantitative basis for antiretroviral therapy for HIV-1 infection. Nat Med. 2012;18:446–51.
20. Sampah MES, Shen L, Jilek BL, Siliciano RF. Dose-response curve slope is a missing dimension in the analysis of HIV-1 drug resistance. Proc Natl Acad Sci U S A. 2011;108:7613–8.
21. Jacks T, Power MD, Masiarz FR, Luciw PA, Barr PJ, Varmus HE. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. Nature. 1988;331:280–3.
22. Erickson-Viitanen S, Manfredi J, Viitanen P, Tribe DE, Tritch R, Hutchison 3rd CA, Loeb DD, Swanstrom R. Cleavage of HIV-1 gag polyprotein synthesized in vitro: sequential cleavage by the viral protease. AIDS Res Hum Retroviruses. 1989;5:577–91.
23. Pettit SC, Lindquist JN, Kaplan AH, Swanstrom R. Processing sites in the human immunodeficiency virus type 1 (HIV-1) Gag-Pro-Pol precursor are cleaved by the viral protease at different rates. Retrovirology. 2005;2:66.
24. Pettit SC, Sheng N, Tritch R, Erickson-Viitanen S, Swanstrom R. The regulation of sequential processing of HIV-1 Gag by the viral protease. Adv Exp Med Biol. 1998;436:15–25.
25. Wiegers K, Rutter G, Kottler H, Tessmer U, Hohenberg H, Krausslich HG. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. J Virol. 1998;72:2846–54.
26. Jager S, Cimermancic P, Gulbahce N, Johnson JR, McGovern KE, Clarke SC, Shales M, Mercenne G, Pache L, Li K, et al. Global landscape of HIV-human protein complexes. Nature. 2012;481:365–70.
27. Ventoso I, Blanco R, Perales C, Carrasco L. HIV-1 protease cleaves eukaryotic initiation factor 4G and inhibits cap-dependent translation. Proc Natl Acad Sci U S A. 2001;98:12966–71.
28. Rhee SY, Taylor J, Fessel WJ, Kaufman D, Towner W, Troia P, Ruane P, Hellinger J, Shirvani V, Zolopa A, et al. HIV-1 protease mutations and protease inhibitor cross-resistance. Antimicrob Agents Chemother. 2010;54:4253–61.
29. Wu TD, Schiffer CA, Gonzales MJ, Taylor J, Kantor R, Chou S, Israelski D, Zolopa AR, Fessel WJ, Shafer RW. Mutation patterns and structural correlates in human immunodeficiency virus type 1 protease following different protease inhibitor treatments. J Virol. 2003;77:4836–47.
30. Velazquez-Campoy A, Vega S, Freire E. Amplification of the effects of drug resistance mutations by background polymorphisms in HIV-1 protease from African subtypes. Biochemistry (Mosc). 2002;41:8613–9.
31. Fun A, Wensing AM, Verheyen J, Nijhuis M. Human Immunodeficiency Virus Gag and protease: partners in resistance. Retrovirology. 2012;9:63.
32. Dam E, Quercia R, Glass B, Descamps D, Launay O, Duval X, Krausslich HG, Hance AJ, Clavel F, Grp AS. Gag mutations strongly contribute to HIV-1 resistance to protease inhibitors in highly drug-experienced patients besides compensating for fitness loss. PLoS Pathog. 2009;5:e1000345.
33. Kolli M, Stawiski E, Chappey C, Schiffer CA. Human immunodeficiency virus type 1 protease-correlated cleavage site mutations enhance inhibitor resistance. J Virol. 2009;83:11027–42.
34. Nijhuis M, van Maarseveen NM, Lastere S, Schipper P, Coakley E, Glass B, Rovenska M, de Jong D, Chappey C, Goedegebuure IW, et al. A novel substrate-based HIV-1 protease inhibitor drug resistance mechanism. PLoS Med. 2007;4:e36.
35. King NM, Prabu-Jeyabalan M, Nalivaika EA, Schiffer CA. Combating susceptibility to drug resistance: lessons from HIV-1 protease. Chem Biol. 2004;11:1333–8.
36. Galiano L, Ding F, Veloro AM, Blackburn ME, Simmerling C, Fanucci GE. Drug pressure selected mutations in HIV-1 protease alter flap conformations. J Am Chem Soc. 2009;131:430–1.
37. Gulnik SV, Suvorov LI, Liu B, Yu B, Anderson B, Mitsuya H, Erickson JW. Kinetic characterization and cross-resistance patterns of HIV-1 protease mutants selected under drug pressure. Biochemistry (Mosc). 1995;34:9282–7.
38. Heaslet H, Kutilek V, Morris GM, Lin YC, Elder JH, Torbett BE, Stout CD. Structural insights into the mechanisms of drug resistance in HIV-1 protease NL4-3. J Mol Biol. 2006;356:967–81.
39. King NM, Prabu-Jeyabalan M, Bandaranayake RM, Nalam MN, Nalivaika EA, Ozen A, Haliloglu T, Yilmaz NK, Schiffer CA. Extreme entropy-enthalpy compensation in a drug-resistant variant of HIV-1 protease. ACS Chem Biol. 2012;7:1536–46.
40. Liu Z, Wang Y, Brunzelle J, Kovari IA, Kovari LC. Nine crystal structures determine the substrate envelope of the MDR HIV-1 protease. Protein J. 2011;30:173–83.
41. Mittal S, Bandaranayake RM, King NM, Prabu-Jeyabalan M, Nalam MN, Nalivaika EA, Yilmaz NK, Schiffer CA. Structural and thermodynamic basis of amprenavir/darunavir and atazanavir resistance in HIV-1 protease with mutations at residue 50. J Virol. 2013;87:4176–84.
42. Muzammil S, Ross P, Freire E. A major role for a set of non-active site mutations in the development of HIV-1 protease drug resistance. Biochemistry (Mosc). 2003;42:631–8.

43. Ohtaka H, Schon A, Freire E. Multidrug resistance to HIV-1 protease inhibition requires cooperative coupling between distal mutations. Biochemistry (Mosc). 2003;42:13659–66.
44. Perryman AL, Lin JH, McCammon JA. HIV-1 protease molecular dynamics of a wild-type and of the V82F/I84V mutant: possible contributions to drug resistance and a potential new target site for drugs. Protein Sci. 2004;13:1108–23.
45. Ragland DA, Nalivaika EA, Nalam MN, Prachanronarong KL, Cao H, Bandaranayake RM, Cai Y, Kurt-Yilmaz N, Schiffer CA. Drug resistance conferred by mutations outside the active site through alterations in the dynamic and structural ensemble of HIV-1 protease. J Am Chem Soc. 2014;136:11956–63.
46. Saskova KG, Kozisek M, Lepsik M, Brynda J, Rezacova P, Vaclavikova J, Kagan RM, Machala L, Konvalinka J. Enzymatic and structural analysis of the I47A mutation contributing to the reduced susceptibility to HIV protease inhibitor lopinavir. Protein Sci. 2008;17:1555–64.
47. Skalova T, Dohnalek J, Duskova J, Petrokova H, Hradilek M, Soucek M, Konvalinka J, Hasek J. HIV-1 protease mutations and inhibitor modifications monitored on a series of complexes. Structural basis for the effect of the A71V mutation on the active site. J Med Chem. 2006;49:5777–84.
48. Wilson SI, Phylip LH, Mills JS, Gulnik SV, Erickson JW, Dunn BM, Kay J. Escape mutants of HIV-1 proteinase: enzymic efficiency and susceptibility to inhibition. Biochim Biophys Acta. 1997;1339:113–25.
49. Yedidi RS, Proteasa G, Martinez JL, Vickrey JF, Martin PD, Wawrzak Z, Liu Z, Kovari IA, Kovari LC. Contribution of the 80s loop of HIV-1 protease to the multidrug-resistance mechanism: crystallographic study of MDR769 HIV-1 protease variants. Acta Crystallogr D Biol Crystallogr. 2011;67:524–32.
50. Prabu-Jeyabalan M, Nalivaika EA, Romano K, Schiffer CA. Mechanism of substrate recognition by drug-resistant human immunodeficiency virus type 1 protease variants revealed by a novel structural intermediate. J Virol. 2006;80:3607–16.
51. Prabu-Jeyabalan M, Nalivaika E, Schiffer CA. Substrate shape determines specificity of recognition for HIV-1 Protease: analysis of crystal structures of six substrate complexes. Structure. 2002;10:369–81.
52. Chellappan S, Kairys V, Fernandes MX, Schiffer C, Gilson MK. Evaluation of the substrate envelope hypothesis for inhibitors of HIV-1 protease. Proteins. 2007;68:561–7.
53. Freedberg DI, Ishima R, Jacob J, Wang YX, Kustanovich I, Louis JM, Torchia DA. Rapid structural fluctuations of the free HIV protease flaps in solution: relationship to crystal structures and comparison with predictions of dynamics calculations. Protein Sci. 2002;11:221–32.
54. Hornak V, Okur A, Rizzo RC, Simmerling C. HIV-1 protease flaps spontaneously open and reclose in molecular dynamics simulations. Proc Natl Acad Sci U S A. 2006;103:915–20.
55. Ishima R, Freedberg DI, Wang YX, Louis JM, Torchia DA. Flap opening and dimer-interface flexibility in the free and inhibitor-bound HIV protease, and their implications for function. Structure. 1999;7:1047–55.
56. Scott WR, Schiffer CA. Curling of flap tips in HIV-1 protease as a mechanism for substrate entry and tolerance of drug resistance. Structure. 2000;8:1259–65.
57. Mittal S, Cai Y, Nalam MN, Bolon DN, Schiffer CA. Hydrophobic core flexibility modulates enzyme activity in HIV-1 protease. J Am Chem Soc. 2012;134:4163–8.
58. Cai Y, Kurt Yilmaz N, Myint W, Ishima R, Schiffer CA. Differential flap dynamics in wild-type and a drug resistant variant of HIV-1 protease revealed by molecular dynamics and NMR relaxation. J Chem Theory Comput. 2012;8:3452–62.
59. Cai Y, Myint W, Paulsen JL, Schiffer CA, Ishima R, Kurt Yilmaz N. Drug resistance mutations alter dynamics of inhibitor-bound HIV-1 protease. J Chem Theory Comput. 2014;10:3438–48.
60. de Vera IMS, Smith AN, Dancel MCA, Huang X, Dunn BM, Fanucci GE. Elucidating a relationship between conformational sampling and drug resistance in HIV-1 protease. Biochemistry (Mosc). 2013;52:3278–88.
61. Kolli M, Lastere S, Schiffer CA. Co-evolution of nelfinavir-resistant HIV-1 protease and the p1-p6 substrate. Virology. 2006;347:405–9.
62. Ozen A, Haliloglu T, Schiffer CA. Dynamics of preferential substrate recognition in HIV-1 protease: redefining the substrate envelope. J Mol Biol. 2011;410:726–44.
63. Ozen A, Haliloglu T, Schiffer CA. HIV-1 protease and substrate coevolution validates the substrate envelope as the substrate recognition pattern. J Chem Theory Comput. 2012;8:703–14.
64. Prabu-Jeyabalan M, Nalivaika EA, King NM, Schiffer CA. Structural basis for coevolution of a human immunodeficiency virus type 1 nucleocapsid-p1 cleavage site with a V82A drug-resistant mutation in viral protease. J Virol. 2004;78:12446–54.
65. Kolli M, Ozen A, Kurt-Yilmaz N, Schiffer CA. HIV-1 protease-substrate coevolution in nelfinavir resistance. J Virol. 2014;88:7145–54.
66. Nijhuis M, Schuurman R, de Jong D, Erickson J, Gustchina E, Albert J, Schipper P, Gulnik S, Boucher CAB. Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. AIDS. 1999;13:2349–59.
67. Rhee SY, Gonzales MJ, Kantor R, Betts BJ, Ravela J, Shafer RW. Human immunodeficiency virus reverse transcriptase and protease sequence database. Nucleic Acids Res. 2003;31:298–303.
68. Maguire MF, Guinea R, Griffin P, Macmanus S, Elston RC, Wolfram J, Richards N, Hanlon MH, Porter DJT, Wrin T, et al. Changes in human immunodeficiency virus type 1 Gag at positions L449 and P453 are linked to I50V protease mutants in vivo and cause reduction of sensitivity to amprenavir and improved viral fitness in vitro. J Virol. 2002;76:7398–406.
69. Pettit SC, Henderson GJ, Schiffer CA, Swanstrom R. Replacement of the P1 amino acid of human immunodeficiency virus type 1 Gag processing sites can inhibit or enhance the rate of cleavage by the viral protease. J Virol. 2002;76:10226–33.
70. Ozen A, Lin KH, Kurt Yilmaz N, Schiffer CA. Structural basis and distal effects of Gag substrate coevolution in drug resistance to HIV-1 protease. Proc Natl Acad Sci U S A. 2014;111:15993–8.
71. King NM, Prabu-Jeyabalan M, Nalivaika EA, Wigerinck P, de Bethune MP, Schiffer CA. Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. J Virol. 2004;78:12012–21.
72. Velazquez-Campoy A, Luque I, Todd MJ, Milutinovich M, Kiso Y, Freire E. Thermodynamic dissection of the binding energetics of KNI-272, a potent HIV-1 protease inhibitor. Protein Sci. 2000;9:1801–9.
73. Velazquez-Campoy A, Todd MJ, Freire E. HIV-1 protease inhibitors: Enthalpic versus entropic optimization of the binding affinity. Biochemistry (Mosc). 2000;39:2201–7.
74. Muzammil S, Armstrong AA, Kang LW, Jakalian A, Bonneau PR, Schmelmer V, Amzel LM, Freire E. Unique thermodynamic response of tipranavir to human immunodeficiency virus type 1 protease drug resistance mutations. J Virol. 2007;81:5144–54.
75. Foulkes-Murzycki JE, Rosi C, Kurt Yilmaz N, Shafer RW, Schiffer CA. Cooperative effects of drug-resistance mutations in the flap region of HIV-1 protease. ACS Chem Biol. 2013;8:513–8.
76. Shen Y, Altman MD, Ali A, Nalam MN, Cao H, Rana TM, Schiffer CA, Tidor B. Testing the substrate-envelope hypothesis with designed pairs of compounds. ACS Chem Biol. 2013;8:2433–41.
77. Nalam MN, Ali A, Reddy GS, Cao H, Anjum SG, Altman MD, Yilmaz NK, Tidor B, Rana TM, Schiffer CA. Substrate envelope-designed potent HIV-1 protease inhibitors to avoid drug resistance. Chem Biol. 2013;20:1116–24.
78. Romano KP, Ali A, Royer WE, Schiffer CA. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc Natl Acad Sci U S A. 2010;107:20986–91.
79. Soumana DI, Ali A, Schiffer CA. Structural analysis of asunaprevir resistance in HCV NS3/4A protease. ACS Chem Biol. 2014;9:2485–90.

HIV-1 Entry and Fusion Inhibitors: Mechanisms and Resistance

36

Colin M. Venner, Annette N. Ratcliff, Mathieu Coutu, Andrés Finzi, and Eric J. Arts



C.M. Venner
Department of Microbiology and Immunology, Western University, London, ON, Canada N6A 3K7
e-mail: earts@uwo.ca

E.J. Arts, Ph.D. (✉)
Canada Research Chair on HIV Pathogenesis and Viral Control, University of Western Ontario, London, ON, Canada

A.N. Ratcliff, Ph.D.
Technology Licensing Manager, Ohio State University, Columbus, OH 43210, USA

M. Coutu • A. Finzi, Ph.D.
Department of Microbiology, Infectiology and Immunology, Canada Research Chair on Retroviral Entry, Université de Montréal, Montreal, QC, Canada

1 Introduction

HIV entry research in the mid-1990s made two key discoveries: (1) formation of a unique six-alpha helix bundle in the viral envelope which is necessary for viral-cell membrane fusion and (2) the identification of two key co-receptors, CXCR4 and CCR5, which permit host cell entry of the syncytium- and non-syncytium-inducing HIV-1 strains, respectively. With these discoveries, entry inhibitors represented the most promising class of new antiretrovirals for prevention and treatment of HIV-1 infection. However, after 10 years of research, only enfuvirtide and maraviroc have been approved by the FDA despite hundreds of preclinical candidates, dozens of potential drugs tested in phase I trials, and three phase III trials.

Drug development had focused on targeting interactions between viral envelope glycoproteins and host cellular receptors, as well as inhibiting the fusion of viral and host cell membranes. Inhibitors showed promise for both treatment of infected individuals and microbicides to prevent or limit new infection. Despite a highly conserved process involved in membrane fusion, targeting and inhibiting the six-alpha helix bundle were difficult if not impossible using small molecules. Thus, most drug screening efforts were focused on development of inhibitors that specifically target CCR5 (or CXCR4) and block its use as coreceptor for HIV entry into host cells. However, due to the extreme diversity between the envelope glycoproteins of HIV-1 isolates, a high bar was set for developing a potent CCR5 antagonist that could block variations in HIV-1 binding affinity and avidity to CCR5. As a consequence, the variations in intrinsic sensitivity to these CCR5 antagonists are higher than those observed with any other antiretroviral drug class.

1.1 Viral Entry

The process of HIV entry into a target cell is divided into three principal events: (1) viral particle attachment to a host cell, (2) binding to a seven-transmembrane chemo-

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_36

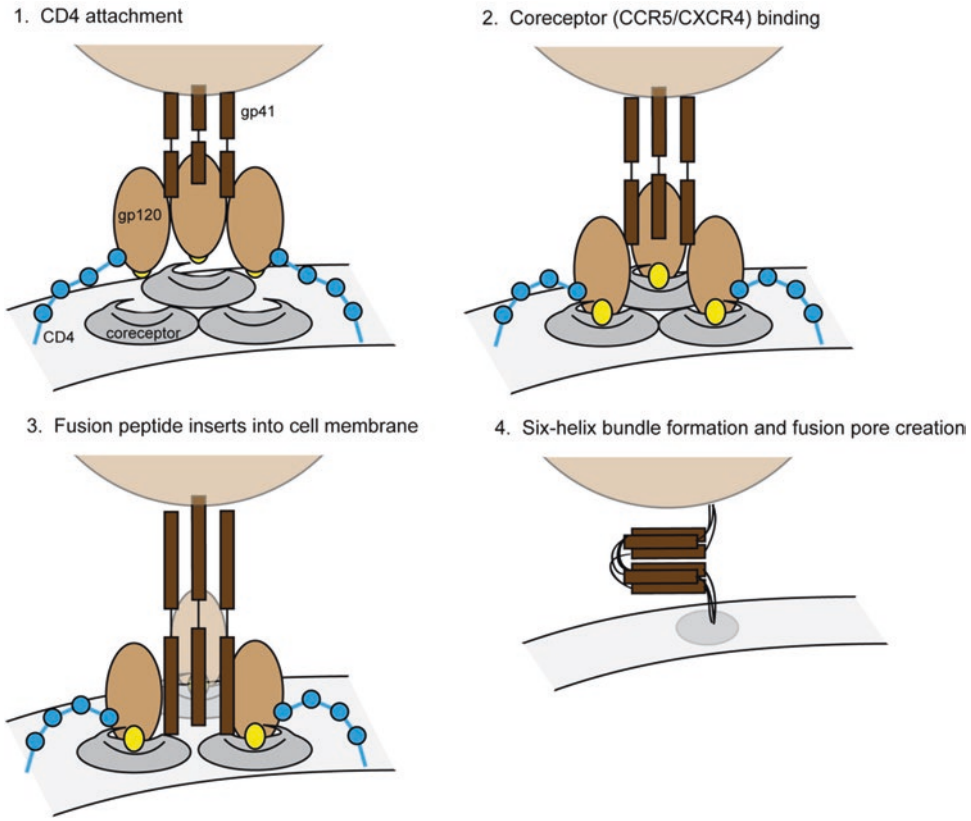


Fig. 36.1 Model of HIV entry. (*1*) HIV entry begins with the attachment of the envelope glycoprotein gp120 to a CD4 molecule on a target cell. Engagement with CD4 induces conformational changes in gp120 which exposes the V3 loop (*yellow*) and coreceptor-binding site. (*2*) Following CD4 attachment, the V3 loop and bridging sheet interact with the N-terminal tail and extracellular loops of a chemokine coreceptor. Coreceptor binding induces further rearrangements leading to the uncovering of the gp41 fusion peptide. (*3*) Once exposed, the fusion peptide inserts into the cellular membrane leading to destabilization of the bilipid membrane. (*4*) To simplify, CD4, coreceptor, and gp120 have been removed. The N-terminal and C-terminal heptad repeat regions of gp41 fold onto each other creating a stable six-helix bundle structure. Folding of this structure brings the viral and cellular membranes into close proximity which allows for lipid mixing and the creation of a fusion pore through which the HIV core enters the target cell cytoplasm

kine coreceptor, and (3) fusion of the viral and cellular membrane (Fig. 36.1). Interactions between the cellular receptors and viral membrane are mediated by the HIV envelope complex. The envelope is comprised of an outer surface glycoprotein (gp120) and a transmembrane glycoprotein (gp41). Gp120 is composed of a bridging sheet and an inner and outer domain where inner domain contains both the N- and C-termini pointing towards gp41 [1]. Three topological layers emanate from the β-sandwich and are linked to the outer domain by the bridging sheet, which are all formed from the conserved regions of gp120 (C1–C5) [1, 2]. Five highly variable regions (V1–V5) span from the outer domain span [3], which, according to recent cryo-EM studies, are essential to the integrity of the envelope complex. The gp41 subunit is composed of a cytoplasmic tail, a transmembrane region, a membrane proximal external region (MPER), and two complementary heptad repeat regions (HR1 and HR2). Noncovalent interactions hold these glycoproteins together to form heterodimeric subunits. A trimer of these heterodimeric subunits comprises an envelope spike on the surface of the virion and functions to mediate entry into host cells.

HIV entry initiates with the attachment of the envelope glycoprotein gp120 to the cellular receptor CD4 [4, 5]. Engagement with CD4 induces a structural rearrangement in gp120 which exposes the coreceptor-binding site. Although over 14 different transmembrane receptors have been shown to support HIV infection in CD4+ cell lines, only CCR5 and CXCR4 have been shown to act as coreceptors in vivo [6–8]. Coreceptor binding initiates further conformational changes in the envelope, permitting insertion of gp41 fusion peptide into the cellular membrane. Through intermediate gp41 structural rearrangements, a fusion pore develops as a result of membrane mixing between the viral and cellular membranes. The capsid core structure, containing the viral genome, is then able to pass through the fusion pore and into the cytoplasm of the cell.

2 Entry Inhibitors and Mechanisms of Resistance

The extensive use of other antiretroviral targets in highly active antiretroviral therapy (HAART), while greatly reducing the mortality rates of infected individuals, also heightened the need for novel drug classes targeting alternative viral processes. Additionally, the emergence of drug-resistant viral strains in treatment-experienced patients and the transmission of these resistant viruses have driven the need for development of more effective treatments. As the first step in HIV infection, the process of viral entry into host cells had long been considered an attractive target for intervention therapy. The intricate process of entry provides ample opportunities to intervene in virus–cell interactions and prevent entry of the viral core into new target cells. In addition to preventing viral propagation within an individual, entry inhibitors also represent a unique opportunity to minimize the transmission of virus between individuals when applied as a microbicide. An arsenal of inhibitors have been developed to target each specific stage of entry and are discussed in the following sections (Table 36.1).

2.1 Attachment Inhibitors

Since CD4 serves as the major receptor required for HIV attachment and entry, it is an appealing target for infection prevention. Multiple approaches have been taken to inhibit viral attachment including targeting the CD4 receptor as well as the CD4-binding site on gp120. Although no CD4-targeting inhibitors are currently approved, some have progressed to clinical trials with promising safety and efficacy results [9–11].

2.1.1 Targeting Viral Proteins

Initial efforts to block the interaction between gp120 and CD4 focused on the soluble form of the CD4 receptor (sCD4) which consists of either all four or the first two extracellular domains. Binding of sCD4 to gp120 in in vitro experiments can have multiple effects on the envelope complex depending on sCD4 concentration and temperature. In addition to competition with membrane-anchored CD4, sCD4 can also inhibit gp120 by inducing various inactivation events. These inactivation events include (1) the decay of the CD4-induced conformation to an inactive conformation and (2) sCD4-induced shedding of gp120 from the envelope trimer [12, 13]. Although sCD4 showed promising efficacy against diverse HIV strains in vitro, therapy with sCD4 in vivo was not able to reduce viral load in HIV-infected patients [14]. It was later determined that higher dosages of sCD4 were required to inhibit the entry of primary isolates than those required to inhibit laboratory-adapted strains [15]. Despite the failure to suppress viral load in vivo, the successful targeting of the CD4-binding site in vitro launched efforts to develop derivatives of CD4 and mimetic compounds with improved treatment profiles.

Developed by Progenics Pharmaceuticals Inc., PRO 542 is a CD4 derivative comprised of a recombinant fusion protein between the D1D2 regions of CD4 and human IgG2 antibody. PRO 542's heterotetrameric structure enables it to bind up to four gp120 subunits simultaneously, blocking gp120 attachment to cellular CD4 and locking gp120 into a nonfunctional conformation [10]. Demonstrating activity

Table 36.1 HIV entry inhibitors

Generic name	Drug action	Manufacturer	Stage of development
CD4 attachment inhibitors			
BMS-378806	Binds gp120	Bristol-Myers Squibb	Clinical trial halted
NBD-556	Binds gp120		Preclinical
CADA	Down-modulates CD4		Preclinical
TNX-355	Binds CD4	TaiMed, Inc.	Phase llb trial
PRO 542	Binds gp120	Progenics Pharmaceuticals, Inc.	
Coreceptor inhibitors			
Maraviroc	Binds CCR5	Pfizer	FDA approved 2007
Aplaviroc	Binds CCR5	GlaxoSmithKline	Phase lla-halted
Vicriviroc	Binds CCR5	Merck	Phase III-halted
Tak-779	Binds CCR5	Takeda	
AMD3100	Binds CXCR4	Genzyme	Marketed for cancer therapy
ALX40-4C	Binds CXCR4		
RANTES derivatives	Bind CCR5		Microbicide studies
PRO 140	Binds CCR5	Progenics Pharmaceuticals, Inc	Phase II trial
Fusion inhibitors			
T20	Binds gp41	Hoffman-La Roche and Trimeris	FDA approved 2003

Development and action of entry inhibitors targeting each stage of viral entry

against a diverse range of HIV strains in cell culture, PRO 542 proceeded to phase I/II clinical trials in 2003. However, further progress has not been reported since the completion of phase II trials in 2005.

Mapping the interaction between CD4 and gp120 has led to the development of mimetic compounds which target the CD4-binding site on gp120. These include the NBD-556 and NBD-557 compounds which were designed to project into the F43 cavity of gp120. Viral replication in both CCR5- and CXCR4-expressing cell lines was inhibited with NBD compounds, indicating that inhibition of viral entry is not dependent upon viral tropism [16]. These compounds were not able to inhibit a CD4-independent viral strain, suggesting their mechanism of action involved blocking the CD4–gp120 interaction. Structural modeling of NBD-556 with gp120 crystal structures indicates that the chloro-phenyl ring of the compound projects even further into the F43 cavity on gp120 than the phenyl ring of F43 of cellular CD4 [17].

Further CD4 mimics, BMS-378806, and related compounds are small molecules that have been developed by Bristol-Myers Squibb. They are believed to inhibit gp120 interaction with CD4 by blocking access to the F43 cavity. Even though it was halted in phase I trials, BMS-378806 is still being pursued as a potential vaginal microbicide. Another compound, BMS-663068, has undergone phase IIb investigations with reports indicating good efficacy and safety profiles [11, 18].

2.1.2 Targeting Host Cell Proteins

An alternative method to block viral attachment is to target the CD4 cellular receptor itself through antibody binding. A humanized monoclonal antibody, ibalizumab (previously TN X-355), targets the CD4 extracellular domain but does not prevent CD4–gp120 binding. Instead it is suspected to block the conformational changes in gp120 typically induced by binding CD4 [19]. Clinical testing in HIV-infected patients of ibalizumab in combination with optimized therapy has proved encouraging with sustained viral load suppression over long-term therapy [9]. Unfortunately, the need for intravenous infusion has limited its development to early phase I trials and with limited prospects for clinical use.

2.1.3 Receptor Downmodulation

Cyclotriazadisulfonamide (CADA) compounds are a novel class of inhibitors which prevent nascent CD4 translocation from the ER membrane, resulting in the selective downmodulation of CD4 on the surface of the cell [20]. Medicinal chemistry efforts have resulted in a panel of CADA analogues with lead compounds demonstrating potent and broad inhibition of diverse HIV-1 strains. HIV-1 escape from the CADA compounds has been associated with changes in the C4 region of gp120 [21]. Although not directly located within the CD4-binding site, the S463P mutation observed by Vermeire et al. may stabilize a liganded conformation of gp120, increasing the ability to scavenge low CD4 surface receptor density. However, this CADA-resistant virus was also found to be more sensitive to neutralization by patient serum. Indeed, increased sensitivity to neutralization has also been described for CD4-independent HIV-1 suggesting that mutations which stabilize CD4-liganded conformations of gp120 do so at the risk of increasing neutralization potential for the virus [22]. In addition to increased sensitivity to neutralizing antibodies, the CADA-resistant virus demonstrated increased replicative fitness, a trait also associated with resistance to the CD4-binding site monoclonal antibody b12 [23].

2.1.4 Attachment Inhibitor Resistance

Targeting the CD4-binding site through CD4 derivatives or mimetics can result in the emergence of resistance via induction of a CD4-bound "activated" conformation of gp120, permitting direct coreceptor binding and potentially enhancing infection. Indeed, some of the first studies noted the ability of viruses to enter CD4-negative cells in the presence of low nanomolar concentrations of sCD4 [24, 25]. Additionally, it has been recently described that gp120 missing part of its three variable regions spontaneously samples the CD4-bound conformation [26]. Switching the major variable regions of gp120 between different viral strains can alter several proprieties of resistance, including evasion of sCD4 and cold neutralization, hinting to a crucial role of the conformation of the Env spike [27]. However, it has also been observed that prolonged exposure to sCD4 induces a transient activated state of gp120 which rapidly decays to a stable, nonfunctional conformation [28].

Resistance to CD4 mimetic compounds primarily involves changes in the CD4-binding site on gp120, as discovered by repeated selection of resistant variants in tissue culture. Mutants resistant to NBD-556 were found to possess two mutations in gp120: S375N in the C3 region and A433T in the C4 region [29]. A previous study indicated that mutations in gp120 residues surrounding or comprising the F43 cavity negatively impacted the inhibitory effect of NBD-556 [17]. Similarly, mutations that rendered a subtype B viral strain resistant to BMS-378806 (M426L and M475I) were situated in the F43-binding cavity [30]. It appears that despite the conserved nature of this cavity, modifications of residues overriding drug inhibition are well tolerated.

2.2 Fusion Inhibitors

In the early 1990s it was discovered that synthetic peptides derived from the helical repeat (HR) regions of gp41 can inhibit HIV replication in vitro [31, 32]. The transitional exposure of the HR regions during pre-hairpin formation presents an opportunity for competitive binding of these synthetic peptides to prevent formation of the six-helix bundle.

Enfuvirtide (T20), marketed as Fuzeon by Hoffmann-La Roche Ltd., was the first entry inhibitor approved for clinical use. This inhibitor is a peptide mimetic of a portion of the HR2 region of gp41 and interferes with conformational changes in gp41 necessary for mediating the fusion of viral and host cell membranes. By mimicking the HR2 region, T20 is able to bind the N-terminal heptad repeat (HR1) and prevent formation of the stable six-helix bundle structure intermediate which aids in the stabilization of fusion pore formation. Delivery of the viral genome into the cell is hindered by T20 and thereby infection is prevented.

Resistance to T20 has been well characterized both in vitro and in vivo, with mutations in the HR1 region of gp41, the site of T20 binding. T20 mutations specifically map to positions 36–45 of gp41 HR1 domain, with a GIV motif from 36 to 39 playing a predominant role in resistance [33, 34]. The amino acid at position 36 has been shown to modulate the fusion kinetics of the envelope [35]. Mutations that reduce T20 binding also appear to reduce six-helix bundle formation and overall fusion rates. Resistance to T20 was also associated with increased sensitivity to neutralization by antibodies targeting gp41 [36].

In addition to T20, new fusion inhibitors were under development including VIR-576, a peptide that targets the gp41 fusion peptide. VIR-576 demonstrated efficacy in a small group of treatment-naïve patients by reducing viral loads an average of 95 % over 10 days of monotherapy [37]. However, it is unlikely that this inhibitor or other peptide fusion inhibitors will be developed for clinical use due to high production costs and intravenous administration. Currently, small-molecule fusion inhibitors targeting gp41 are under investigation but suitable leads have been documented.

2.3 Inhibitors of gp120–CXCR4 Interaction

Targeting the CXCR4 chemokine coreceptor to inhibit X4 tropic HIV-1 is especially difficult given the key role of CXCR4 in human physiology. Genetic deletion of the *cxcr4* gene or its ligand, CXCL12 (previously SDF-1), severely impairs development and causes embryonic lethality in mice [38, 39]. In adults, CXCL12 is a strong chemotactic signal and through CXCR4 signaling regulates hematopoietic stem cell development and migration.

Attempts to target CXCR4 as an inhibitor of X4 HIV-1 have resulted in multiple small-molecule inhibitors; however, none are currently approved for clinical use due to poor bioavailability and toxicity issues. The bicyclam AMD3100 efficiently inhibits X4 tropic virus entry with IC_{50}s in the nanomolar range and is used as a research tool to block CXCR4 virus entry [40]. Multiple derivatives of AMD3100, in addition to other CXCR4-targeting inhibitors, were in development but have significant in vivo effects. In general, the gp120 envelope binds to the N-terminus and second extracellular loop, a large interface on CXCR4 (or CCR5) which also occludes the binding sites on CXCR4 for the natural ligand such as CXCL12 [40, 41]. Most inhibitors that bind to hydrophobic pocket at the base of the extracellular loops of CXCR4 induce a significant conformation change that disrupt HIV-1 gp120 binding as well as CXCL21 binding, receptor internalization, and signaling. These effects are not manifested in cytotoxicity but rather in native CXCL21–CXCR4 functions [41]. Thus, CXCR4 antagonists have been largely abandoned for HIV therapy but are still being considered for cancer chemotherapy based on the CXCR4 involvement in early metastasis [42].

2.4 Inhibitors of gp120–CCR5 Interaction

Early indications that CCR5 was a viable target for HIV therapeutic intervention arose from the discovery of a 32 base-pair deletion in the *ccr5* gene resulting in dysfunctional surface receptor expression in individuals homozygous for the *ccr5Δ32* allele [43]. Homozygosity bestows relative protection against HIV infection by R5 tropic virus strains, although infection by X4 or dual-tropic virus is still permissible [44]. Individuals heterozygous for *ccr5Δ32* exhibit lower receptor expression and those infected with HIV tend to progress less rapidly to disease [44]. Despite lack of CCR5 expression on the cell surface, homozygotes for *ccr5Δ32* have no apparent disadvantageous immunologic effect [45]. Observations like these led the way for the development of inhibitors that could target and block CCR5 and HIV envelope interactions.

2.4.1 Chemokine Analog Inhibitors

The earliest attempts to block gp120–CCR5 interaction focused on the development of chemokine analogs by N-terminal modification of the natural CCR5 ligand RANTES [46]. Chemokine ligand binding to receptor induces signaling cascades resulting in lymphocyte activation and chemotaxis. The goal of using a modified form of the RANTES chemokine was to limit the induction of signal transduction cascades that would result in immune activation while retaining high affinity binding to the receptor. The N-terminus was chosen for modification because the N-terminus of chemokine ligands contributes to initiation of the signaling cascade while chemokine receptor specificity is attributed to the core of the ligand [47]. An aminooxypentane (AOP) addition to the N-terminus of RANTES resulted in an analog with increased potency against HIV replication compared to the native ligand [46]. Although this AOP-RANTES derivative did not induce chemotaxis upon binding it did induce calcium flux, possibly leading to activation of HIV replication [48]. Traces of G protein signaling also led to an activation of R5 HIV-1 replication when CD4+ T cells were preincubated with high concentrations of AOP-RANTES [49], similar to stimulatory effects of HIV replication observed with native β chemokines in the absence of

entry inhibition [40, 50]. This potential for activation limits the efficacy of these chemokine analogs, demonstrating the need for additional modification of the RANTES N-terminus to prevent signaling [51]. These investigations resulted in an analog with even higher potency than AOP-RANTES [52]. The PSC-RANTES analog has been shown to block vaginal HIV transmission in the SHIV-macaque model and is being developed as a potential microbicide [53].

Chemokine ligand binding not only induces immune-activating cascades but also results in receptor internalization through a clatherin-dependent endocytic pathway [54]. Similar to the natural ligand, RANTES derivatives are likewise able to induce internalization of CCR5, though intracellular receptor sequestration was prolonged for PSC-RANTES. Furthermore, receptor internalization is believed to be the primary mechanism of action for PSC-RANTES and is likely the mode of inhibition in single-cycle drug sensitivity assays. However, recent studies have shown differential sensitivity to this inhibitor in multiple-cycle assays lending support to a competitive inhibition model [55–57].

PSC-RANTES resistance was described in the context of an SHIV used in vaginal transmission challenge of macaque monkeys [55]. Mutations responsible for resistance were identified in the ectodomain of gp41 (N640D) as well as in the V3 loop (K315R). The PSC-RANTES-resistant virus was also less sensitive to inhibition by the allosteric inhibitor TAK-779 than was the control virus. The mutation in the V3 loop is located in the region of gp120 that binds to the same site on CCR5 that PSC-RANTES does and could therefore alter gp120 interactions with CCR5 at this site. The mutation in gp41 is located within the HR2 region and may influence the kinetics of viral fusion. Following this report, Nedellec et al. [58] indicated that the K315R/M640D in the same HIV-1 strain did not confer PSC-RANTES resistance but these assays were performed in single-cycle assay conditions where PSC-RANTES only mediated CCR5 receptor down-regulation. Subsequent mechanistic studies on PSC-RANTES inhibition (as compared to inhibition by CCR5 antagonists) revealed that CCR5 was desensitized to down-regulation by PSC-RANTES but that this ligand remained bound to the receptor and primarily blocked HIV-1 infection through competitive binding [59]. Following the return of the CCR5–PSC complex to the cell surface, the PSC-RANTES-resistant K325R/M640D HIV-1 could compete for CCR5 binding with PSC-RANTES better than the wild-type HIV-1 leading to better host cell entry, replication, and evidence of PSC-RANTES resistance [59].

2.4.2 Small-Molecule Antagonists

Small-molecule antagonists of CCR5 show the highest potency of HIV-1 inhibition when compared to antiretroviral drugs of all other classes. Maraviroc (MVC) inhibits HIV at the low nanomolar to high picomolar concentrations in tissue culture. In vivo, MVC was first approved as a salvage therapy by FDA in 2007 but only with pretesting for circulating CXR4-tropic HIV-1 within the patient. However, a recent study has revealed the efficacy of MVC as compared to efavirenz (EFV), as the backbone for long-term combination therapy in treatment-naïve patients. The proportion of patients maintaining viral load below 50 copies/mL was similar (~50 %) between the MVC and EFV treatment arms throughout the study (up to 240 weeks). Interestingly, patients receiving MVC had a greater increase in mean CD4 cell counts during the course of the study. Finally, MVC-treated patients reported fewer adverse events [60]. Despite this observed efficacy, there are less than 50,000 patients receiving MVC whereas EFV is the backbone of >5 million treatment regimens of HIV-infected individuals.

The anti-HIV CCR5 antagonists function through an allosteric mechanism where binding to the receptor induces altered conformations of the extracellular loops (ECLs) and prevents HIV envelope recognition and coreceptor engagement. Maraviroc and other CCR5 antagonists including vicriviroc (VVC), aplaviroc (APL), and TAK-779 all share a binding site in the transmembrane cavity of CCR5 (Fig. 36.2). Alanine scanning mutagenesis of the transmembrane domains of CCR5 revealed that key residues in ECLs 1, 2, 3, and 7 comprise the small-molecule binding cavity [61]. This binding site does not overlap the binding site of either CCR5 agonists or the HIV envelope; rather, binding induces receptor conformations that are not recognized by either CCR5 ligands or the HIV envelope glycoproteins. Binding of each respective inhibitor to the ECLs is predicted to cause different conformational changes [62]. This may explain why some viral strains resistant to one inhibitor have been shown to retain sensitivity to another inhibitor in this class.

Initially identified in a screen of a Pfizer compound library for binding to the CCR5 chemokine receptor maraviroc is an imidazopyridine that antagonized β-chemokine binding and signaling with IC_{50}s in the nanomolar range (Fig. 36.3) [63]. Maraviroc also inhibited RANTES-, MIP-1α-, and MIP-1β-induced signaling of intracellular calcium redistribution but did not trigger calcium signaling or receptor internalization upon binding. Reductions in basal γ-S-GTP binding suggested some inverse agonist activity for maraviroc, potentially due to the formation of inactive states of CCR5. In the same study, maraviroc was shown to have potent antiviral activity against a diverse panel of primary R5 HIV-1 isolates with a mean IC_{90} of 2 nM. However, following FDA approval, several studies showed a high level of variable MVC inhibition of primary R5 HIV-1 isolates with IC50 values ranging from 0.1 to 4.5 nM [59] which was similar to that observed with other CCR5 agonists and antagonists.

The MOTIVATE 1 (conducted in North America) and MOTIVATE 2 (conducted in Europe, Australia, and the USA) phase III clinical trials sought to study the safety and efficacy of maraviroc in treatment-experienced patients. MOTIVATE stands for Maraviroc versus Optimized Therapy In Viremic

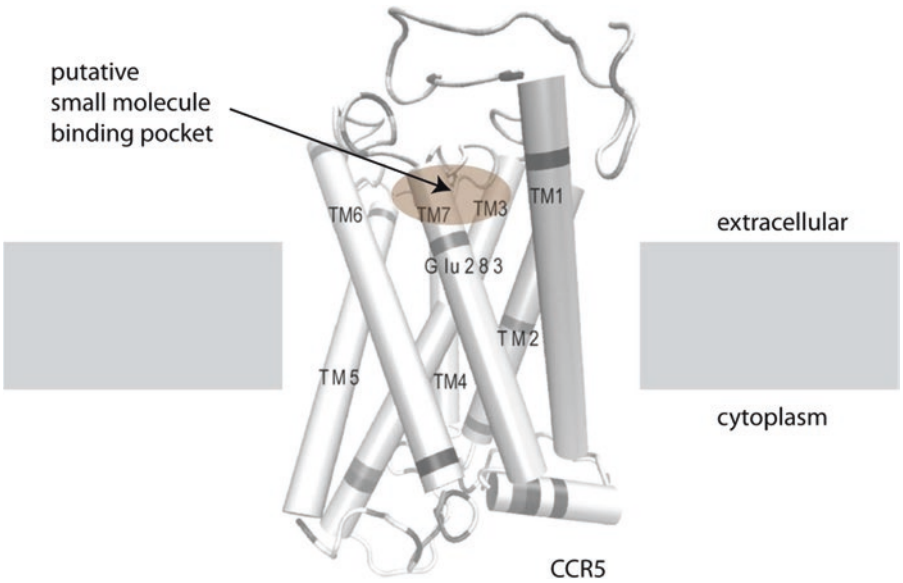


Fig. 36.2 Model of CCR5 structure and antagonist-binding site. Cartoon representation of predicted CCR5 structure. Transmembrane helices are numbered and marked. The putative small-molecule binding site is indicated (*brown*). Adapted with permission from Wang and Duan [82]

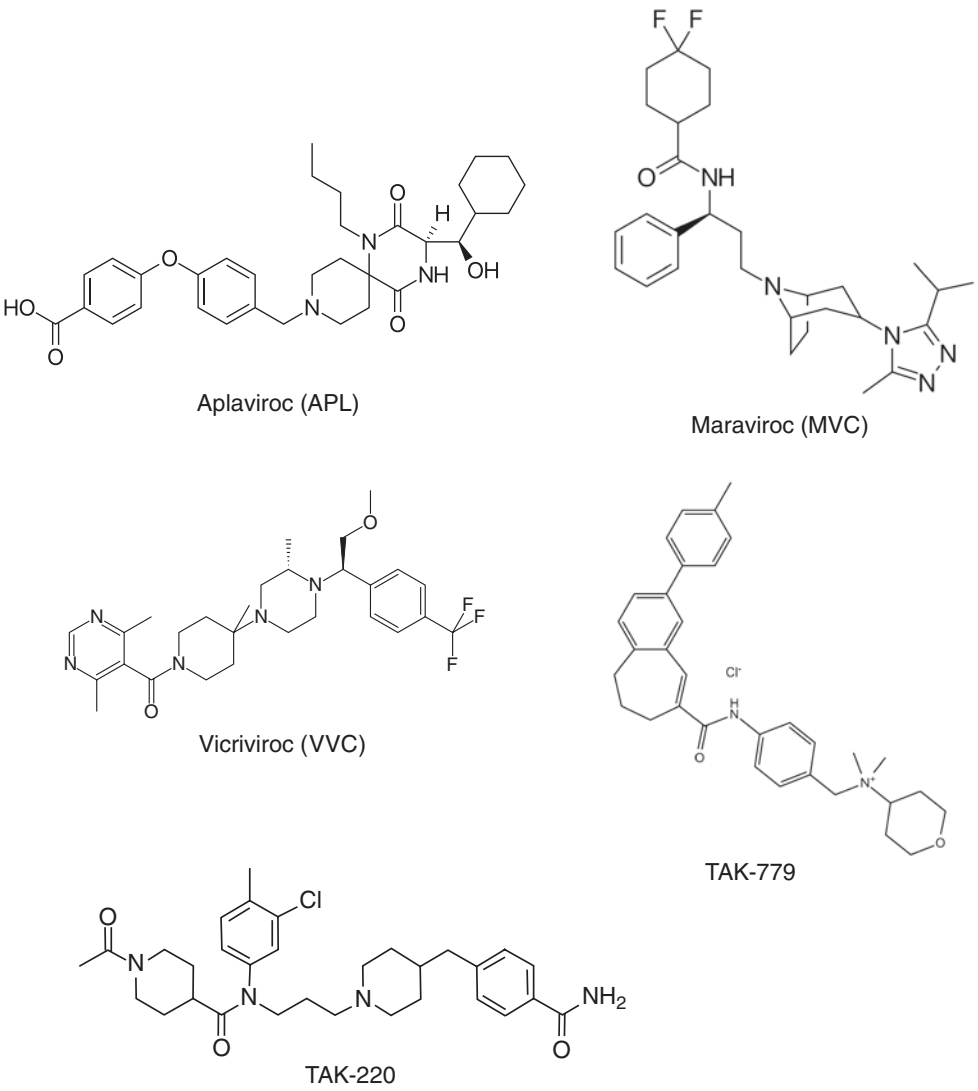


Fig. 36.3 Chemical structure of small-molecule CCR5 antagonists. The chemical structure of five CCR5 antagonists is shown. Reprinted with permission from American Society for Pharmacology and Experimental Therapeutics [62], copyright (2008)

Antiretroviral Treatment Experienced patients. Results from these trials published in 2008 indicated that patients receiving maraviroc with an optimized background therapy (OBT) showed significant reductions in HIV-1 RNA levels and increases in CD4 cell counts versus patients receiving OBT alone [64, 65]. Preliminary data resulted in FDA approval in 2007 for clinical use in salvage therapy regimens. Since then the indication has expanded to allow use in first-line drug regimens in combination with nucleoside analogs. It is currently marketed as Selzentry by Pfizer, Inc.

In addition to maraviroc, the CCR5 antagonists vicriviroc and aplaviroc have both been shown to inhibit HIV replication in humans. However, development of vicriviroc was discontinued after preliminary phase III clinical data while hepatic toxicity issues halted development of aplaviroc [66].

2.4.3 CCR5 Antagonist Resistance

Since entry inhibitors bind to host receptors and not directly to viral proteins, unique and complex resistance profiles are likely to emerge. These potential pathways of resistance include (1) alternative coreceptor usage (utilization of CXCR4 instead of CCR5 for entry), (2) enhanced entry kinetics, (3) increased receptor affinity, and (4) utilization of inhibitor-bound receptor for entry.

A primary concern in targeting CCR5 was the emergence of resistance as a change in coreceptor usage for entry. With rare exceptions, R5 HIV virus establishes new infections and predominates in asymptomatic stages of disease. However, in approximately half of patients, X4 variants emerge during the course of disease. Whether the emergence of X4 variants is a consequence or cause of AIDS of disease progression remains unclear but many ex vivo studies have shown that X4 HIV have higher replication rates, are more cytopathogenic, and result in cell syncythia formation. In vitro selections of entry inhibitor-resistant viruses have largely utilized PBMC cultures which express both CCR5 and CXCR4 [67]. When dual/X4 tropism was not preexisting in the viral swarm, mutations conferring altered coreceptor usage were not the favored resistance pathway within patients. Resistant viruses retained R5 tropism and were not able to infect CXCR4+/CD4+ cell lines or any other cell not expressing CCR5. Clinically, early reports from the MOTIVATE trials describing failure to maraviroc were attributed to coreceptor switching; however, advancements in the sensitivity of tropism testing revealed that in nearly all patients, X4 or dual-tropic HIV-1 preexisted at some low level in the intrapatient HIV swarm prior to the start of treatment [64]. Of the 133 patients who failed maraviroc treatment, 76 patients had dual/mixed or X4 tropic virus. Interestingly, the level of dual/X4 virus diminished rapidly when these patients stopped all treatment or when MVC was replaced in the combination treatment regimen [67]. Furthermore, MVC had no benefit in patients with non-R5 tropic virus and still receiving maraviroc treatment versus selected optimized background therapy [68]. Given the uncertainty regarding negative consequences of X4 virus in patients, i.e., accelerating disease progression, these studies recommended maraviroc treatment be limited to patients with only R5 HIV. As a result, tropism testing is required by the FDA for all patients under consideration for maraviroc therapy.

In the MOTIVATE study, only 76 of 133 patients had an emergence of X4 HIV-1 resulting in MVC resistance whereas 57 patients may have failed by an alternative resistance mechanism. In the absence of X4/dual-tropic virus mixed with R5 virus, in vitro resistance to CCR5 antagonist is caused primarily by altered affinity or avidity of R5 HIV-1 to CCR5. These findings also indicate that the switch to X4 usage by R5 HIV-1 is likely driven by a complex pattern of mutations with more drastic fitness loss (or "valley") than that related to MVC resistance due to mutations altering CCR5 binding. MVC resistance in patients is more related to an emergence of X4 HIV-1 rather than an R5-to-X4 switch and selection during MVC selection pressure. Two models for resistance to entry inhibitors have been proposed: competitive and noncompetitive.

In the competitive resistance model (Fig. 36.4a), gp120 binds to CCR5 with a given affinity. Inhibitors like PSC-RANTES that bind nearby regions of CCR5 demonstrate higher binding affinity for those regions than gp120 which can effectively prevent envelope engagement. Resistance to these inhibitors manifests through acquired mutations in gp120 which increase coreceptor affinity and promote inhibitor displacement. Competitive resistance is exhibited in drug sensitivity assays as increases in inhibitor concentration required to achieve half-maximal inhibition (IC_{50}) [57]. This shift in IC_{50} value is typical of resistance to most other antiretroviral classes such as reverse transcriptase and protease inhibitors. However, resistance to inhibitors like small-molecule antagonists that bind allosteric regions of CCR5 are predicted to follow noncompetitive resistance pathways (Fig. 36.4b). The binding of allosteric inhibitors such as maraviroc alter the conformation of the coreceptor, preventing gp120 from recognizing and binding CCR5. Mutations in gp120 that confer resistance to this type of CCR5 antagonist inhibitors (e.g., MVC) may permit gp120 recognition and binding of inhibitor-bound forms of the coreceptor [69–71]. An HIV envelope capable of utilizing inhibitor-bound receptor could maintain a level of entry despite increasing concentrations of drug, exhibiting a plateau effect where the maximum inhibition achieved remains steady at high drug concentrations but never reaches 100 %. The highest level of inhibition achieved is termed maximal percent inhibition (MPI). The MPI level is modulated based on the efficiency with which the viral envelope is able to use the inhibitor-bound versus inhibitor-free forms of the coreceptor.

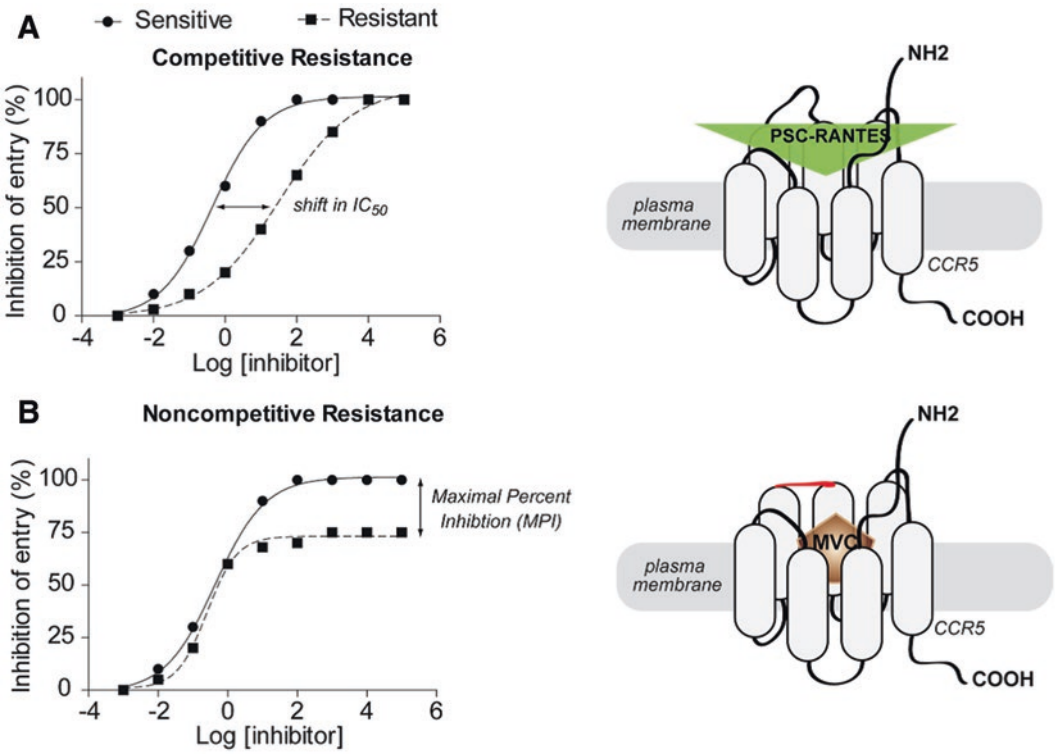


Fig. 36.4 Mechanisms of resistance to entry inhibitors. (**a**) Competitive resistance model in which shifts in IC50 concentration are indicative of resistance. Resistance to inhibitors that bind the same region of CCR5 as ligands and HIV gp120 (e.g., PSC-RANTES) is predicted to follow competitive resistance pathways. (**b**) Noncompetitive resistance model in which maximal inhibition is not achievable despite increasing inhibitor concentrations. The maximal level of inhibition achieved is called maximal percent inhibition (MPI). Inhibitors that bind to allosteric regions (e.g., maraviroc) and induce altered receptor conformations are predicted to follow noncompetitive resistance pathways

Despite these clear resistance mechanisms in several in vitro studies, MVC resistance in patients may be more complex than just the noncompetitive resistance resulting in an MPI effect. Only 4 of 38 patients failing MVC in the MOTIVATE study had MVC resistance related to an MPI effect [72]. Interestingly, this characterization of MVC resistance was limited to single-cycle assays utilizing virus pseudotyped with the patient's Env gene cloned into an expression vector [72]. Several studies have now shown that HIV sensitivity to MVC and other CCR5 antagonists/agonists can be affected by cell type, level of CCR5 expression, cytokines/chemokines in the media, and use of single- versus multiple-cycle assays [55–57, 59, 73, 74]. For example, Westby et al. have shown that some HIV-1 strains resistant to CCR5 antagonists may have a preferential use of altered CCR5 conformation, either utilizing the drug-bound form of the receptor for entry or engaging a subpopulation of CCR5 that do not efficiently bind the antagonist [67, 75]. Other studies have shown that use of different chemokines (MIP-1alpha, MCP-1, RANTES) and cytokines (IL-7, IL-15, IL-2) can dramatically impact sensitivity of R5 HIV-1 to MVC inhibition in primary T cells. The HIV-1 envelope as well as various chemokines (e.g., RANTES/CCL5) can also induce a signaling cascade in primary T cells that "primes" the cell for more efficient and higher levels of replication [49, 50]. Thus, the timing of drug addition in relation to endogenous or exogenous cytokines/chemokines has significant impact on the level of "resistance" to these CCR5 antagonists. Finally, the vast majority of MVC-resistant viruses were not identified in single-cycle assays but required a multiple-cycle virus infection assay in primary T cells or specific CD4+/CCR5+ cell line cultures to demonstrate high-level MVC resistance. In contrast, the same studies showed that HIV-1 variants resistant to other antiretroviral drugs (e.g., T20, 3TC, NVP) demonstrated the same level of drug resistance in both single-cycle or multiple-cycle infection assays. Together, these complex properties of MVC resistance, aside from the noncompetitive MPI effect, could explain for the remaining 34 MVC failures in the MOTIVATE study.

The overall kinetic rate of the entry process, as well as the dynamic relationship between CD4 and coreceptor binding affinity, plays a major role in the sensitivity of primary isolates to entry inhibitors. Studying the specific contributions of these processes separately has proven difficult; however general observations and inferences can be made. The intrinsic sensitivity of primary isolates to entry inhibitors can vary as much

as 1000-fold in IC_{50} values [74], a level not observed for other antiretrovirals such as PIs or RTIs. Factors such as the extreme diversity in the *env*-coding region, envelope glycoprotein conformational flexibility, differential affinity for CD4 and CCR5, and variable rates of six-helix bundle formation may account for some of this variation. Indeed, differential sensitivity to the fusion inhibitor T-20 as well as the CCR5 antagonist TAK-779 have been attributed to kinetic fusion rate nuances [76], while V3 loop polymorphisms have been shown not only to contribute to coreceptor affinity and viral fitness differences but also differences in sensitivity to PSC-RANTES [56].

In addition to influencing sensitivity to entry inhibitors, receptor affinity and entry kinetics are thought to contribute significantly to viral replicative fitness. Fitness is defined as the replication capacity of a virus in a given environment. Viral fitness in vivo is influenced by many parameters including host immune response, virus mutation/turnover rates, and presence of antiretrovirals. Reduced fitness is often associated with resistance to RTIs and PIs when measured in ex vivo competitions in PBMCs [77]. However, viral entry plays a major role in determining overall replication efficiency and therefore significantly impacts overall fitness. Mutations in the envelope that influence receptor affinity and entry rates, which might be acquired in the development of resistance to entry inhibitors, may also significantly influence viral fitness [69]. For example, a virus able to outcompete inhibitors such as PSC-RANTES for receptor occupancy would demonstrate reduced sensitivity to this inhibitor but may also demonstrate increased rates of entry and increased viral fitness. Indeed, studies of primary HIV-1 isolates have described a direct relationship between replicative fitness and sensitivity to entry inhibitors [55, 56]. Viral envelopes taken from individuals with elite suppression displayed reduced entry efficiency, slower entry kinetics, and increased entry inhibitor sensitivity [78]. Likewise, a strong correlation between replicative fitness and sensitivity to T-20, PSC-RANTES, and the CCR5 antibody 2D7 was described in viruses containing V3 loop polymorphisms [56]. In studies where resistance to CCR5 antagonists was selected in vitro, many of the gp120 mutations resulting in resistance were associated with increases in replicative fitness [69]. Finally, the PSC-RANTES-resistant SHIV variant infecting macaques vaginally exposed to this drug was more fit than the inoculating $SHIV_{SF162\text{-}P3}$ virus [55]. These data would suggest that competitive binding and overall rates of the entry process not only account for differences in sensitivity to entry inhibitors, but also influence overall replicative fitness. Selection of more fit, drug-resistant HIV-1 isolates, be they X4 or R5 tropic, following treatment with CCR5 antagonists is alarming considering that replicative fitness is a direct correlate of disease progression [78–81]. In contrast, resistance to nearly every other antiretroviral drug results in mutated HIV-1 strains that are less fit than the parental HIV-1.

3 Conclusion

HIV-1 entry is a highly cooperative process involving complex interactions between viral envelope glycoproteins and host cell receptors. These envelope-receptor interactions play a critical role in target cell tropism and influence viral replicative fitness, transmission, and ultimately disease progression. Extensive study of the mechanisms involved in the entry process has led to greater understanding of transmission, disease progression, and pathogenesis which has contributed to the development of inhibitors which may improve clinical treatment of HIV-1 infection. Each stage in attachment and entry provide numerous opportunities for inhibition by a diverse array of compounds. These compounds have roles in limiting viral spread both within a given individual and between individuals when used as a microbicide. Possibly the greatest focus of this class of HIV inhibitors is on drugs targeting CCR5 and its use as a viral coreceptor necessary for entry. These drugs are able to prevent the interaction between gp120 and CCR5 through a number of different mechanisms resulting in an inability to fuse the viral and cellular membranes, preventing the transfer of the viral core into the host cytoplasm.

References

1. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature. 1998;393:648–59.
2. Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S. Molecular architecture of native HIV-1 gp120 trimers. Nature. 2008;455:109–13.
3. Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Sparks ES, Parks WP, Joseph SF, Gallo RC, Wong-Staal F. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell. 1986;45(5):637–48.
4. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature. 1984;312:763–7.
5. Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell. 1986;47:333–48.
6. Cilliers T, Willey S, Sullivan WM, Patience T, Pugach P, Coetzer M, Papathanasopoulos M, Moore JP, Trkola A, Clapham P, Morris L. Use of alternate coreceptors on primary cells by two HIV-1 isolates. Virology. 2005;339:136–44.
7. Lee S, Tiffany HL, King L, Murphy PM, Golding H, Zaitseva MB. CCR8 on human thymocytes functions as a human immunodeficiency virus type 1 coreceptor. J Virol. 2000;74:6946–52.
8. Sharron M, Pohlmann S, Price K, Lolis E, Tsang M, Kirchhoff F, Doms RW, Lee B. Expression and coreceptor activity of STRL33/Bonzo on primary peripheral blood lymphocytes. Blood. 2000;96:41–9.
9. Kuritzkes DR, Jacobson J, Powderly WG, Godofsky E, DeJesus E, Haas F, Reimann KA, Larson JL, Yarbough PO, Curt V, Shanahan Jr WR. Antiretroviral activity of the anti-CD4 monoclonal antibody

TNX-355 in patients infected with HIV type 1. J Infect Dis. 2004;189:286–91.
10. Mukhtar M, Parveen Z, Pomerantz RJ. Technology evaluation: PRO-542, Progenics Pharmaceuticals inc. Curr Opin Mol Ther. 2000;2:697–702.
11. Ray N, Wind-Rotolo M, Healy M, Hwang C, Huang S, Whitcomb J, Zhou N, Krystal M, Hanna G. Lack of resistance development to the HIV-1 attachment inhibitor BMS-626529 during short-term mono-therapy with its pro-drug BMS-663068. In: 19th conference on retroviruses and opportunistic infections, 2012.
12. Moore JP, McKeating JA, Norton WA, Sattentau QJ. Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1 virions: gp120 dissociation and its implications for virus-cell binding and fusion reactions and their neutralization by soluble CD4. J Virol. 1991;65:1133–40.
13. Moore JP, McKeating JA, Weiss RA, Sattentau QJ. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science. 1990;250:1139–42.
14. Daar ES, Li XL, Moudgil T, Ho DD. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc Natl Acad Sci U S A. 1990;87:6574–8.
15. Orloff SL, Kennedy MS, Belperron AA, Maddon PJ, McDougal JS. Two mechanisms of soluble CD4 (sCD4)-mediated inhibition of human immunodeficiency virus type 1 (HIV-1) infectivity and their relation to primary HIV-1 isolates with reduced sensitivity to sCD4. J Virol. 1993;67:1461–71.
16. Zhao Q, Ma L, Jiang S, Lu H, Liu S, He Y, Strick N, Neamati N, Debnath AK. Identification of N-phenyl-N′-(2,2,6,6-tetramethyl-piperidin-4-yl)- oxalamides as a new class of HIV-1 entry inhibitors that prevent gp120 binding to CD4. Virology. 2005;339:213–25.
17. Madani N, Schon A, Princiotto AM, Lalonde JM, Courter JR, Soeta T, Ng D, Wang L, Brower ET, Xiang SH, Kwon YD, Huang CC, Wyatt R, Kwong PD, Freire E, Smith III AB, Sodroski J. Small-molecule CD4 mimics interact with a highly conserved pocket on HIV-1 gp120. Structure. 2008;16:1689–701.
18. Lataillade M. Safety profile of HIV-1 attachment inhibitor prodrug BMS-663068 in antiretroviral-experienced subjects: week 24 analysis. IDWeek 2014. Idsa, 2014.
19. Moore JP, Sattentau QJ, Klasse PJ, Burkly LC. A monoclonal antibody to CD4 domain 2 blocks soluble CD4-induced conformational changes in the envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) and HIV-1 infection of CD4+ cells. J Virol. 1992;66(8):4784–93.
20. Vermeire K, Zhang Y, Princen K, Hatse S, Samala MF, Dey K, Choi HJ, Ahn Y, Sodoma A, Snoeck R, Andrei G, De CE, Bell TW, Schols D. CADA inhibits human immunodeficiency virus and human herpes virus 7 replication by down-modulation of the cellular CD4 receptor. Virology. 2002;302:342–53.
21. Vermeire K, Van LK, Janssens W, Bell TW, Schols D. Human immunodeficiency virus type 1 escape from cyclotriazadisulfonamide induced CD4-targeted entry inhibition is associated with increased neutralizing antibody susceptibility. J Virol. 2009;83:9577–83.
22. Hoffman TL, LaBranche CC, Zhang W, Canziani G, Robinson J, Chaiken I, Hoxie JA, Doms RW. Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. Proc Natl Acad Sci U S A. 1999;96:6359–64.
23. Bunnik EM, van Gils MJ, Lobbrecht MS, Pisas L, Nanlohy NM, van Baarle D, van Nuenen AC, Hessell AJ, Schuitemaker H. Emergence of monoclonal antibody b12-resistant human immunodeficiency virus type 1 variants during natural infection in the absence of humoral or cellular immune pressure. J Gen Virol. 2010;91:1354–64.
24. Clapham PR, McKnight A, Weiss RA. Human immunodeficiency virus type 2 infection and fusion of CD4-negative human cell lines: induction and enhancement by soluble CD4. J Virol. 1992;66:3531–7.
25. Sullivan N, Sun Y, Binley J, Lee J, Barbas III CF, Parren PW, Burton DR, Sodroski J. Determinants of human immunodeficiency virus type 1 envelope glycoprotein activation by soluble CD4 and monoclonal antibodies. J Virol. 1998;72:6332–8.
26. Do Kwon Y, Finzi A, Wu X, Dogo-Isonagie C, Lee LK, Moore LR, Schmidt SD, Stuckey J, Yang Y, Zhou T, Zhu J, Vicic DA, Debnath AK, Shapiro L, Bewley CA, Mascola JR, Sadroski JG, Kwong PD. Unliganded HIV-1 gp120 core structures assume the CD4-bound conformation with regulation by quaternary interactions and variable loops. Proc Natl Acad Sci U S A. 2012;109(15):5663–8.
27. Medjahed H, Pacheco B, Désormeaux A, Sodroski J, Finzi A. The HIV-1 gp120 major variable regions modulate cold inactivation. J Virol. 2013;87(7):4103–11.
28. Haim H, Si Z, Madani N, Wang L, Courter JR, Princiotto A, Kassa A, DeGrace M, McGee-Estrada K, Mefford M, Gabuzda D, Smith III AB, Sodroski J. Soluble CD4 and CD4-mimetic compounds inhibit HIV-1 infection by induction of a short-lived activated state. PLoS Pathog. 2009;5:e1000360.
29. Yoshimura K, Harada S, Shibata J, Hatada M, Yamada Y, Ochiai C, Tamamura H, Matsushita S. Enhanced exposure of human immunodeficiency virus type 1 primary isolate neutralization epitopes through binding of CD4 mimetic compounds. J Virol. 2010;84:7558–68.
30. Lin PF, Blair W, Wang T, Spicer T, Guo Q, Zhou N, Gong YF, Wang HG, Rose R, Yamanaka G, Robinson B, Li CB, Fridell R, Deminie C, Demers G, Yang Z, Zadjura L, Meanwell N, Colonno R. A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc Natl Acad Sci U S A. 2003;100:11013–8.
31. Wild C, Oas T, McDanal C, Bolognesi D, Matthews T. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc Natl Acad Sci U S A. 1992;89:10537–41.
32. Wild CT, Shugars DC, Greenwell TK, McDanal CB, Matthews TJ. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc Natl Acad Sci U S A. 1994;91:9770–4.
33. Derdeyn CA, Decker JM, Sfakianos JN, Zhang Z, O'Brien WA, Ratner L, Shaw GM, Hunter E. Sensitivity of human immunodeficiency virus type 1 to fusion inhibitors targeted to the gp41 first heptad repeat involves distinct regions of gp41 and is consistently modulated by gp120 interactions with the coreceptor. J Virol. 2001;75:8605–14.
34. Rimsky LT, Shugars DC, Matthews TJ. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. J Virol. 1998;72:986–93.
35. Kinomoto M, Yokoyama M, Sato H, Kojima A, Kurata T, Ikuta K, Sata T, Tokunaga K. Amino acid 36 in the human immunodeficiency virus type 1 gp41 ectodomain controls fusogenic activity: implications for the molecular mechanism of viral escape from a fusion inhibitor. J Virol. 2005;79:5996–6004.
36. Reeves JD, Lee FH, Miamidian JL, Jabara CB, Juntilla MM, Doms RW. Enfuvirtide resistance mutations: impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity, and virus neutralization. J Virol. 2005;79:4991–9.
37. Forssmann WG, The YH, Stoll M, Adermann K, Albrecht U, Tillmann HC, Barlos K, Busmann A, Canales-Mayordomo A, Gimenez-Gallego G, Hirsch J, Jimenez-Barbero J, Meyer-Olson D, Munch J, Perez-Castells J, Standker L, Kirchhoff F, Schmidt RE. Short-term monotherapy in HIV-infected patients with a virus entry inhibitor against the gp41 fusion peptide. Sci Transl Med. 2010;2:63re3.
38. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell

lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature. 1996;382:635–8.
39. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature. 1998;393:591–4.
40. Schols D, Este JA, Henson G, De CE. Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antiviral Res. 1997;35:147–56.
41. Hatse S, Princen K, De Clercq E, Rosenkilde MM, Schwartz TW, Hernandez-Abad PE, Skerlj RT, Bridger GJ, Schols D. AMD3465, a monomacrocyclic CXCR4 antagonist and potent HIV entry inhibitor. Biochem Pharmacol. 2005;70:752–61.
42. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res. 2002;62(6):1832–7.
43. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996;86:367–77.
44. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature. 1996;382:722–5.
45. Lim JK, Glass WG, McDermott DH, Murphy PM. CCR5: no longer a 'good for nothing' gene–chemokine control of west Nile virus infection. Trends Immunol. 2006;27:308–12.
46. Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, Buser R, Wells TN, Proudfoot AE. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science. 1997;276:276–9.
47. Blanpain C, Doranz BJ, Bondue A, Govaerts C, De LA, Vassart G, Doms RW, Proudfoot A, Parmentier M. The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle. J Biol Chem. 2003;278:5179–87.
48. Elsner J, Mack M, Brühl H, Dulkys Y, Kimmig D, Simmons G, Clapham PR, Schlöndorff D, Kapp A, Wells TN, Proudfoot AE. Differential activation of CC chemokine receptors by AOP-RANTES. J Biol Chem. 2000;275:7787–94.
49. Marozsan AJ, Torre VS, Johnson M, Ball SC, Cross JV, Templeton DJ, Quinones-Mateu ME, Offord RE, Arts EJ. Mechanisms involved in stimulation of human immunodeficiency virus type 1 replication by aminooxypentane RANTES. J Virol. 2001;75:8624–38.
50. Popik W, Hesselgesser JE, Pitha PM. Binding of human immunodeficiency virus type 1 to CD4 and CXCR4 receptors differentially regulates expression of inflammatory genes and activates the MEK/ERK signaling pathway. J Virol. 1998;72:6406–13.
51. Rodriguez-Frade JM, Vila-Coro AJ, Martin A, Nieto M, Sanchez-Madrid F, Proudfoot AE, Wells TN, Martinez A, Mellado M. Similarities and differences in RANTES- and (AOP)-RANTES-triggered signals: implications for chemotaxis. J Cell Biol. 1999;144:755–65.
52. Hartley O, Gaertner H, Wilken J, Thompson D, Fish R, Ramos A, Pastore C, Dufour B, Cerini F, Melotti A, Heveker N, Picard L, Alizon M, Mosier D, Kent S, Offord R. Medicinal chemistry applied to a synthetic protein: development of highly potent HIV entry inhibitors. Proc Natl Acad Sci U S A. 2004;101:16460–5.
53. Lederman MM, Veazey RS, Offord R, Mosier DE, Dufour J, Mefford M, Piatak Jr M, Lifson JD, Salkowitz JR, Rodriguez B, Blauvelt A, Hartley O. Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. Science. 2004;306:485–7.
54. Signoret N, Pelchen-Matthews A, Mack M, Proudfoot AE, Marsh M. Endocytosis and recycling of the HIV coreceptor CCR5. J Cell Biol. 2000;151:1281–94.
55. Dudley DM, Wentzel JL, Lalonde MS, Veazey RS, Arts EJ. Selection of a simian-human immunodeficiency virus strain resistant to a vaginal microbicide in macaques. J Virol. 2009;83:5067–76.
56. Lobritz MA, Marozsan AJ, Troyer RM, Arts EJ. Natural variation in the V3 crown of human immunodeficiency virus type 1 affects replicative fitness and entry inhibitor sensitivity. J Virol. 2007;81:8258–69.
57. Lobritz MA, Ratcliff AN, Marozsan AJ, Dudley DM, Tilton JC, Arts EJ. Multifaceted mechanisms of HIV inhibition and resistance to CCR5 inhibitors PSC-RANTES and Maraviroc. Antimicrob Agents Chemother. 2013;57:2640–50.
58. Nedellec R, Coetzer M, Lederman MM, Offord RE, Hartley O, Mosier DE. "Resistance" to PSC-RANTES revisited: two mutations in human immunodeficiency virus type 1 HIV-1SF162 or simian-human immunodeficiency virus SHIVSF162-p3 do not confer resistance. J Virol. 2010;84(11):5842–5.
59. Ratcliff AN, Shi W, Arts EJ. HIV-1 resistance to maraviroc conferred by a CD4 binding site mutation in the envelope glycoprotein gp120. J Virol. 2013;87(2):923–34.
60. Cooper DA, Heera J, Ive P, Botes M, Dejesus E, Burnside R, Clumeck N, Walmsley S, Lazzarin A, Mukwaya G, Saag M, van Der Ryst E. Efficacy and safety of maraviroc vs. efavirenz in treatment-naive patients with HIV-1: 5-year findings. AIDS 2014;28(5):717.
61. Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP, Moore JP. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc Natl Acad Sci U S A. 2000;97:5639–44.
62. Kondru R, Zhang J, Ji C, Mirzadegan T, Rotstein D, Sankuratri S, Dioszegi M. Molecular interactions of CCR5 with major classes of small molecule anti-HIV CCR5 antagonists. Mol Pharmacol. 2008;73:789–800.
63. Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, Mori J, Rickett G, Smith-Burchnell C, Napier C, Webster R, Armour D, Price D, Stammen B, Wood A, Perros M. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob Agents Chemother. 2005;49:4721–32.
64. Fatkenheuer G, Nelson M, Lazzarin A, Konourina I, Hoepelman AI, Lampiris H, Hirschel B, Tebas P, Raffi F, Trottier B, Bellos N, Saag M, Cooper DA, Westby M, Tawadrous M, Sullivan JF, Ridgway C, Dunne MW, Felstead S, Mayer H, van der Ryst E. Subgroup analyses of maraviroc in previously treated R5 HIV-1 infection. N Engl J Med. 2008;359:1442–55.
65. Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, Horban A, Nadler J, Clotet B, Karlsson A, Wohlfeiler M, Montana JB, McHale M, Sullivan J, Ridgway C, Felstead S, Dunne MW, van der Ryst E, Mayer H. Maraviroc for previously treated patients with R5 HIV-1 infection. N Engl J Med. 2008;359:1429–41.
66. Nichols WG, Steel HM, Bonny T, Adkison K, Curtis L, Millard J, Kabeya K, Clumeck N. Hepatotoxicity observed in clinical trials of aplaviroc (GW873140). Antimicrob Agents Chemother. 2008;52:858–65.
67. Westby M, Lewis M, Whitcomb J, Youle M, Pozniak AL, James IT, Jenkins TM, Perros M, van der Ryst E. Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. J Virol. 2006;80:4909–20.
68. Saag M, Goodrich J, Fatkenheuer G, Clotet B, Clumeck N, Sullivan J, Westby M, van der Ryst E, Mayer H. A double-blind, placebo-

controlled trial of maraviroc in treatment-experienced patients infected with non-R5 HIV-1. J Infect Dis. 2009;199:1638–47.
69. Anastassopoulou CG, Marozsan AJ, Matet A, Snyder AD, Arts EJ, Kuhmann SE, Moore JP. Escape of HIV-1 from a small molecule CCR5 inhibitor is not associated with a fitness loss. PLoS Pathog. 2007;3:e79. 07-PLPA-RA-0149R2.
70. Berro R, Sanders RW, Lu M, Klasse PJ, Moore JP. Two HIV-1 variants resistant to small molecule CCR5 inhibitors differ in how they use CCR5 for entry. PLoS Pathog. 2009;5:e1000548.
71. Strizki JM, Xu S, Wagner NE, Wojcik L, Liu J, Hou Y, Endres M, Palani A, Shapiro S, Clader JW, Greenlee WJ, Tagat JR, McCombie S, Cox K, Fawzi AB, Chou CC, Pugliese-Sivo C, Davies L, Moreno ME, Ho DD, Trkola A, Stoddart CA, Moore JP, Reyes GR, Baroudy BM. SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo. Proc Natl Acad Sci U S A. 2001;98:12718–23.
72. Mori J, Mosley M, Lewis M, Simpson P, Toma J, Huang J, Whitcomb J, Ciaramella G, Westby M. (2007) Characterization of maraviroc resistance in patients failing treatment with CCR5-tropic HIV-1 in MOTIVATE 1 and 2. In: XVI international HIV drug resistance workshop, Barbados, 12–16 June 2007.
73. Ketas TJ, Klasse PJ, Spenlehauer C, Nesin M, Frank I, Pope M, Strizki JM, Reyes GR, Baroudy BM, Moore JP. Entry inhibitors SCH-C, RANTES, and T-20 block HIV type 1 replication in multiple cell types. AIDS Res Hum Retroviruses. 2003;19:177–86.
74. Torre VS, Marozsan AJ, Albright JL, Collins KR, Hartley O, Offord RE, Quinones-Mateu ME, Arts EJ. Variable sensitivity of CCR5-tropic human immunodeficiency virus type 1 isolates to inhibition by RANTES analogs. J Virol. 2000;74:4868–76.
75. Pugach P, Marozsan AJ, Ketas TJ, Landes EL, Moore JP, Kuhmann SE. HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. Virology. 2007;361:212–28.
76. Reeves JD, Gallo SA, Ahmad N, Miamidian JL, Harvey PE, Sharron M, Pohlmann S, Sfakianos JN, Derdeyn CA, Blumenthal R, Hunter E, Doms RW. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc Natl Acad Sci U S A. 2002;99:16249–54.
77. Quinones-Mateu ME, Arts EJ. Fitness of drug resistant HIV-1: methodology and clinical implications. Drug Resist Updat. 2002;5:224–33.
78. Lassen KG, Lobritz MA, Bailey JR, Johnston S, Nguyen S, Lee B, Chou T, Siliciano RF, Markowitz M, Arts EJ. Elite suppressor-derived HIV-1 envelope glycoproteins exhibit reduced entry efficiency and kinetics. PLoS Pathog. 2009;5:e1000377.
79. Blaak H, van't Wout AB, Brouwer M, Hooibrink B, Hovenkamp E, Schuitemaker H. In vivo HIV-1 infection of CD45RA+ CD4+ T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4+ T cell decline. Proc Natl Acad Sci U S A. 2000;97:1269–74.
80. Quiñones-Mateu ME, Ball SC, Marozsan AJ, Torre VS, Albright JL, Vanham G, van der Groen G, Colebunders RL, Arts EJ. A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. J Virol. 2000;74:9222–33.
81. Troyer RM, Collins KR, Abraha A, Fraundorf E, Moore DM, Krizan RW, Toossi Z, Colebunders RL, Jensen MA, Mullins JI, Vanham G, Arts EJ. Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. J Virol. 2005;79:9006–18.
82. Wang T, Duan Y. HIV co-receptor CCR5: structure and interactions with inhibitors. Infect Disord Drug Targets. 2009;9:279–88.

HIV-1 Resistance to Integrase Inhibitors

37

Ying-Shan Han, Thibault Mesplède, and Mark A. Wainberg

1 Introduction

Over the past three decades, new antiretroviral drugs have been rapidly developed and expanded for use in the clinic. Antiretroviral therapy (ART) generally combines at least three different drugs for treatment of HIV-infected patients. As more new antiretroviral drugs (ARVs) belonging to different classes have become available, ART has greatly decreased the death rate due to HIV-1 infection [1]. To date, 29 antiretroviral drugs have been approved by the Food and Drug Administration (FDA) and are available for treatment of HIV-1 infections. These drugs are classified into six distinct classes based on their molecular mechanism and resistance profiles: nucleoside-analog reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), fusion inhibitors, and coreceptor antagonists. However, HIV can rapidly mutate and develop resistance to all currently used anti-HIV drugs [2].

Human immunodeficiency virus type 1 (HIV-1) integrase, an essential enzyme in the viral life cycle, catalyzes multistep reactions to incorporate viral DNA into the genome of host cells. These steps include a 3′-processing reaction and a strand transfer reaction. The HIV integrase enzyme therefore is a significant therapeutic target that can be blocked by integrase inhibitors. Since there is no human homolog, HIV-1 integrase is a specific and effective HIV drug target with excellent tolerability and minimal toxicity. Integrase strand transfer inhibitors (INSTIs) prevent the HIV-1 integrase-catalyzed strand transfer step from inserting viral DNA into human host chromosomal DNA. INSTIs are the latest class of anti-HIV drugs.

So far three INSTIs, raltegravir (RAL, Isentress), elvitegravir (EVG, part of the combination termed Stribild), and dolutegravir (DTG, Tivicay) have been approved by the FDA for use in clinic. Numerous clinical studies have shown that INSTIs are highly effective in treatment of HIV patients and support the use of INSTIs in first-line regimens [3–7].

However, HIV resistance to RAL and EVG emerges relatively rapidly both in vitro and in patients, in the absence of other active ARVs. Cross-resistance between RAL and EVG/c has also been observed [7–11]. RAL and EVG share similar resistance profiles since the primary resistance mutations associated with each of these drugs are located near the active site of HIV-1 integrase. Both drugs have a relatively low genetic barrier to resistance in that only one or two mutations in integrase are capable of causing significantly reduced susceptibility to RAL and EVG. In contrast, DTG, a second-generation INSTI, has shown a better resistance profile than RAL or EVG [12–14]. DTG can also inhibit RAL- and EVG-resistant viruses either completely or partially in vitro and in vivo [8, 15, 16]. It is notable that DTG is the only anti-HIV drug that HIV has not developed resistance mutations upon virologic rebound in previously treatment-naïve patients in clinical practice until now [17].

Drug resistance is caused by primary mutations that reduce drug susceptibility. Often a combination of primary mutations with secondary mutations further decreases virus susceptibility but also compensates for the decreased fitness associated with the primary mutations. HIV resistance as well as cross-resistance are observed to varying extents among the INSTIs. In this review, we summarize the latest findings on resistance mutations to INSTIs, and the underlying mechanisms involved, and discuss new perspectives pertaining to the use of INSTIs in therapy.

2 HIV Resistance to RAL and EVG

The three most common mutations associated with resistance to RAL are Q148H/K/R, N155H, and Y143C/H/R (Table 37.1), which are associated with virologic failure and reduced sus-

Y.-S. Han, Ph.D. • T. Mesplède, Ph.D. • M.A. Wainberg, Ph.D. (✉)
Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University AIDS Centre, Montreal, QC, Canada
e-mail: mark.wainberg@mcgill.ca

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_37

Table 37.1 Major resistance pathways to RAL, EVG, and DTG

	Mutational pathways	Fold resistance		
		RAL	EVG	DTG
Y143 pathway	Y143C	<10	<2	<2
	Y143R	<50	<2	<2
	T97A/Y143C	>100	<2	<2
	T97A/Y143R	>100	<2	<2
	L74M/T97A/Y143G	<50	ND	<2
	L74M/T97A/E138A/Y143C	<20	ND	<2
N155 pathway	N155N	<50	<50	<2
	E92Q/N155H	<100	>100	<10
	L74M/N155H	<50	<50	<2
Q148 pathway	Q148H	<20	<10	<2
	Q148K	<100	<100	<2
	Q148R	<50	<100	<2
	E138K/Q148H	<10	<20	<2
	E138K/Q148K	>100	>100	<10
	E138K/Q148R	>100	>100	<10
	G140S/Q148H	>100	>100	<20
	G140S/Q148K	<10	<100	<2
	G140S/Q148R	>100	>100	<10
	E138A/G140S/Y143H/Q148H	>100	ND	<50
R263K pathway	R263K	<1	3	4
	R263K/H51Y	3–5	3	4–6
G118R pathway	G118R	10–17	>5	>8
	G118R/H51Y	ND	ND	ND
	G118R/E138K	4–20	4–5	8–13

ND not detected

ceptibility to RAL [18]. These primary mutations are often found in combination with one or more secondary mutations that either augment resistance or restore viral fitness or both. Some of the accessory mutations include L74I, T97A, and E138K. Substitutions at Q148, located on the active site of HIV-1 integrase, are often accompanied by secondary mutations such as G140S/A or E138K. The addition of such secondary mutations can improve viral replication capacity as well as strand transfer and 3′ processing activities.

Substitutions at N155H, close to the active site of integrase, usually result in lower RAL resistance than those on Q148. This mutation reduces viral replication capacity by impairing strand transfer and 3′ processing activities to varying degrees. The addition of different secondary mutations to N155H can cause a broad range of reductions in susceptibility to RAL. For example, the addition of E92Q to N155H results in additional decreases in both replication capacity and RAL susceptibility. The addition of Q95K to N155H caused increased RAL resistance and partially restored replication capacity. Mutations at Y143 in the HIV-1 integrase gene increase resistance to RAL while reducing viral replication capacity. Biochemical studies showed that Y143R/C severely impaired strand transfer but only moderately impaired 3′ processing activity [19].

Major primary mutations for EVG are predominantly E92Q, followed by Q148H/K/R, N155H, and T66I (Table 37.1). The E92Q mutation greatly reduces EVG susceptibility (FC = 33), while the T66I mutation reduced susceptibility 15-fold. There is a high level of cross-resistance between EVG and RAL. The E92Q and T66I mutations reduce RAL susceptibility by 6.0-fold and 1.4-fold, respectively. In addition, these primary resistance mutations were often accompanied by secondary mutations in integrase. The secondary mutations at H51Y, S147G, and E157Q were found to accompany the E92Q mutation, while F121Y (a mutation also associated with reduced RAL susceptibility), S153Y, and R263K accompanied the primary T66I mutation. These secondary mutations caused additional reduced EVG susceptibility. Since there is a similar binding mechanism for RAL and EVG at the active site of integrase, extensive cross-resistance between the two inhibitors has been attributed to mutations at positions 155 and 148.

3 HIV Resistance to DTG

DTG is a second-generation integrase inhibitor with unique properties: unboosted daily dosing, a high barrier to resistance, and low cross-resistance to the first-generation INSTIs RAL and EVG. DTG is now a preferred ART regimen for HIV therapy in both treatment-naïve and treatment-experienced patients [14, 20, 21].

Initially, DTG was specifically designed to have a novel resistance profile to avoid cross-resistance with the first-generation INSTIs and to maintain a high barrier to resistance [22]. Clinical studies (VIKING) demonstrated that DTG maintains activity against RAL- and EVG-resistant virus [23]. However, DTG showed reduced potency against variants with Q148 plus two or more additional mutations (e.g., Q148+G140+E138) in treatment-experienced participants [24, 25].

To this time, no major resistance mutations against DTG have been identified. Some mutational positions in HIV-1 integrase that are potentially involved in HIV resistance to DTG and that were selected in vitro and in vivo include F121, S153, G118, E138, and R263 (Table 37.1) [12]. In vitro selection studies in cell culture under pressure with DTG caused changes in HIV-1 integrase at positions E92, L101, T124, S153, and G193, which are associated with a moderate reduction in susceptibility (fold change, FC<2.5) [22]. In vitro selection studies revealed that R263K, the most common mutation to emerge, conferred only low-level resistance to DTG in culture (FC=2.3), with greatly impaired strand transfer activity and reduced viral replication capacity [26]. R263K was often observed together with H51Y in cell culture selections with DTG. H51Y alone had no effect on resistance to DTG, while the addition of H51Y to R263K increased resistance to DTG approximately fivefold, while dramatically decreasing viral replication capacity by ~90% and enzyme strand transfer activity by ~80% [27]. Further studies showed that R263K even in combination with additional mutations such as M50I, G118R, H51Y, E138K, T66I, N155H, or M184I/V only slightly increased resistance to DTG, but did not restore viral replication capacity [12].

R263K has been reported to be present in several treatment-experienced, INSTI-naïve patients in the SAILING clinical trial [28]. A recent study reported that the G118R is another common mutation observed in cell culture selection with DTG. The G118R mutation caused low-level resistance to DTG (FC=3.1) with greatly decreased strand transfer activity. The addition of H51Y or E138K to G118R yielded no significant increase in the level of resistance (FC=3.4) [29]. These studies showed that R263K or G118R, alone or in combination with secondary mutations, slightly increases resistance to DTG in tissue culture, but does not restore the diminished viral fitness associated with the R263K or G118R mutations. These combinations result in a virus with limited cross-resistance. The R263K resistance pathway often severely impairs virus replication capacity and may represent an evolutionary dead end [30, 31]. This may explain why primary resistance to DTG is so rare in clinical practice.

4 Mechanisms of HIV Resistance to INSTIs

There has been considerable progress in studies on mechanisms of HIV resistance to INSTIs [12]. It is generally believed that the ability of DTG to maintain a high genetic barrier to HIV resistance is due to its slower dissociation rate from integrase-DNA complexes than that of either RAL or EVG [32]. Biochemical studies suggest that INSTIs inhibit integrase by binding to and sequestering essential active-site magnesium ions. Mutations in HIV-1 integrase may lead to faster INSTI dissociation kinetics that contribute to the development of integrase resistance by perturbing metal binding to the active site [14, 33, 34]. DTG has an extended linker which allows its difluorophenyl group to enter farther into the pocket within the integrase active site than the other INSTIs. DTG also has the ability to adjust its structure and conformation in response to structural changes within the active sites of RAL- and EVG-resistant integrases, compared to RAL and EVG (Fig. 37.1) [8, 35, 36]. Biochemical studies have shown that the R263K mutation in integrase results in

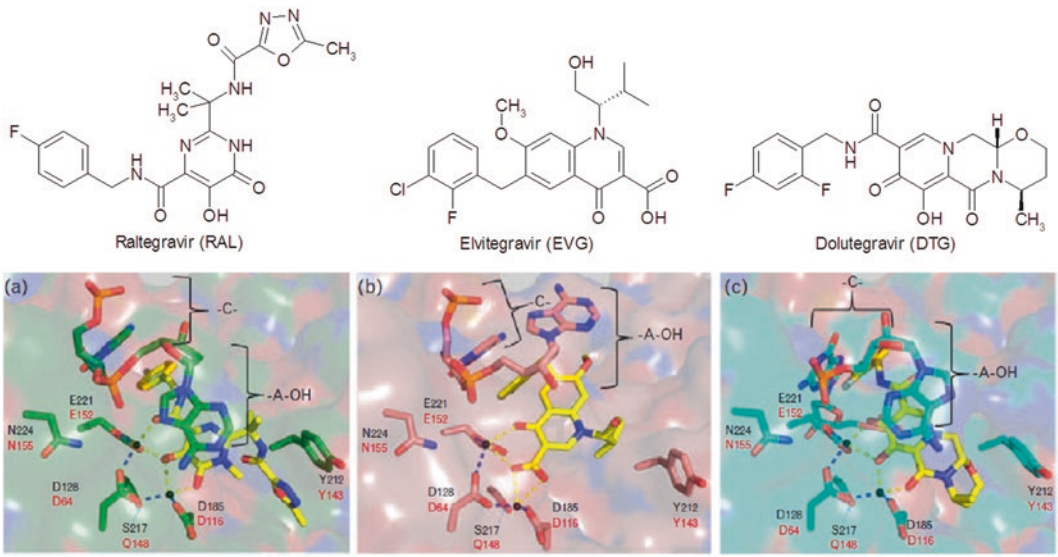


Fig. 37.1 Chemical structures of (**a**) RAL, (**b**) EVG, and (**c**) DTG and their binding modes to the prototype foamy virus (PFV) integrase active site

decreases in 3′-processing and strand transfer activities. Homology modeling of intasomes and strand transfer complexes from wild-type and R263K-containing integrases reveals altered interactions in integrase-DNA. In addition, an integrase-DNA binding assay showed that the R263K mutation decreased integrase-viral DNA binding [26]. Similarly, biochemical studies have shown that the G118R mutation in integrase greatly decreases strand transfer activity but does not affect 3′-processing activity [29].

To understand the molecular mechanism of cross-resistance conferred by E138K/Q148K to RAL, EVG, and DTG, a homology modeling of the constructed tetrameric HIV-1 intasome was conducted. The molecular dynamics simulation and residue interaction network (RIN) analysis showed that residue P145 in the 140S loop (G140–G149) of the intasome has strong hydrophobic interactions with INSTIs and is involved in a conformational rearrangement at the active site of the HIV-1 intasome. A systematic RIN analysis demonstrated that communications between the residues in the resistant mutant are increased compared with those of the wild-type HIV-1 intasome. In addition, the chelating ability of the oxygen atoms in INSTIs (e.g., RAL and EVG) to Mg^{2+} in the active site of the resistant integrase was reduced due to conformational change and this is most likely responsible for the cross-resistance [37]. A computational analysis of the G118R and F121Y mutations, conferring high-level resistance to RAL, EVG, and DTG, showed that these substitutions were associated with reduced binding affinities to each of the INSTIs and with a decreased number of hydrogen bonds compared with the wild-type complexes [38]. These studies provide valuable information on the mechanism of resistance to INSTIs and will be useful for structure-based design of novel INSTIs that may possess superior resistance profiles compared to currently available drugs.

5 Transmission of Integrase Inhibitor-Resistant HIV Mutants

Transmitted drug resistance, i.e., the primary acquisition of an HIV variant already resistant to antiretrovirals, remains a challenge for all new infections. The first case of transmitted INSTI resistance was reported in an ART treatment-naïve man who harbored an INSTI-resistant and four-drug-class-resistant HIV-1 variant. The virus contained INSTI drug resistance substitutions at Q148H and G140S along with multiple RT and PI resistance mutations [39]. However, it was also recently reported that transmitted resistance to INSTIs is very rare. In a study on 1090 patient samples in California between March 2013 and June 2015, NNRTI resistance was found to be the most common, at a prevalence of 13 %, 23 %, and 10 % during the years 2013, 2014, and 2015, respectively. This was followed by prevalence of resistance to NRTIs, INSTIs, and PIs. Notably, no transmitted integrase inhibitor resistance was observed, even though INSTI resistance is slightly more common in treated persons than is PI resistance [40]. It is believed that failure to detect transmitted INSTI-resistant virus must be due to both low rates of treatment failure and low replication capacity of INSTI-resistant virus. The results are highly reassuring and further reinforce the potential benefits of INSTI-based treatments. Obviously INSTI resistance still needs to be evaluated in many more HIV-1-infected patients. As INSTIs become more widely used, continuous surveillance of primary INSTI resistance and monitoring of INSTI resistance transmission are needed in many more HIV-1-infected patients who are treated with INSTI-based regimens [41–43].

6 Conclusion and Perspectives

Although resistance mutations continue to be observed in vitro and in patients, INSTIs are highly potent drugs. DTG has a higher barrier to HIV drug resistance compared to RAL and EVG, and shows very limited cross-resistance compared to RAL and EVG. So far, no resistance mutations to DTG in treatment-naïve patients have been identified. In vitro cell culture selection with DTG has yielded only two mutations that confer low-level resistance to this drug, accompanied by a significant drop in viral replication capacity. No secondary compensatory mutations that might augment resistance and restore viral replication capacity have been observed in selection experiments in cell culture for more than 5 years. These results may explain the fact that viruses containing DTG resistance mutations are relatively replication impaired and may be unlikely to efficiently replicate in patients. If this is true, the development of low-level resistance to DTG in first-line therapy might not have adverse clinical consequences. Furthermore, it might make sense to use DTG in treatment as prevention (TasP) protocols to reduce viral load on a population level, as this may eventually result in diminished rates of HIV transmission [12, 27].

7 Future Research

It is conceivable that DTG might eventually be employed as monotherapy in treatment-naïve patients, since R263K-containing viruses are not able to replicate well and might not be able to survive in patients, due to impaired replication capacity. If DTG treatment regimens are interrupted, viruses might begin to replicate from the latent reservoirs as has been observed in other ART treatment interruption trials. However, re-initiation of DTG monotherapy might then convert these viruses into DTG-resistant attenuated forms, i.e., replication-impaired viruses. Logically, a number of cycles

of DTG treatment interruption followed by re-initiation of DTG monotherapy could conceivably convert all the HIV in the body, even that within latent reservoirs, to replication-impaired forms. As viral reservoirs decay over a number of cycles, wild-type viruses in reservoirs might not be able to rebound once interruption of ART takes place [44, 45]. It is speculated that this could conceivably provide an approach that might lead to a functional cure of HIV or remission of HIV disease.

HIV is able to develop mutations that confer resistance to all currently available antiretrovirals. Further studies and monitoring of HIV-1 resistance to INSTIs, both in vitro and in the clinic, are important. More sensitive assays are needed, such as next-generation sequencing for the detection of low-level viremia and minority resistance variants. Nonhuman primate models are important tools to study issues of drug resistance as well as the persistence and transmission of drug-resistant viruses [46, 47] and studies on DTG monotherapy in the SIV macaque model are urgently needed to support possible future clinical studies.

Acknowledgements Research performed in our laboratory is funded by the Canadian Institutes for Health Research.

References

1. Broder S. The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. Antiviral Res. 2010;85(1):1–18.
2. Wainberg MA, Zaharatos GJ, Brenner BG. Development of antiretroviral drug resistance. N Engl J Med. 2011;365(7):637–46.
3. Abdel-Magid AF. HIV integrase inhibitors for treatment of HIV infections and AIDS. ACS Med Chem Lett. 2014;5(2):102–3.
4. Mesplede T, Wainberg MA. Integrase strand transfer inhibitors in HIV therapy. Infect Dis Ther. 2013;2(2):83–93.
5. Park TE, et al. Review of integrase strand transfer inhibitors for the treatment of human immunodeficiency virus infection. Expert Rev Anti Infect Ther. 2015;13(10):1195–212.
6. Blanco JL, et al. HIV integrase inhibitors: a new era in the treatment of HIV. Expert Opin Pharmacother. 2015;16(9):1313–24.
7. White KL, Raffi F, Miller MD. Resistance analyses of integrase strand transfer inhibitors within phase 3 clinical trials of treatment-naive patients. Viruses. 2014;6(7):2858–79.
8. Quashie PK, Mesplede T, Wainberg MA. Evolution of HIV integrase resistance mutations. Curr Opin Infect Dis. 2013;26(1):43–9.
9. Grobler JA, Hazuda DJ. Resistance to HIV integrase strand transfer inhibitors: in vitro findings and clinical consequences. Curr Opin Virol. 2014;8c:98–103.
10. Geretti AM, Armenia D, Ceccherini-Silberstein F. Emerging patterns and implications of HIV-1 integrase inhibitor resistance. Curr Opin Infect Dis. 2012;25(6):677–86.
11. Mesplede T, Wainberg MA. Resistance against integrase strand transfer inhibitors and relevance to HIV persistence. Viruses. 2015;7(7):3703–18.
12. Wainberg MA, Han YS. Will drug resistance against dolutegravir in initial therapy ever occur? Front Pharmacol. 2015;6:90.
13. Osterholzer DA, Goldman M. Dolutegravir: a next-generation integrase inhibitor for treatment of HIV infection. Clin Infect Dis. 2014;59(2):265–71.
14. Dow DE, Bartlett JA. Dolutegravir, the second-generation of integrase strand transfer inhibitors (INSTIs) for the treatment of HIV. Infect Dis Ther. 2014;3:83–103.
15. Hurt CB, et al. Resistance to HIV integrase strand transfer inhibitors among clinical specimens in the United States, 2009–2012. Clin Infect Dis. 2014;58(3):423–31.
16. Fourati S, et al. Cross-resistance to elvitegravir and dolutegravir in 502 patients failing on raltegravir: a French national study of raltegravir-experienced HIV-1-infected patients. J Antimicrob Chemother. 2015;70(5):1507–12.
17. Mesplede T, Wainberg MA. Is resistance to dolutegravir possible when this drug is used in first-line therapy? Viruses. 2014;6(9):3377–85.
18. Cooper DA, et al. Subgroup and resistance analyses of raltegravir for resistant HIV-1 infection. N Engl J Med. 2008;359(4):355–65.
19. Mbisa JL, Martin SA, Cane PA. Patterns of resistance development with integrase inhibitors in HIV. Infect Drug Resist. 2011;4:65–76.
20. Cruciani M, Malena M. Combination dolutegravir-abacavir-lamivudine in the management of HIV/AIDS: clinical utility and patient considerations. Patient Prefer Adherence. 2015;9:299–310.
21. Taha H, Das A, Das S. Clinical effectiveness of dolutegravir in the treatment of HIV/AIDS. Infect Drug Resist. 2015;8:339–52.
22. Kobayashi M, et al. In vitro antiretroviral properties of S/GSK1349572, a next-generation HIV integrase inhibitor. Antimicrob Agents Chemother. 2011;55(2):813–21.
23. Eron JJ, et al. Safety and efficacy of dolutegravir in treatment-experienced subjects with raltegravir-resistant HIV type 1 infection: 24-week results of the VIKING Study. J Infect Dis. 2013;207(5):740–8.
24. Castagna A, et al. Dolutegravir in antiretroviral-experienced patients with raltegravir- and/or elvitegravir-resistant HIV-1: 24-week results of the phase III VIKING-3 study. J Infect Dis. 2014;210(3):354–62.
25. Cavalcanti Jde S, et al. High frequency of dolutegravir resistance in patients failing a raltegravir-containing salvage regimen. J Antimicrob Chemother. 2015;70(3):926–9.
26. Quashie PK, et al. Characterization of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir. J Virol. 2012;86(5):2696–705.
27. Mesplede T, et al. Viral fitness cost prevents HIV-1 from evading dolutegravir drug pressure. Retrovirology. 2013;10:22.
28. Cahn P, et al. Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naive adults with HIV: week 48 results from the randomised, double-blind, non-inferiority SAILING study. Lancet. 2013;382(9893):700–8.
29. Quashie PK, et al. Biochemical analysis of the role of G118R-linked dolutegravir drug resistance substitutions in HIV-1 integrase. Antimicrob Agents Chemother. 2013;57(12):6223–35.
30. Fantauzzi A, Mezzaroma I. Dolutegravir: clinical efficacy and role in HIV therapy. Ther Adv Chronic Dis. 2014;5(4):164–77.
31. Wainberg MA, Mesplede T, Raffi F. What if HIV were unable to develop resistance against a new therapeutic agent? BMC Med. 2013;11:249.
32. Hightower KE, et al. Dolutegravir (S/GSK1349572) exhibits significantly slower dissociation than raltegravir and elvitegravir from wild-type and integrase inhibitor-resistant HIV-1 integrase-DNA complexes. Antimicrob Agents Chemother. 2011;55(10):4552–9.
33. Grobler JA, Stillmock KA, Miller MD, Hazuda DJ. Mechanism by which the HIV integrase active site mutation N155H confers resistance to raltegravir. In: XVII HIV drug resistance workshop, Sitges, Spain.
34. Hare S, et al. Molecular mechanisms of retroviral integrase inhibition and the evolution of viral resistance. Proc Natl Acad Sci U S A. 2010;107(46):20057–62.

35. Hare S, et al. Structural and functional analyses of the second-generation integrase strand transfer inhibitor dolutegravir (S/GSK1349572). Mol Pharmacol. 2011;80(4):565–72.
36. DeAnda F, et al. Dolutegravir interactions with HIV-1 integrase-DNA: structural rationale for drug resistance and dissociation kinetics. PLoS One. 2013;8(10):e77448.
37. Xue W, et al. Exploring the molecular mechanism of cross-resistance to HIV-1 integrase strand transfer inhibitors by molecular dynamics simulation and residue interaction network analysis. J Chem Inf Model. 2013;53(1):210–22.
38. Malet I, et al. New raltegravir resistance pathways induce broad cross-resistance to all currently used integrase inhibitors. J Antimicrob Chemother. 2014;69(8):2118–22.
39. Young B, et al. Transmission of integrase strand-transfer inhibitor multidrug-resistant HIV-1: case report and response to raltegravir-containing antiretroviral therapy. Antivir Ther. 2011;16(2):253–6.
40. Volpe JM, Walworth CM, et al. Absence of integrase inhibitor resistant HIV-1 transmission in the California AIDS healthcare foundation network. In: ICAAC 2015 conference, San Diego, 2015. Abstract no LB3389.
41. Casadella M, et al. Primary resistance to integrase strand-transfer inhibitors in Europe. J Antimicrob Chemother. 2015;70(10):2885–8.
42. Jeanfreau K, et al. Prevalence of integrase strand inhibitor resistance (INSTI)in New Orleans, Louisiana, 2012–2013. J La State Med Soc. 2015;167(1):19–20.
43. Stekler JD, et al. Lack of resistance to integrase inhibitors among antiretroviral-naive subjects with primary HIV-1 infection, 2007–2013. Antivir Ther. 2015;20(1):77–80.
44. Buzon MJ, et al. Long-term antiretroviral treatment initiated at primary HIV-1 infection affects the size, composition, and decay kinetics of the reservoir of HIV-1-infected CD4 T cells. J Virol. 2014;88(17):10056–65.
45. Hocqueloux L, et al. Long-term antiretroviral therapy initiated during primary HIV-1 infection is key to achieving both low HIV reservoirs and normal T cell counts. J Antimicrob Chemother. 2013;68(5):1169–78.
46. Hassounah SA, et al. Characterization of the drug resistance profiles of integrase strand transfer inhibitors in SIVmac239. J Virol. 2015;89:12002–13.
47. Wares M, et al. Simian-tropic HIV as a model to study drug resistance against integrase inhibitors. Antimicrob Agents Chemother. 2015;59(4):1942–9.

The Hepatitis B Virus and Antiviral Drug Resistance: Causes, Patterns and Mechanisms

38

Stephen A. Locarnini

1 Introduction

The hepatitis B virus (HBV) is a DNA-containing virus that belongs to the family Hepadnaviridae. Selection of HBV quasispecies with mutations in the viral reverse transcriptase (rt) during antiviral therapy can result in progression of liver disease and, in some cases, significant clinical deterioration. The development of antiviral drug resistance depends on a number of parameters such as the magnitude and rate of viral replication, the fidelity of the viral polymerase, the selective pressure of the drug, the amount of replication space in the liver and the fitness of the resistant virus. Thus, in the treatment of chronic hepatitis B, the development of drug resistance is not unexpected if viral replication continues in the setting of ongoing therapy. Prevention of resistance requires the adoption of strategies that effectively control virus replication, and in its simplest form this is using antiviral drugs that are potent and have a high genetic barrier, namely entecavir or tenofovir. This chapter briefly reviews the major aspects of the molecular virology and replication of HBV and summarises the viral mutants of clinical significance that are associated with drug resistance. Also, the factors and mechanisms of drug resistance in hepatitis B are discussed. Finally, strategies to prevent the emergence of drug resistance are addressed.

2 Background

The hepatitis B virus (HBV) is a DNA-containing virus (Fig. 38.1a, b) and belongs to the family *Hepadnaviridae*. Under normal circumstances, viral infection and subsequent replication within the hepatocyte do not result directly in cell death. The inability of the host's immune response to clear HBV from infected hepatocytes within the liver and the subsequent inappropriate attempts at immune clearance are the basis for the ensuing progressive liver disease. Since most patients once chronically infected do not resolve their infection, the course and clinical outcome of chronic hepatitis B (CHB) infection are determined by the generation and selection of viral escape mutants. Serial unsuccessful attempts by the host's immune response to clear wild-type and escape mutants of HBV from infected hepatocytes lead to a cycle of ongoing necroinflammation and viral replication resulting in the liver damage recognised as CHB [1]. The emergence of these 'immune-escape' mutants as dominant populations during active HBV replication may have important consequences for the severity of disease [2–7], such as hepatitis Be antigen (HBeAg)-negative CHB. Similarly, selection of HBV quasispecies with mutations in the viral reverse transcriptase (rt) during antiviral therapy can result in further progression of liver disease and, in some cases, significant clinical deterioration [8, 9].

The hepatitis B virus utilizes reverse transcription of the pregenomic RNA intermediate, to copy its DNA, thereby generating mutant viral genomes at a much higher rate than other DNA viruses. Particular selection pressures, both endogenous (host immune clearance via innate and adaptive responses) and exogenous (vaccines and antivirals), readily select out these escape mutants. Not surprisingly then, the introduction of nucleoside/nucleotide analogue (NA) therapy has resulted in the emergence of primary antiviral drug resistance to every approved agent, except tenofovir, thereby limiting drug efficacy [10, 11]. Factors determining treatment failure include patient compliance, drug regimen potency and the drug's inherent genetic barrier to resistance. The development of antiviral drug resistance depends on a number of parameters such as the magnitude and rate of viral replication, the fidelity of the viral polymerase, the selective pressure of the drug, the amount of replication space in the

S.A. Locarnini, MBBS, BSc, (Hon), PhD, FRCPath. (✉)
Research and Molecular Development, Victorian Infectious Diseases Reference Laboratory, Doherty Institute,
Level 2, 792 Elizabeth Street, Melbourne, VIC 3000, Australia
e-mail: Stephen.Locarnini@mh.org.au

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_38

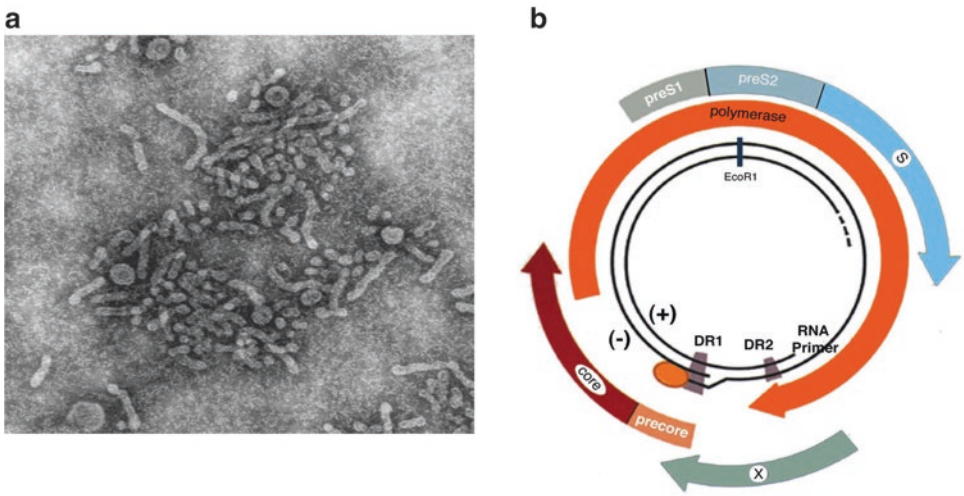


Fig. 38.1 (**a**) Electron micrograph of the HBV virons (42 nm), filamentous structures and 22 nm small particles of HBsAg can be seen. (**b**) The genetic organisation of the HBV genome. The four major open reading frames are shown (see text). The nicked and gapped molecules of DNA are held in a relaxed circular (RC) arrangement by the direct repeat 1 (DRI) and DR-2 regions

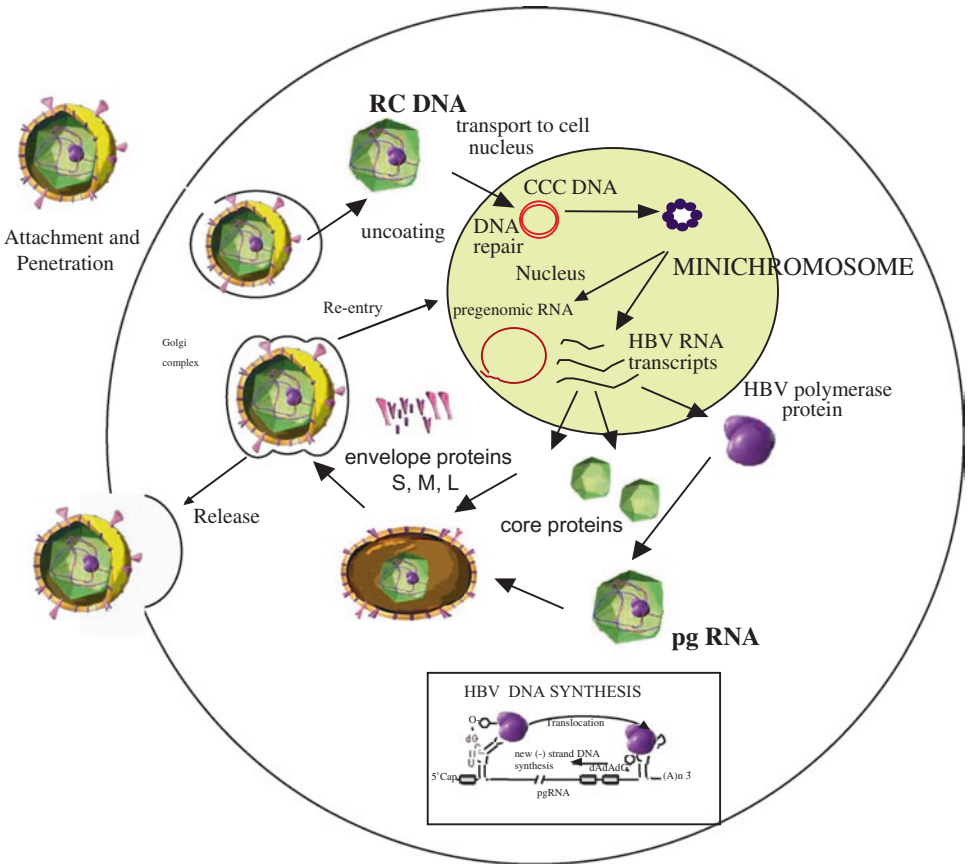


Fig. 38.2 HBV life cycle. The major processes involved in (HBV) genome replication: conversion of replication complex (RC) DNA, reverse transcription of cccDNA to produce pregenomic RNA(pgRNA), reverse transcription of pgRNA to make minus HBV DNA and HBV DNA polymerase activity to make the RC DNA, completing the cycle

liver and the fitness of the resistant virus [10, 11]. Thus, in the treatment of chronic hepatitis B, the development of drug resistance is not unexpected if viral replication continues in the setting of ongoing treatment. Prevention of resistance will require the adoption of strategies that effectively control virus replication.

3 Molecular Virology and Life Cycle (Fig. 38.2)

HBV is distantly related to the retroviruses and replicates its genome by the reverse transcription of an RNA intermediate, referred to as pregenomic RNA (pgRNA) (see Fig. 38.2).

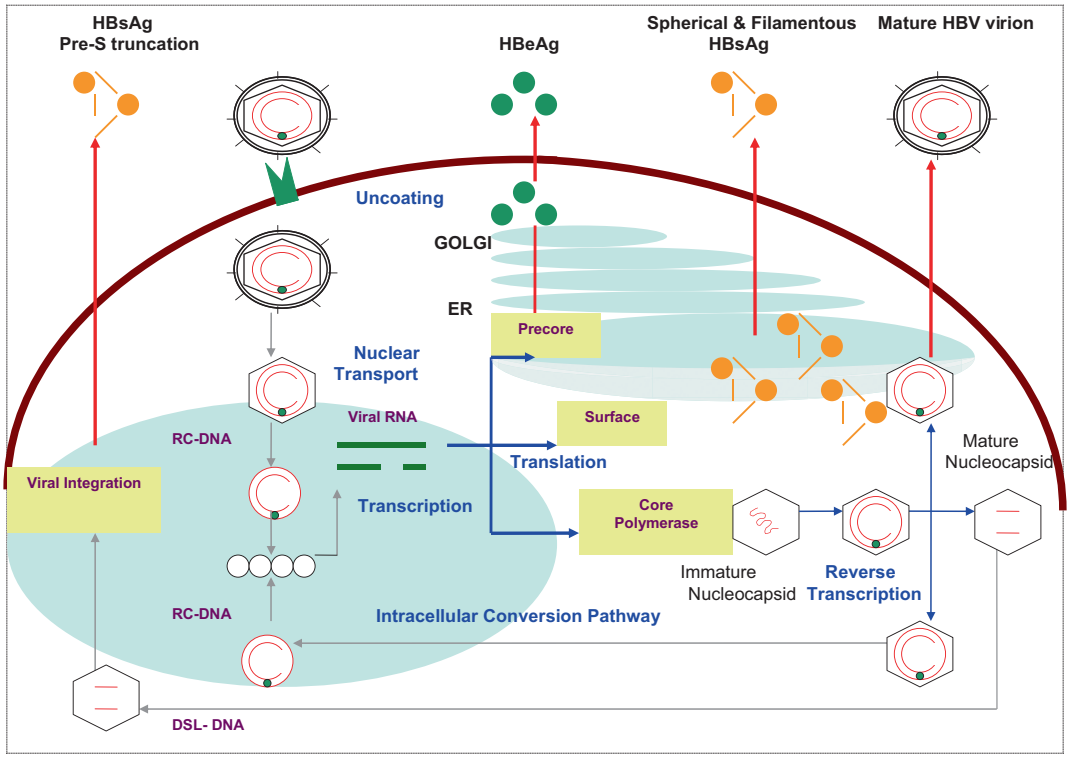


Fig. 38.3 An overview of HBV replication highlighting cccDNA generation and processing and reverse transcription and yield progeny HBV. Also shown is the DSL HBV DNA pathway leading to HBV DNA integration which can be associated with extra HBsAg production

The 3.2 kb double-stranded DNA HBV genome is organised into four overlapping and frame-shifted open reading frames (ORFs) (Fig. 38.1b). The longest of these encodes the viral polymerase (Pol ORF). The second ORF, referred to as the envelope ORF, encodes the viral surface proteins and is located within the Pol ORF but in a frame-shifted manner. Two smaller ORFs that encode the precore/core proteins and the X protein partially overlap the Pol ORF. The viral life cycle of HBV has been well characterised [9] and the detailed molecular steps are shown in Fig. 38.3.

3.1 Attachment, Penetration and Uncoating

The first stage of infection involves attachment to a susceptible hepatocyte and the penetration of HBV into the cell cytoplasm following the binding of the HBV envelope to its specific cellular high affinity the sodium taurocholate cotransporting polypeptide (NTCP) [12], and the low-affinity binding to heparan sulphate proteoglycans (HSPG) on the hepatocyte surface [13]. The subsequent events of penetration and uncoating are not well defined but it has been assumed by most investigators that a process of receptor-mediated endocytosis is responsible for delivery of the DNA-containing cores to inside the cell.

3.2 Conversion of Genomic RC DNA into cccDNA and Transcription of the Viral Minichromosome

Following viral penetration and envelope uncoating, the cytoplasmic viral nucleocapsids are transported to the nuclear membrane, where they uncoat [14]. The genomic relaxed circular [15] DNA (RC DNA in Figs. 38.2 and 38.3) is released into the nucleus and then converted into covalently closed circular DNA (cccDNA) using a number of host cell enzymes, resulting in the formation of the viral minichromosome, the major template of HBV that is used for the transcription of all the viral mRNAs involved in viral protein production and replication [16, 17].

Using this transcriptional template, five major unspliced RNA species, two of 3.5 kb, and one each of 2.4, 2.1 and 0.7 kb, are generated (Fig. 38.3). The transcripts can be classified into two classes: subgenomic and genomic [18]. Both classes contain heterogeneous transcripts that are of positive orientation, are capped at the 5′ end and are polyadenylated at the 3′ end. The synthesis of these transcripts is controlled by the enhancer II/basal core (BCP), large surface antigen (Pre-S1), major surface antigen (S) and enhancer I/X gene promoters [18].

The smaller, subgenomic transcripts, which measure 2.4, 2.1 and 0.7 kb, function exclusively as mRNAs for the

translation of the viral envelope proteins (Pre-S1, Pre-S2 and S) and the accessory protein, X (Fig. 38.3). The 2.4 and 2.1 kb mRNAs translate the large (Pre-S1), middle (Pre-S2) and small (S) envelope proteins. Both the Pre-S2 and S envelope proteins are translated from the 2.1 kb mRNAs. The Pre-S1 is translated from the 2.4 kb transcript and is required for the formation of the virions as well as the filamentous forms of the hepatitis B surface antigen (HBsAg). The S protein forms the small 22 nm spherical particles of HBsAg. The 0.7 kb mRNA translates the X protein, a modest transactivator of transcription that also appears to have a regulatory function in viral replication [19]. The X protein is regarded as an accessory protein of HBV.

The greater than genomic transcripts measure 3.5 kb, are greater than one genome in length and serve as the pgRNA and precore RNAs. The pgRNA encodes the viral nucleocapsid (core protein, HBcAg), and the HBV polymerase (Pol), and also acts as a template for reverse transcription. The precore RNA is slightly longer than the pgRNA at the 5′ end and encodes the second accessory protein of the HBV, HBeAg.

3.3 Viral Reverse Transcription

The process of reverse transcription used by HBV to convert its pgRNA into double-stranded DNA (dsDNA) has been extensively reviewed [20–22]. Reverse transcription is initiated upon binding of the viral polymerase to the encapsidation signal (epsilon) on the pgRNA. This then signals the binding of core protein dimers to form nucleocapsids and the basic replication complex of the HBV genome. A series of interactions including the involvement of host chaperone proteins results in the synthesis of minus-strand DNA strand, followed by positive-strand synthesis and circularisation of the genome [18]. The viral envelope, the small particles and the filamentous forms are synthesised and assembled at the endoplasmic reticulum (ER) membranes and then bud into its lumen. The HBcAg protein is synthesised in the cytosol and assembled independently [20] of the enveloped proteins [18, 20–23].

3.4 Assembly and Release

The assembly of nucleocapsids containing mature relaxed circular DNA (RC DNA) occurs in the cytosol, and these nucleocapsids are selectively enveloped before exiting the cell [18]. Minus-strand DNA synthesis appears to be coupled to phosphorylation of the nucleocapsid (replication complex), which is required for envelopment to occur (see Figs. 38.2 and 38.3). Incomplete dsDNA/RNA genomes that have completed minus-strand DNA synthesis and at least started plus-strand synthesis can readily be found in the blood as secreted virions. Also, failure to translocate the (+) strand primer for second-strand synthesis results in the formation of a double-stranded linear (DSL) intermediate which has been linked to an integration precursor of HBV [24] (see Fig. 38.3). However, it should be noted that integration is not required for a productive viral life cycle but is associated with an increased liver cancer risk [25].

3.5 Replication and Diversity of HBV Genomes

The unique replication strategy of HBV provides it with at least two selective advantages. First, the HBV cccDNA minichromosome that acts as the major transcriptional template for the virus is very stable. Second, the error-prone HBV reverse transcriptase generates a high rate of mutations, resulting in a population of viral quasispecies. The high mutation rate of HBV rt has resulted in substantial diversity in the nucleotide sequence of HBV. Currently, ten major genotypes, A through to J, have been identified based on nucleotide (nt) diversity of ≥8 % at the whole-genome level [26, 27]. These genotypes typically have a distinct global geographic distribution with A and D mainly found in Europe and North America, B and C in Asia and F and H in Latin America, and E in Africa. This geographical clustering is now starting to merge reflecting the substantial population migrations that have occurred from Africa and Asia over the last 50–100 years.

4 Antiviral Drug Resistance

Antiviral drug resistance reflects the reduced susceptibility of a virus to the inhibitory effect of a drug, and results from a process of adaptive mutations under the selection pressure of antiviral therapy. Approved and/or available medications for CHB include lamivudine (LMV), a synthetic deoxycytidine analogue with an unnatural L-conformation, and related L-nucleosides, including emtricitabine (FTC) and telbivudine (LdT). A second group of nucleos(t)ide analogues is the acyclic phosphonates which include adefovir dipivoxil (ADV), a prodrug for the acyclic 2′-deoxyadenosine monophosphate (dAMP) analogue adefovir, and the structurally similar tenofovir disoproxil fumarate (TDF), which is also approved for the treatment of patients with the human immunodeficiency virus (HIV) infection. A third group of agents that is also approved for the therapy of CHB which contains a cyclopentane/cyclopentene sugar moiety and includes the most potent anti-HBV drug discovered to date is the deoxyguanosine analogue, entecavir (ETV) [28].

Two types of mutations have been identified that have been associated with treatment failure for these agents:

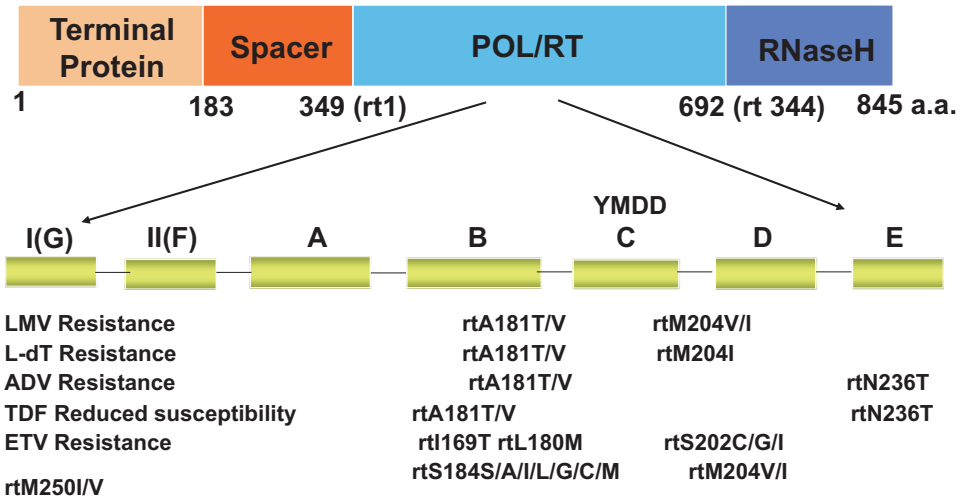


Fig. 38.4 The location of major drug resistance mutations on the HBV polymerase. According to convention and for consistent identification of mutations conferring resistance to antiviral nucleos(t)ide analogues, amino acids are numbered from the beginning of the Pol/RT (rt1–rt344) domain [30]. Mutations associated with resistance to lamivudine (LMV), telbivudine (LdT), adefovir (ADV), tenofovir (TDF) and entecavir (ETV) are indicated

Table 38.1 Annual prevalent resistance rates for lamivudine, adefovir, entecavir, emtricitabine and telbivudine

	Resistance at year of therapy expressed as percentage of patients				
Drug	1	2	3	4	5
Lamivudine[a]	23	46	55	71	80
Adefovir[b]	0	3	6	18	29
Entecavir[c] (naïve)	0.1	0.4	1.1	–	–
Entecavir[c] (LAM resistant)	6	14	32	–	–
Emtricitabine[c]	9–16	19–37	–	–	–
Telbivudine[d]	4[d]	–	–	–	–
Tenofovir	0	0	0	0	0

[a]Modified and updated from Lai et al. [37], and Leung et al. [87]
[b]From Locarnini et al. [88]
[c]From Perrillo et al. [89], Colonno et al. [48]
[d]In the LAM comparator arm, the percentage was only 8 % based on a complex case definition of antiviral drug resistance/treatment failure. One would thus expect a comparable relative level of 10–12 % based on genotypic resistance compared with lamivudine (25 % per annum)

primary resistance mutations (Fig. 38.4), which are directly responsible for the associated drug resistance, and secondary or compensatory mutations, which probably occur in order to promote or enhance replication competence. Compensatory mutations emerge because the selection of genetic resistance is usually associated with some cost in replication fitness for the virus. Compensatory mutations are important as they 'fix' the discriminatory primary drug-resistant mutations into the genetic archive of the HBV minichromosome, thus providing quasispecies memory [29].

4.1 Lamivudine Resistance-Associated Mutations (L-Nucleosides)

Antiviral resistance to LMV has been mapped to the YMDD locus in the catalytic or C domain (Fig. 38.4) of HBV Pol [30]. The primary resistance mutations within the Pol gene that have been selected during LMV therapy are designated rtM204I/V/S (domain C)+/−rtL180M (domain B) [31]. Other primary mutations include rtA181T/V [32] (Fig. 38.4). Compensatory mutations can be found in other domains of the HBV Pol, such as rtL80V/I [33], rtI169T [34], rtV173L [35], rtT184S/G, rtS202I and rtQ215S [36], that enhance viral replication levels.

Lamivudine resistance increases progressively during treatment at rates of 14–32 % annually, exceeding 70 % after 48 months of treatment [37] (Table 38.1). Factors that increase the risk of development of resistance include high pretherapy serum HBV DNA and ALT levels and the incomplete suppression of viral replication [37, 38]. The main LMV resistance mutations rtM204V/I do not confer cross-resistance to ADV (Table 38.2), but the rtA181T/V does [36]. The rtI169T, rtT184S/G and rtS202I contribute to entecavir resistance [34] (Fig. 38.4). The rtM204V/I is cross-resistant with all other L-nucleoside analogues tested such as

Table 38.2 Pathways of antiviral resistance in chronic hepatitis B

Pathway	Primary resistance mutation	Associated resistance
L-nucleoside	rtM204V/I	Lamivudine (LMV)
		Emtricitabine (FTC)
		Telbivudine (LdT)
Acyclic phosphonate	rtN236T	Adefovir (ADV)
		Tenofovir (TFV)
"Shared"	rtA181T/V	L-nucleosides (see above)
		Acyclic phosphonates (see above)
Double	rtA181T/V + rtN23T	L-nucleosides
		Acyclic phosphonates (see above)
Naïve entecavir resistance	rtL180M + rtM204V with one of rtI169T, rtT184, rtS202 or rtM250	Entecavir (ETV)
Multidrug resistance	Complex patterns, e.g. rtA181T + rtN236T + rtM250L	Multidrug

Modified from Zoulim and Locarnini [10, 11]

emtricitabine (FTC) and telbivudine (LdT) (see Table 38.2 and Fig. 38.4).

Mutations that confer LMV resistance decrease in vitro sensitivity to LMV from at least 100-fold to >1000-fold. The rtM204I substitution has been detected in isolation, but rtM204V and rtM204S are found only in association with other changes in the A or B domains [39]. Five common patterns of resistance have been identified and include (1) rtM204I, (2) rtL180M + rtM204V, (3) rtL180M + rtM204I, (4) rtV173L + rtL180M + rtM204V and (5) rtL80V/I ± rtL180M + rtM204I. The dominance of particular patterns tends to be influenced by the HBV genotype [40]. The molecular mechanism of LMV resistance is steric hindrance caused by the β-branched side group of the valine or isoleucine amino acids colliding with the oxathiolane ring of LMV with the deoxynucleotide triphosphate (dNTP)-binding site [41]. This results in a >100-fold increase in EC_{50}.

4.2 Adefovir Dipivoxil and Tenofovir Resistance-Associated Mutations (Acyclic Phosphonates)

Resistance to ADV was initially associated with mutations in the B (rtA181T) and D (N236T) domains of the enzyme [42] (Fig. 38.4). HBV resistance to ADV occurs less frequently than resistance to LMV, with a prevalence of around 2–3 % after 2 years, 4–6 % after 3 years, 18 % after 4 years and 29 % after 5 years [43] (Table 38.1). These ADV-associated mutations in HBV Pol result in only a modest (3–8-fold) increase in the concentration of the drug required for 50 % inhibition for viral replication in vitro (EC_{50}), and are partially cross-resistant with TDF, probably because the molecular mechanism of resistance may be similar in both with indirect perturbation of the triphosphate-binding site between the A and D domains being proposed [41, 44]. The rtN236T does not significantly affect sensitivity to LMV [42], but the rtA181T/V changes are partially cross-resistant to LMV (Fig. 38.4). Recently, another mutation (rtI233V) mapped to the reverse transcriptase domain has been identified that was claimed to confer resistance to ADV [45]. In clinical studies, the rtI233V mutation occurred in approximately 2 % of all patients with CHB [45, 46]. However, the final significance of this mutation will need independent confirmation since other groups have not found an association between the rtI233V and ADV resistance [47].

To date, no primary resistance-associated mutations to TDF have been found; when patients are switched to TDF after ADV failure due to resistance (reA181T/V +/− rtN236T), the virological response is slow and regarded as reduced sensitivity [10, 11].

4.3 Entecavir Resistance-Associated Mutations (Cyclopenta(e)ne Sugar)

Resistance to ETV has been observed in patients who are naïve to therapy [48] and are also LMV resistant [34]. Mutations in the viral polymerase associated with the emergence of ETV resistance were mapped to the B domain (rtI169T, rtL180M and/or rtS184G), C domain (rtS202I and rtM204V) and E domain (rtM250V) of HBV Pol (Fig. 38.4 and Table 38.2). In the absence of LMV-associated mutations, the rtM250V causes a ninefold increase in IC_{50}, whereas the rtT184G + rtS202I changes have only a modest effect [34, 49–53]. The mechanism of ETV resistance for the rtT184G + rtS202I is an allosteric change with altered geometry of the nucleotide-binding pocket and DNA template binding of the polymerase near the YMDD site [53]. The molecular mechanism of resistance for the rtM250V change is thought to be an alteration of the binding interaction between the DNA primer strand and DNA template strand with the incoming dNTP [53].

Recent clinical experience with ETV failure has indicated that at least three mutations, rtL180M ⊕ rtM204V and either

rtT184G/S or rtS202I, are required in the HBV Pol for ETV resistance to develop (Fig. 38.4). This accounts for the low rate of resistance in treatment-naïve patients after 1 year (0.1 %), 2 years (0.4 %) and 3 years (1.1 %) of ETV monotherapy (Table 38.1). In contrast, in LMV-experienced patients, it should be noted that as well as rtL180M and rtM204V/I, changes at codon 184 occur in 4.5 % of patients and the frequency of ETV genotypic resistance changes in LMV-experienced patients is 6 % (year 1), 14 % (year 2) and 32 % (year 3) (see Table 38.1). In this group, viral breakthrough as well as genotypic resistance occur in 1 % (year 1), 10 % (year 2) and 25 % (year 3) [54] of patients.

4.4 Multidrug Resistance

Recently, multidrug-resistant HBV has been reported in patients who have received sequential treatment with NA monotherapies [34, 55–58]. The development of multidrug resistance will certainly have implications on the efficacy of rescue therapy, as in the case of multidrug-resistant human immunodeficiency virus [59, 60]. Successive evolution of different patterns of resistance mutations has been reported during long-term LMV monotherapy [32, 61]. The isolates of HBV with these initial mutations appear to be associated with decreased replication fitness compared with wild-type HBV; however, additional mutations that can restore replication fitness are frequently detected as treatment is continued [35, 62].

A study by Yim et al. [63], characterised multidrug-resistant HBV in more detail in six patients receiving alternating monotherapies, typically LMV and ADV. Using conventional cloning techniques with subsequent PCR sequencing, the majority of the clones sequenced (85 %) had mutations to both therapies on the same genome. The remainder had LMV-resistant clones only. In three of the patients, analysis of successive samples revealed progressive evolution from the clones with LMV-resistant HBV mutations only to mixtures of clones that had multidrug-resistant mutations. These studies strongly support the role for combination therapy in managing patients with CH-B [64] (see below).

4.5 Summary

The paradigm of antiviral therapy is the suppression and maintenance of viremia below the limit of detection. Emergence of resistance is heralded by an increasing HBV DNA viral load (≥1.0 log IU/mL), identification of known genotypic markers of drug resistance within the polymerase (Fig. 38.4), increasing serum ALT levels and finally clinical deterioration. These events are summarised in Fig. 38.5. The pathways of antiviral resistance in chronic hepatitis B are summarised in Table 38.2 and cross-resistance profiles are summarised in Table 38.3.

5 Why HBV Antiviral Drug-Resistant Mutants Are Selected

Antiviral drug resistance depends on at least five factors: (1) magnitude and rate of virus replication, (2) the fidelity of the viral polymerase, (3) selective pressure of the drug, (4) amount of replication space in the liver and (5) replication fitness of the drug-resistant virus, and these are discussed below.

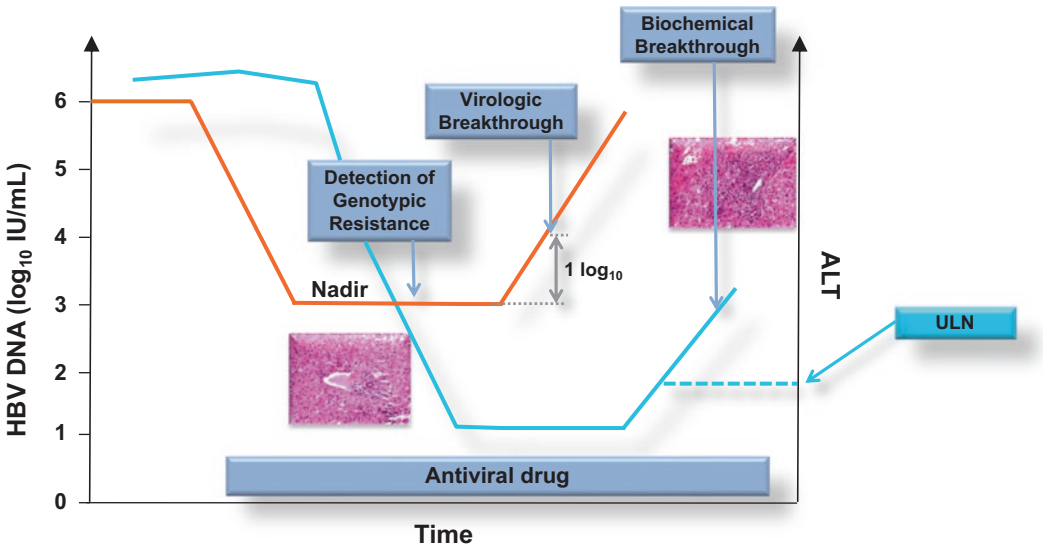


Fig. 38.5 A typical patient undergoing HBV drug resistance. The sequence of events: detection of genotypic resistance, virological breakthrough and then biochemical breakthrough are highlighted. Modified from Locarnini et al. [67] and Zoulim and Locarnini [10]

Table 38.3 Pathways of antiviral resistance in chronic hepaitis B

Pathway	Primary resistance mutation	Associated resistance
L-nucleoside	rtM204V/I	Lamivudine (LMV)Emtricitabine (FTC)Telbivudine (LdT)
Acyclic phosphonate	rtN236T	Adefovir (ADV)Tenofovir (TFV)
"Shared"	rtA181T/V	L- nucleosides (see above)Acyclic phosphonates (see above)
Double	rtA181T/V + rtN23T	L-nucleosidesAcyclic phosphonates (see above)
Naïve entecavir resistance	rtL180M + rtM204V with one of rtI169T, rtT184, rtS202 or rtM250	Entecavir (ETV)
Multi-drug resistance	Complex patterns e.g. rtA181T + rtN236T + rtM250L	Multi-drug

Modified from Zoulim and Locarnini [1, 2]

5.1 Magnitude and Rate of Virus Replication

The natural history of CHB is highly variable, but generally can be divided into four phases: immune tolerant (high replicative), immune elimination (intermediate replicative), non-replicative phase and a 'reactivation phase' generally associated with the development of HBeAg-negative CHB [43, 65]. During the HBeAg-positive immune-tolerant phase there is a very high daily virion production of virions, approximately $10^{12–13}$ [66], whilst over the remaining phases of CHB, the HBV replication rate is considered to be approximately 10^{11} virions per day [66]. This substantial daily production coupled with the mutational frequency of the HBV Pol (see below) equates to at least 10^{10} point mutations produced per day in individuals who have a high level of replication. HBV genomes typically contain approximately 3200 base pairs; thus all possible single-base changes can be produced each day [67]. HBV thus exists in an infected individual as different populations of HBV called quasispecies. However, the organisation of the ORFs into a frame-shifted overlapping arrangement within the HBV genome does place some restriction on the final number of viable mutants that are actually generated. The stability of the predominate HBV within the quasispecies pool is maintained by particular selection pressures from the host's innate and adaptive immune system and viability and replication competence of the selected virus.

5.2 Fidelity of the Viral Polymerase

The HBV mutation frequency has been estimated to be approximately $1.4–3.2 \times 10^{-5}$ nucleotide substitutions per site per year [68, 69]. This rate is approximately tenfold higher than that for other DNA viruses and more in keeping with the RNA viruses such as retroviruses. Unlike cellular polymerases, the HBV Pol is a reverse transcriptase that lacks proofreading function. As discussed above, the mutation rate of HBV is also influenced by the clinical phase of the patient, such as whether the patient is in the immune-tolerant phase (low error rate) or the immune-elimination phase (higher rate), HBeAg-negative CHB, and by clinical settings such as immunosuppression and transplantation [31]. Thus, prior to antiviral therapy, because of the quasispecies pool, variants carrying single and double mutations potentially associated with drug resistance pre-exist [70].

5.3 Selective Pressure of the Drug

The probability of a mutation associated with drug resistance being selected out during therapy depends on the efficacy of that drug; the probability has been depicted graphically as a bell-shaped curve [71]. Hence, a drug with low antiviral activity does not exert significant selection pressure on the virus and the risk of drug resistance emerging is not high. Conversely, complete suppression of viral replication allows almost no opportunity for resistance to emerge because as highlighted above, mutagenesis is replication dependent [67]. Because monotherapies exert varying degrees of antiviral activity directed at one single target site, they result in the highest probability of selecting for drug resistance. The ideal treatment regimen exerts antiviral activity targeted at different sites in the viral life cycle to reduce the risk significantly of selecting drug-resistant quasispecies. Resistance emerges when replication occurs in the presence of drug-selection pressure. The corollary of this is that 'no replication' translates into 'no resistance'.

5.4 Amount of Replication Space in the Liver

Replication space for HBV has been described as the potential of the liver to accommodate new transcriptional templates or molecules of cccDNA [24, 72]. This indicates that the eventual takeover by a mutant virus is dependent upon

the loss of the original wild-type virus, and is governed by factors such as replication fitness as well as the turnover and proliferation of hepatocytes [24, 72]. Hepatocyte turnover in the normal liver is slow displaying a typical half-life of over 100 days [66]. This can be reduced to less than 10 days in the setting of increased necroinflammatory activity or associated toxicity [66]. In a fully infected liver, synthesis of new HBV cccDNA molecules can only occur if uninfected cells are generated by normal growth within the liver, hepatocyte proliferation and turnover or loss of wild-type (dominant) cccDNA from existing infected hepatocytes [73, 74]. The enrichment of one species over another suggests that the expanding virus has augmented its population through an expansion of cccDNA synthesis [73, 74]. In other words, the expansion of a (drug-) resistant mutant in the infected liver can be possible only with the creation of new replication space [74].

5.5 Replication Fitness of the Drug-Resistant Virus

Replication fitness has been defined as the ability to produce offspring in the setting of natural selection [70]. This is not a yield measurement of viral replication, and can be measured using in vitro coinfection competition assays. Unfortunately, this cannot be conveniently done with HBV because of the lack of a suitable cell culture system for viral infectivity that supports such competitive coinfection experiments. The recent identification of the NTCP as the receptor for HBV [12] should change this.

Several clinical observations demonstrate the fitness of lamivudine-resistant HBV. Thibault et al. [75] were the first to document the transmissibility of LMV-resistant HBV from patient to patient [75]. Several groups have described the persistence of LMV-resistant HBV as co-dominant quasispecies with wild-type HBV post-treatment for at least 3 months [76], or as a minor quasispecies with wild-type HBV post-treatment for almost 1 year [77].

5.6 Other Factors

Host factors affecting antiviral therapy include previous drug experience, compliance, host genetic factors (e.g. inborn errors of metabolism) and the ability to efficiently convert the nucleos(t)ide analogue to its active metabolite via several intracellular phosphorylations (intrahepatic salvage enzymes) [78, 79]. In addition, there are sequestered sites/sanctuaries of viral replication that may not be accessible to the antiviral agent, and HBV replicative intermediate, and as the cccDNA form is typically recalcitrant to conventional therapy [17, 80].

6 Strategies to Overcome Resistance

Currently, interferon, tenofovir and entecavir can all be considered as first-line therapy for individuals with noncirrhotic liver disease [81]. In the context of rescue or salvage therapy, mutations that confer resistance to lamivudine confer cross-resistance to other L-nucleosides including telbivudine, and reduce sensitivity to entecavir but not to adefovir or tenofovir (Tables 38.2, 38.3, and Fig. 38.4). Generally, mutants that are resistant to adefovir and tenofovir remain sensitive to L-nucleosides and entecavir (Tables 38.2 and 38.3). Multiple mutations are required for high-level resistance to entecavir (Tables 38.2 and 38.3) [34, 54]. The lower risk of resistance to tenofovir and entecavir (Table 38.1) supports their use in high-risk situations such as pre-emptive immunosuppressive therapy, in liver transplantation patients and in patients with cirrhosis or decompensated liver disease, given that development of drug resistance is more likely to precipitate clinical deterioration in these individuals [67]. The complete lack of primary resistant associated changes to tenofovir strongly supports the use of this drug in this setting unless there is a renal or other contraindication.

7 Public Health Implications of the Polymerase Envelope Genes Overlap

The polymerase gene overlaps the envelope gene completely and changes in the HBV Pol selected during antiviral resistance can cause concomitant changes to the overlapping reading frame of the envelope (see Fig. 38.6). Thus, the major resistance mutations associated with LMV, LdT, ADV and ETV-failure would also have the potential of altering the C-terminal region of HBsAg (see Table 38.4). For example, changes associated with LMV resistance such as the rtM204V result in a change at sI195M in the surface antigen, whilst the rtM204I change is associated with three possible changes, sW196S, sW196L or a termination codon. To date, there has been only one published study that has examined the effect of the main LMV resistance mutations on the altered antigenicity of HBsAg [82]. One of the common HBV quasispecies that is selected during LMV treatment is rtV173L + rtL180M + rtM204V that result in change in the HBsAg at sE164D + sI195M. Approximately 20 % of HIV–HBV-coinfected individuals [83] and 10 % of mono-infected individuals encode this ‘triple-Pol mutant’ [35]. In binding assays, HBsAg expressing these LMV-resistant associated residues had reduced anti-HB binding [82]. This reduction was similar to the classical vaccine escape mutant, sG145R. This variant was later shown to superinfect vaccine-immunised chimpanzees [84], establishing its public health

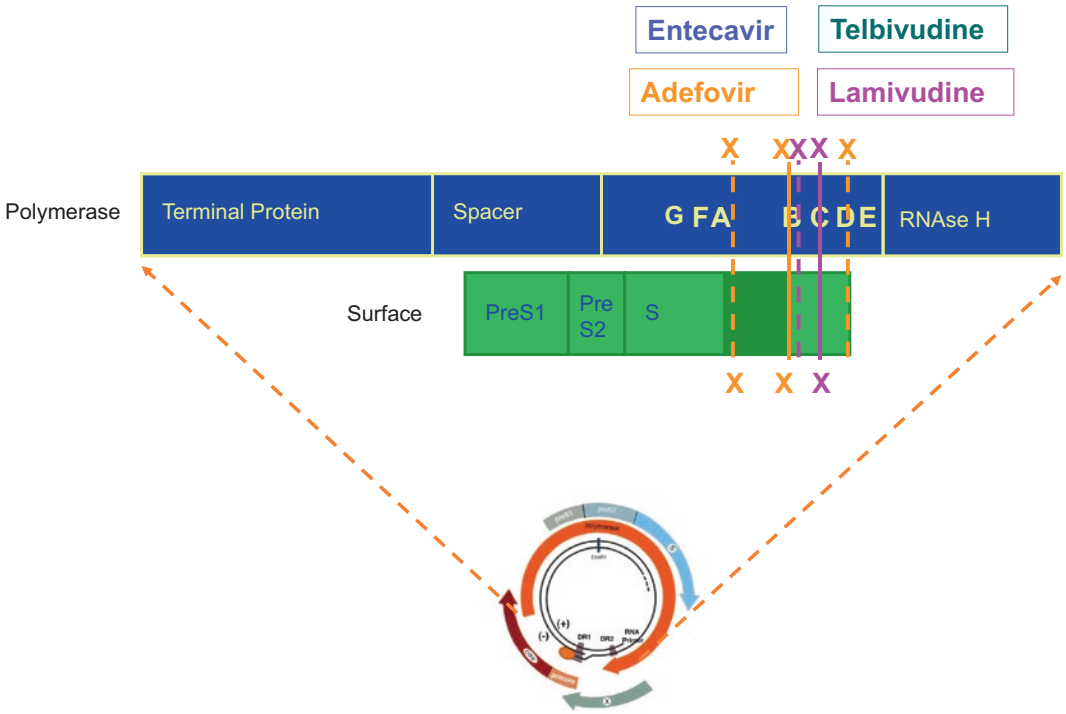


Fig. 38.6 Stylised diagram showing the polymerase-HBsAg overlap and how drug-resistant mutations in polymerase can alter the neutralisation domain ('a' determinant) of HBsAg. Modified from Zoulim and Locarnini [10, 11]

Table 38.4 Patterns and pathways of antiviral drug reisntace in chroinc hepaitis B in the context of cross-resitance

Pathway	Amino acid substitutions in the rt domain	LMV	LdT	ETV	ADV	TFV
	Wild-type	S	S	S	S	S
L-Nucleoside (LMV/LdT)	M204I/V	*R*	*R*	I	S	S
Acyclic phosphonate (ADV)	N236T	S	S	S	*R*	I
Shared (LMV, LdT, ADV)	A181T/V	*R*	*R*	S	*R*	I
Double (ADV, TFV)	A181T/V + N236T	*R*	*R*	S	*R*	*R*
D-Cyclopentane (ETV)	L180M+M204V/I ± I169 ± T184 ± S202 ± M250	*R*	*R*	*R*	S	S

Abbreviations: *I* intermediate sensitivity, *R* resistant, *S* sensitive based on cell culture and clinical responses
Modified from: Zoulim, F. and S. Locarnini, Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology, 2009. 137(5): p. 1593-608 e1–2. Zoulim, F. and S. Locarnini, Management of treatment failure in chronic hepatitis B. J Hepatol, 2012. 56 Suppl 1: p. S112–22.

potential to disrupt the current hepatitis B immunisation program [85].

The ADV resistance mutation rtN236T does not affect the envelope gene and overlaps with the stop codon at the end of the envelope gene. The rtA181T mutation selected by ADV and/or LMV/LdT results in a stop codon mutation at sW172stop (Table 38.4). The ADV-resistant mutation at rtA181V results in a significant amino acid change at sL173F. HBV with mutations that result in a stop codon in the envelope gene such as those for LMV and ADV would be present in association with a low percentage of wild type to enable viral packaging.

The ETV-resistant-associated change at rtI169T, rtS184G and rtS202I also affects HBsAg and results in changes at sF161L, sL/V176G and sV194F (Table 38.4). The rtM250V is located after the end of HBsAg. The sF161L is located within the region that was defined as the 'a' determinant or major hydrophilic region (MHR), which includes amino acids 90–170 of the HBsAg [86]. This region is a highly conformational epitope, characterised by multiple disulphide bonds formed from sets of cysteines at residues 107–138, 137–149 and 139–147 [86]. Thus, distal substitutions such as sE164D significantly affect anti-HB binding [83]. The influence of other changes to HBsAg, such as sF161L, needs further investigation to determine the effect on the envelope structure and subsequent anti-HB binding. A summary of the HBsAg changes selected during emergence of resistance to NA is shown in Table 38.4 with the stop codons highlighted.

Whilst evidence for the spread of transmission of antiviral resistant HBV is limited, there has been a report of the transmission of LMV-resistant HBV to an HIV patient undergoing LMV as part of antiretroviral therapy [75]. Clearly, in the well-established high-risk settings for HBV transmission, increased surveillance will establish whether or not these antiviral drug-associated potential vaccine escape mutants or ADAPVEMs [85] represent a genuine public health threat.

8 Conclusions

Antiviral drug resistance should no longer pose a major problem in the management of patients with CHB. If first-line NA entecavir and tenofovir are used in naïve patients, then minimal or no resistance should be the outcome in the medium term (5–7 years). However, the economic reality is that low genetic barrier drugs such as lamivudine are still used as first line and lamivudine use results in frequent resistance, subsequently compromising the rescue options. As a guiding principle, the probability that viral resistance will develop is directly proportional to the potency of the drug regimen and the diversity of quasispecies. Inhibition of HBV replication should be able to prevent the development of drug resistance, mainly because mutagenesis is replication dependent. If viral replication can be suppressed for a sufficient length of time, viral load will theoretically decline to a point where the continued production of quasispecies with the potential for resisting new drug treatments is no longer possible. Whether this end point also translates to other benefits such as recovery of the host immune response allowing HBeAg seroconversion, sustained virological suppression with histological improvement or even HBsAg seroconversion, is presently being investigated in clinical trials.

References

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol. 1995;13:29–60.
2. Chen BF, et al. High prevalence and mapping of pre-S deletion in hepatitis B virus carriers with progressive liver diseases. Gastroenterology. 2006;130(4):1153–68.
3. Chen CH, et al. Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. J Clin Microbiol. 2005;43(12):6000–6.
4. Kao JH, et al. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology. 2003;124(2):327–34.
5. Lin CL, et al. Basal core-promoter mutant of hepatitis B virus and progression of liver disease in hepatitis B e antigen-negative chronic hepatitis B. Liver Int. 2005;25(3):564–70.
6. Song BC, et al. Comparison of full length sequences of hepatitis B virus isolates in hepatocellular carcinoma patients and asymptomatic carriers of Korea. J Med Virol. 2005;75(1):13–9.
7. Yotsuyanagi H, et al. Precore and core promoter mutations, hepatitis B virus DNA levels and progressive liver injury in chronic hepatitis B. J Hepatol. 2002;37(3):355–63.
8. Bartholomeusz A, Locarnini SA. Antiviral drug resistance: clinical consequences and molecular aspects. Semin Liver Dis. 2006;26(2):162–70.
9. Locarnini S. Molecular virology and the development of resistant mutants: implications for therapy. Semin Liver Dis. 2005;25 Suppl 1:9–19.
10. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology 2009;137(5):1593–608. e1–2.
11. Zoulim F, Locarnini S. Management of treatment failure in chronic hepatitis B. J Hepatol. 2012;56 Suppl 1:S112–22.
12. Yan H, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife. 2012;1:e00049.
13. Sureau C, Salisse J. A conformational heparan sulfate binding site essential to infectivity overlaps with the conserved hepatitis B virus a-determinant. Hepatology. 2013;57(3):985–94.
14. Rabe B, et al. Nuclear import of hepatitis B virus capsids and release of the viral genome. Proc Natl Acad Sci U S A. 2003;100(17):9849–54.
15. Locarnini S, Birch C. Antiviral chemotherapy for chronic hepatitis B infection: lessons learned from treating HIV-infected patients. J Hepatol. 1999;30(3):536–50.
16. Bock CT, et al. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. Virus Genes. 1994;8(3):215–29.
17. Newbold JE, et al. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J Virol. 1995;69(6):3350–7.
18. Ganem D, Schneider R. Hepadnaviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. Fields virology. Philadelphia: Lippincott-Raven; 2001. p. 2923–70.
19. Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Virol. 2004;78(23):12725–34.
20. Bartenschlager R, Schaller H. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J. 1992;11(9):3413–20.
21. Will H, et al. Replication strategy of human hepatitis B virus. J Virol. 1987;61(3):904–11.
22. Zoulim F, Seeger C. Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. J Virol. 1994;68(1):6–13.
23. Kann M, Gerlich W. Hepadnaviridae: structure and molecular virology. In: Zuckerman A, Thomas H, editors. Viral hepatitis. London: Churchill Livingstone; 1998. p. 77–105.
24. Zhang YY, Summers J. Enrichment of a precore-minus mutant of duck hepatitis B virus in experimental mixed infections. J Virol. 1999;73(5):3616–22.
25. Chemin I, Zoulim F. Hepatitis B virus induced hepatocellular carcinoma. Cancer Lett. 2009;286(1):52–9.
26. Norder H, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology. 2004;47(6):289–309.
27. Stuyver L, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol. 2000;81(Pt 1):67–74.
28. Shaw T, Locarnini S. Entecavir for the treatment of chronic hepatitis B. Expert Rev Anti Infect Ther. 2004;2(6):853–71.
29. Domingo E. Quasispecies and the development of new antiviral strategies. Prog Drug Res. 2003;60:133–58.
30. Stuyver LJ, et al. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology. 2001;33(3):751–7.

31. Gunther S, et al. Naturally occurring variants of hepatitis B virus. Adv Virus Res. 1999;52:25–137.
32. Yeh CT, et al. Clearance of the original hepatitis B virus YMDD-motif mutants with emergence of distinct lamivudine-resistant mutants during prolonged lamivudine therapy. Hepatology. 2000;31(6):1318–26.
33. Ogata N, et al. Novel patterns of amino acid mutations in the hepatitis B virus polymerase in association with resistance to lamivudine therapy in japanese patients with chronic hepatitis B. J Med Virol. 1999;59(3):270–6.
34. Tenney DJ, et al. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. Antimicrob Agents Chemother. 2004;48(9):3498–507.
35. Delaney WE, et al. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. J Virol. 2003;77(21):11833–41.
36. Bartholomeusz A, et al. Mechanistic basis for hepatitis B virus resistance to acyclic nucleoside phosphonate analogues. Adefovir and Tenofovir Hepatology. 2005;42 Suppl 1:594A.
37. Lai CL, et al. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clin Infect Dis. 2003;36(6):687–96.
38. Leung N. Clinical experience with lamivudine. Semin Liver Dis. 2002;22 Suppl 1:15–21.
39. Delaney WEt, Locarnini S, Shaw T. Resistance of hepatitis B virus to antiviral drugs: current aspects and directions for future investigation. Antivir Chem Chemother. 2001;12(1):1–35.
40. Zollner B, et al. Viral features of lamivudine resistant hepatitis B genotypes A and D. Hepatology. 2004;39(1):42–50.
41. Bartholomeusz A, Tehan BG, Chalmers DK. Comparisons of the HBV and HIV polymerase, and antiviral resistance mutations. Antivir Ther. 2004;9(2):149–60.
42. Angus P, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology. 2003;125(2):292–7.
43. Hadziyannis S. Hepatitis B e antigen negative chronic hepatitis B: from clinical recognition to pathogenesis and treatment. Viral Hepat Rev. 1995;1:7–36.
44. Bartholomeusz A, et al. Molecular modelling of hepatitis B virus polymerase and adefovir resistance identifies three clusters of mutations. Hepatology. 2004;40 Suppl 1:246A.
45. Schildgen O, et al. Variant of hepatitis B virus with primary resistance to adefovir. N Engl J Med. 2006;354(17):1807–12.
46. Chang TT, Lai CL. Hepatitis B virus with primary resistance to adefovir. N Engl J Med. 2006;355(3):322–3. Author reply 323.
47. Yuen L, et al. SEQHEPB: a sequence analysis program and relational database system for chronic hepatitis B. Antiviral Res. 2007;75(1):64–74. Epub 2006 Dec 22.
48. Colonno RJ, et al. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology. 2006;44(6):1656–65.
49. Angus P, Locarnini S. Lamivudine-resistant hepatitis B virus and ongoing lamivudine therapy: stop the merry-go-round, it's time to get off! Antivir Ther. 2004;9(2):145–8.
50. Chin R, et al. In vitro susceptibilities of wild-type or drug-resistant hepatitis B virus to (-)-beta-D-2,6-diaminopurine dioxolane and 2′-fluoro-5-methyl-beta-L-arabinofuranosyluracil. Antimicrob Agents Chemother. 2001;45(9):2495–501.
51. Levine S, et al. Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob Agents Chemother. 2002;46(8):2525–32.
52. Ono-Nita SK, et al. Novel nucleoside analogue MCC-478 (LY582563) is effective against wild-type or lamivudine-resistant hepatitis B virus. Antimicrob Agents Chemother. 2002;46(8):2602–5.
53. Warner N, et al. Molecular modelling of entecavir resistant mutations in the hepatitis B virus polymerase selected during therapy. Hepatology 2004;40(4 (Suppl. 1)):245A.
54. Colonno RJ, et al. Assessment at three years shows high barrier to resistance is maintained in entecavir-treated nucleoside naive patients while resistance emergence increases over time in lamivudine refractory patients. In: 57th annual meeting of American Association for the Study of Liver Disease. 2006. Abstract 110.
55. Brunelle MN, et al. Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. Hepatology. 2005;41(6):1391–8.
56. Fung SK, et al. Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. J Hepatol. 2005;43(6):937–43.
57. Mutimer D, et al. Selection of multiresistant hepatitis B virus during sequential nucleoside-analogue therapy. J Infect Dis. 2000;181(2):713–6.
58. Villet S, et al. Sequential antiviral therapy leads to the emergence of multiple drug resistant hepatitis B virus. Hepatology 2005;42(Suppl. 1)(4):581A.
59. Gonzales MJ, et al. Colinearity of reverse transcriptase inhibitor resistance mutations detected by population-based sequencing. J Acquir Immune Defic Syndr. 2003;34(4):398–402.
60. Shafer RW, et al. Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients. Ann Intern Med. 1998;128(11):906–11.
61. Natsuizaka M, et al. Long-term follow-up of chronic hepatitis B after the emergence of mutations in the hepatitis B virus polymerase region. J Viral Hepat. 2005;12(2):154–9.
62. Ono SK, et al. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest. 2001;107(4):449–55.
63. Yim HJ, et al. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology. 2006;44(3):703–12.
64. Shaw T, Locarnini S. Combination chemotherapy for hepatitis B virus: the path forward? Drugs. 2000;60(3):517–31.
65. Lok A. Natural history and control of perinatally acquired hepatitis B virus infection. Dig Dis. 1992;10(1):46–52.
66. Nowak MA, et al. Viral dynamics in hepatitis B virus infection. Proc Natl Acad Sci U S A. 1996;93(9):4398–402.
67. Locarnini S, et al. Management of antiviral resistance in patients with chronic hepatitis B. Antivir Ther. 2004;9(5):679–93.
68. Girones R, Miller RH. Mutation rate of the hepadnavirus genome. Virology. 1989;170(2):595–7.
69. Okamoto H, et al. Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through maternofetal transmission. Jpn J Exp Med. 1987;57(4):231–6.
70. Richman DD. The impact of drug resistance on the effectiveness of chemotherapy for chronic hepatitis B. Hepatology. 2000;32(4 Pt 1):866–7.
71. Richman DD. The implications of drug resistance for strategies of combination antiviral chemotherapy. Antiviral Res. 1996;29(1): 31–3.
72. Zhang YY, Summers J. Low dynamic state of viral competition in a chronic avian hepadnavirus infection. J Virol. 2000;74(11):5257–65.
73. Doo E, Liang TJ. Molecular anatomy and pathophysiologic implications of drug resistance in hepatitis B virus infection. Gastroenterology. 2001;120(4):1000–8.
74. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev. 2000;64(1):51–68.
75. Thibault V, et al. Primary infection with a lamivudine-resistant hepatitis B virus. AIDS. 2002;16(1):131–3.
76. Niesters HG, et al. Identification of a new variant in the YMDD motif of the hepatitis B virus polymerase gene selected during lamivudine therapy. J Med Microbiol. 2002;51(8):695–9.
77. Lok AS, et al. Monitoring drug resistance in chronic hepatitis B virus (HBV)-infected patients during lamivudine therapy: evaluation of performance of INNO-LiPA HBV DR assay. J Clin Microbiol. 2002;40(10):3729–34.

78. Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. J Hepatol. 2006;44(3): 593–606.
79. Shaw T, Locarnini SA. Preclinical aspects of lamivudine and famciclovir against hepatitis B virus. J Viral Hepat. 1999;6(2):89–106.
80. Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. J Hepatol. 2006;44(2):422–31.
81. Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. Hepatology. 2009;50(3):661–2.
82. Torresi J, et al. Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the "fingers" subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. Virology. 2002;299(1):88–99.
83. Matthews GV, et al. Characteristics of drug resistant HBV in an international collaborative study of HIV-HBV-infected individuals on extended lamivudine therapy. AIDS. 2006;20(6):863–70.
84. Kamili S, et al. Efficacy of hepatitis B vaccine against antiviral drug-resistant hepatitis B virus mutants in the chimpanzee model. Hepatology. 2009;49(5):1483–91.
85. Clements CJ, Coghlan B, Creati M, Locarnini S, Tedder RS, Torresi J. Global control of hepatitis B virus: does treatment-induced antigenic change affect immunization? Bull World Health Organ. 2010;88(1):66–73. doi: 10.2471/BLT.08.065722.
86. Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. J Viral Hepat. 1997;4 Suppl 1:11–20.
87. Leung NW, et al. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology. 2001;33(6):1527–32.
88. Locarnini S, et al. Incidence and predictors of emergence of adefovir resistant HBV during four years of adefovir dipivoxil (ADV) therapy for patients with chronic hepatitis B (CHB). J Hepatol. 2005;42 Suppl 2:A17.
89. Perrillo RP. Current treatment of chronic hepatitis B: benefits and limitations. Semin Liver Dis. 2005;25 Suppl 1:20–8.

HCV Drug Resistance

39

Bianca Heinrich and John P. Bilello

1 Introduction to HCV

Treatment of hepatitis C virus (HCV) infection has rapidly evolved since the initial interferon-alfa (IFN-α)-based therapy was introduced in 1989 [1]. Improved formulations of IFN-α and coadministration of ribavirin (RBV) resulted in HCV-sustained viral response (SVR) rates of approximately 50 %, which is dependent on HCV genotype (Fig. 39.1) [2]. The approval of the first direct-acting antiviral (DAA) protease inhibitors (PIs) in 2010, when used in combination with IFN-α and RBV, further improved SVR rates to 70–80 % (Fig. 39.1) [2]. However, a low barrier to resistance associated with the protease inhibitors allowed for frequent emergence of treatment-associated resistance variants. Rapid emergence of resistance-associated variants (RAVs) was also observed with the superbly potent class of NS5A replication inhibitors, precluding their use as a single agent and for specific genotype variants. Although nucleoside inhibitors were one of the first classes of HCV DAAs in preclinical development, approval of a nucleotide prodrug-based HCV treatment regimen with IFN-α and RBV was secondary to the protease inhibitors. Therapies that include nucleotide prodrugs can achieve SVR rates of nearly 100 % depending on the genotypes and DAA drug combinations assessed. Clinical trials are focusing mainly on combination regimens that include a nucleotide prodrug and at least one other DAA class, with the focus residing in prevention of RAV emergence and shorter treatment duration. Knowledge of treatment-emergent RAVs will either guide clinicians in their selection of combination therapies for HCV or serve as a historical perspective of resistance once observed prior to a single-pill combination regimen that does not select resistant variants [3].

1.1 Epidemiology of HCV Infection

Exposure to HCV via blood-borne routes results in an acute infection, which can be naturally cleared by the immune system in only 20 % of cases [4]. However, the vast majority of patients exposed to HCV become chronically infected with the virus, which can lead to subsequent health detriments later in life. Worldwide estimates of chronic HCV infections range from 140 to 185 million people, representing approximately 3 % of the global population [5]. In the USA, 60–75 % of the 3–4 million chronic HCV infections are genotype (GT) 1, while GT2 and 3 comprise the most of the remainder of chronically infected people [5–7]. Genotypes 1a and 1b comprise 36–55 % and 23–25 % of HCV infections in the USA, respectively, and the prevalence of chronic infection is higher in black and male populations. Other more common risk factors of HCV-infected people in the USA include coinfection with HIV and a history of intravenous drug use. Worldwide, genotypes 1, 3, and 4 are the most prevalent, representing 42 %, 26 %, and 17 %, respectively. Genotype distribution can be regionalized to certain geographical areas [8, 9]. Genotype 5, for example, is almost exclusively detected in the South sub-Saharan Africa, where its prevalence is >50 % of the genotypes [10]. Chronic HCV infection leads to chronic liver disease in 60–70 % of patients and cirrhosis of the liver in approximately 5–20 % of patients, which can progress to hepatocellular carcinoma (HCC) in 1–5 % of patients [3, 11]. HCV is a direct risk factor for the development of HCC, accounting for approximately 20 % of all new cases each year, which increases with additional risk factors such as excess alcohol consumption, marijuana or steroid usage, age, gender, and HIV coinfection [4, 9].

B. Heinrich, Ph.D.
Genomic Assays, Abcam, Inc., 1400W 3rd Floor, One Kendall Square, Cambridge, MA 02139, USA
e-mail: bianca.heinrich@abcam.com

J.P. Bilello, Ph.D. (✉)
Infectious Diseases Biology-Discovery, Merck, West Point, PA, USA
e-mail: john.bilello@merck.com

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_39

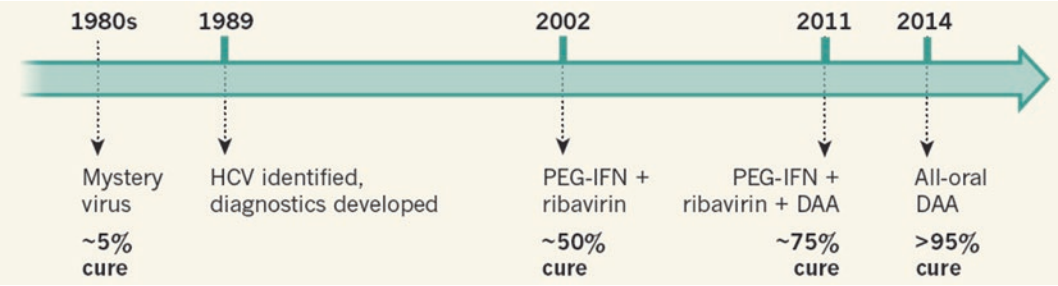


Fig. 39.1 HCV trajectory. From Rice and Saeed, Nature (2014) 510(7503):43–44

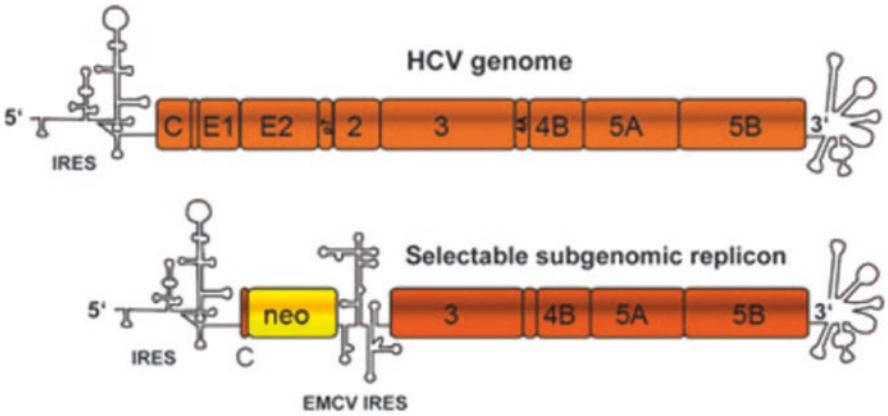


Fig. 39.2 Structure of the HCV genome/replicon. From Bühler and Bartenschlager (2012)

1.2 HCV Biology

HCV is a member of the flaviviridae family of single-stranded positive RNA genome viruses. The HCV hepacivirus genome consists of a single 9.6-kb open reading frame flanked by 5′- and 3′-untranslated regions (UTRs) and encodes a polyprotein of approximately 3020 amino acids. The HCV polyprotein is posttranslationally cleaved into ten proteins: four amino-terminal structural proteins and six nonstructural (NS) C-terminal proteins (Fig. 39.2) [12]. Among the structural proteins, the two envelope glycoproteins E1 and E2 and the 63-residue p7 ion channel protein are membranous proteins targeted for vaccine development and/or antiviral therapy [13–19]. The core protein, the most amino-terminal structural protein, is the main component of the HCV nucleocapsid [13, 14, 20]. While not a well-studied antiviral target, the core protein may also play roles in cellular signaling and proliferation pathways as well as immunoevasion. With the exception of the NS2 protease responsible for autoproteolysis of itself from NS3, antiviral therapies have been developed for each of the nonstructural proteins (NS3/4A, NS4B, NS5A, and NS5B) [15, 21]. Only recently has a class of direct-acting small-molecule inhibitors of NS2 autoproteolytic function been identified [22]. While the structural proteins are released from the HCV polyprotein by cellular peptidases, the NS3 protease is responsible for cleavage of most of the nonstructural proteins into individual functional components. Several drugs are available for the treatment of HCV that target the NS3 proteolytic activity. In addition to its serine proteinase activity localized to its N-terminus, NS3 also has a helicase domain at its C-terminal region responsible for RNA binding and unwinding as well as for NTP hydrolysis. NS4A forms a complex with NS3 and mediates its association with NS4B and NS5A to facilitate formation of the HCV replication complex where the HCV genome is replicated. The NS4B protein forms a membranous web at the endoplasmic reticulum that serves as an assembly point for the HCV replication complex and virus progeny formation. NS5A is a three-domain multifunctional protein with a more structured N-terminal domain that is phosphorylated connected to two amorphous domains. In addition to being an essential protein for HCV virus proliferation, NS5A has been shown to mediate the interferon antiviral response and affect multiple cellular

signaling cascades. Inhibitors of NS5A-mediated HCV replication remain the most potent class of direct-acting antivirals (DAAs) to enter clinical trials. NS5B is the most C-terminal protein of the HCV polyproteins and encodes the viral RNA-dependent RNA polymerase (RdRp) solely responsible for genome replication. Although part of the HCV replication complex, NS5B can replicate the HCV genome in the absence of other protein cofactors, which has facilitated the in vitro development of DAAs targeting NS5B with multiple mechanisms of action, including several allosteric and active-site inhibitors of the NS5B RdRp activity [13, 14]. Development of numerous DAAs targeting the p7 structural protein and each nonstructural protein class has uncovered a wide range of resistance mutations that emerge upon anti-HCV therapy, necessitating a combination therapy approach to prevent frequent treatment failures.

2 Methods to Examine Viral Resistance

2.1 Standard Sanger Method

Sanger sequencing is currently the gold standard method for sequencing virus populations, including HCV. It is cost effective and readily available and the data are easily interpreted without the requirement of sophisticated software tools. However, this method has inherent disadvantages when analyzing viral "quasispecies." Like many other RNA viruses, HCV exists as a population of closely related, but distinct, variants within an infected individual [23]. This is the result of an RdRp that lacks proofreading activity. In combination with a replication turnover rate of ~10^{12} virions/day [24], this allows the virus to quickly adapt to environmental changes. Analyzing HCV genomes therefore always requires a reasonably good idea of the detection limit of the individual approach used. Direct sequencing of viral PCR products obtained from blood serum samples can only provide insights into variants that are present above ~20 % of frequency of the viral population. As such, this methodology allows for identification of the representative genomes within a given viral population and is therefore known as population or bulk sequencing. It reliably detects major resistance variants that arise in response to drug exposure in patient serum samples. To identify variants that are present below 20 % of frequency, sequencing chromatograms need to be manually scanned for minor peaks. While sensitivity can be increased dramatically by cloning and transforming PCR-amplified viral genome fragments into bacteria that are subsequently isolated and sequenced, such clonal sequencing of a viral population is labor intensive and has poor scalability. However, this approach can uncover minority variants with a detection limit of approximately 5 %, depending on how many clones are analyzed. Clonal sequencing should be performed when resistance patterns are complex and to reveal linkage of variants, though linkage should always be verified by multiclonal analysis from multiple, independent PCR reactions. Linkage of RAVs can be of relevance because resistance levels to an antiviral drug may increase significantly when specific RAVs are located together on the same RNA molecule. This has clearly been demonstrated through cell-based HCV replicon assays where double mutants confer increased resistance to a drug compared to each mutation alone [25, 26]. Linkage may also influence of how long such variants persist in a patient after the end of antiviral treatment [27, 28].

2.2 Ultra-Deep Sequencing Methods

Low-cost next-generation sequencing (NGS) technologies allow for massive parallel sequencing of individual genomic fragments and for the detection of rare variants in mixed viral populations down to <1 % of frequency. However, the current error rates also lie within this range and therefore thorough steps have to be taken to ensure the reliability of the data (i.e., careful threshold determination through plasmid sequencing). Obtaining reliable data with sensitivity levels below 0.5 % may be problematic with the current technologies available. Major challenges include PCR amplification bias, sequencing errors, and software analysis of the large amounts of data produced. The number of reads at any given position, typically hundreds to several thousand, determines the depth of the sequencing run and the overall data quality. A variety of different NGS platforms are currently on the market, each of them with their own inherent advantages and disadvantages (cost per base, read length, speed, accuracy, etc.). The four major platforms that dominate the market are 454, Illumina, Ion Torrent, and PacBio. The question of which platform to use depends very much on the goal of the project. The PacBio technology, for example, allows for linkage analysis due to the long read length, but intrinsic sequence error rates are higher compared to any of the other platforms. Much smaller fourth-generation devices are now in development that further aim to increase run speeds and decrease sequencing costs. Such advances will most likely also improve point-of-care testing abilities of this technology. One of the exciting novel innovations the deep sequencing field is particularly looking forward to is the sequencing of nucleic acids in real time via translocation through so-called nanopores [29]. Deep sequencing has proven invaluable for the characterization of within-host evolution of HCV, especially in the context of drug resistance, and several studies have uncovered low-frequency DAA drug resistance mutations through this methodology [30–34]. These studies demonstrated that RAVs exist at low frequencies before drug exposure within a virus population. One study determined through deep sequencing that

naturally occurring variants to protease inhibitors were present in over 80% of patients [35]. In contrast, population sequencing detects NS3 and NS5A variants only in about 3–10% of patients across different genotypes [36, 37]. Even though deep sequencing allows for the detection of many more RAVs in any given quasispecies, it is currently unclear at what threshold the presence of such preexisting variants is still clinically meaningful. This is complicated by the fact that preexisting mutations do not prevent successful treatment regardless of their frequency. Three out of eight patients treated with the DAAs daclatasvir (an NS5A inhibitor) and asunaprevir (an NS3 inhibitor), for example, were shown to contain daclatasvir-resistant variants ranging from 0.9 to 99% of the overall viral population, but still achieved SVR [33]. The presence of baseline-resistant variants also did not substantially affect SVR rates in a large cohort of DAA-naïve patients infected with GT1 [37]. Our current understanding of the relevance of preexisting RAVs with respect to resistance development and successful DAA treatment is, as such, still limited.

Even though it has become clear over the past years that the presence of baseline variants cannot reliably predict clinical outcome, a common trend has emerged, suggesting that baseline RAVs do slightly influence SVR rates across the board. This is especially true for patients with poor response to IFN/RBV [38] and for genotype 1a patients receiving simeprevir and carrying the NS3 Q80K variant [39]. While clinically relevant sensitivity thresholds remain to be determined, NGS technologies do provide unique insights into the heterogeneity of viral quasispecies. In addition, DAA classes with higher genetic barriers such as the NS5B nucleos(t)ide analogs may require highly sensitive technologies to better understand their resistance mechanisms as the frequency of RAVs is directly related to viral replication capacity. For example, for the currently approved nucleos(t)ide inhibitor, sofosbuvir, resistance appears to be of fairly little concern in the clinic so far. The S282T substitution is the primary resistance mutation identified in vitro, but due to its extremely impaired fitness it is very rarely identified in patients either upon treatment with sofosbuvir or as a preexisting variant [40, 41]. To effectively examine the evolution of resistance variants in patients in response to sofosbuvir or other novel nucleos(t)ide inhibitors, deep sequencing is without doubt a highly valuable approach. This is also true for monitoring the long-term persistence of RAVs as their frequencies can be expected to decline continuously over time. At the point when RAVs become undetectable by population sequencing, they are most likely still present as minor variants within the viral quasispecies. To determine true decay rates of treatment-emergent RAVs following the removal of the selection pressure, deep sequencing is certainly a compelling option.

2.3 Phenotypic Testing

Phenotypic analysis is an effective tool to assess antiviral effects on replication and drug susceptibility by analyzing clinical isolates in a transient assay system. In this approach, PCR products of HCV isolates are inserted into replicon vectors to replace entire target genes with patient sequence. RNA is then transcribed from the linearized subgenomic vectors and transiently transfected into permissive cell lines. This way, naturally occurring polymorphism and treatment-emergent variants from patient samples collected at baseline or at the time of treatment failure can be assayed to better understand HCV heterogeneity and antiviral response. The HCV Drug Development Advisory Group (HCV DRAG) recommends that phenotypic analysis should be clonal if RAVs are present as mixtures or linkage is unknown [42]. As previously mentioned, population sequencing does not adequately describe linkage between resistance-associated variants (at least not if their frequencies lie below 50%), and deep sequencing only allows for linkage analysis when reads are sufficiently long. As such, phenotypic systems have been established for common HCV drug targets such as NS3, NS4B, NS5A, and NS5B [43–47]. Because infectious clones and stable cell lines containing HCV replicons often harbor adaptive mutations to enhance their replication efficiencies in vitro, the ability to measure antiviral activities within the context of naturally occurring patient sequences has enormous merit. Ideally, phenotypic results (EC_{50} values) from a patient following drug exposure should always be compared to a baseline sample from the same subject so that meaningful fold-change values can be derived. One major challenge with this methodology is the establishment of clinical significant cutoff values, which are defined by technical parameters such as assay variability and sensitivity as well as natural variations in drug susceptibility. Despite these drawbacks, phenotypic testing can help to derive the most efficient drug regimen for an individual's virus and is a pivotal method to uncover drug resistance pathways.

2.4 In Vitro Models

In vitro subgenomic HCV replicon models have been utilized to examine anti-HCV activity of putative antivirals since soon after the Bartenschlager lab demonstrated the propagation of a genotype 1b replicon in hepatic cell lines back in 1999 [48, 49]. Typically, HCV replicons are themselves positive single-stranded RNA composed of the 5′-UTR preceding an antibiotic resistance gene and the NS3–NS5b subgenomic regions, followed by the 3′-UTR (Fig. 39.2) [12]. Often a reporter gene, such as luciferase, is fused to the antibiotic resistance gene to facilitate easy detection of the levels of the self-replicating HCV replicon. HCV replicons of additional HCV genotypes have been gradually developed through the years to represent single-genotype clones of GTs 1a, 2a, 3a, 4a, and

now also 5a [50–55]. Genotype 6a, as well as HCV nonstructural sequences derived from HCV-infected patient samples, has been studied through the use of intergenotypic replicons. Intergenotypic replicons may contain single or multiple HCV nonstructural genes from less robust genotypes and specimens inserted into more replication-competent laboratory genotype strains [56–58]. These genotype-specific and intergenotypic replicon clones are used for evaluation of antiviral drug resistance in addition to potency. Several full-length individual or hybrid genotype self-replicating hepatitis C viruses can also be utilized for resistance studies [59–61]; however, much of the in vitro resistance profiling of antiviral drugs is performed in the HCV replicions. The full-length lab-adapted HCV viral clones remain an invaluable tool to study the potency and resistance mechanisms of viral entry inhibitors and mediators of p7 and core protein activity [53].

Resistance analysis of anti-HCV drugs is routinely performed during the discovery stage of development and continues throughout clinical trials and postdrug approval to monitor the possible emergence and longevity of resistance mutations. Antiviral drug resistance is most often evaluated using the in vitro replicon systems discussed above; however, much of the resistance analysis may also be applied to full-length replicating virus clones. The most prevalent in vitro resistance analyses involve either targeted site-directed mutagenesis or drug selection of mutants using existing laboratory genotype replicon clones [62–67]. Antiviral drug candidates are often tested for their altered potency against a panel of predefined mutations genetically engineered within the nonstructural genes of the HCV replicon. These site-directed mutant replicon clones are transfected in parallel to wild-type replicons into permissible hepatic (non-exclusively) cells and treated with drug for several days. Comparison of the inhibition of replicon propagation between the mutant and wild-type replicons allows a prediction of putative mutations that may arise in the clinic as well as allows the assessment of possible cross-resistance between two antiviral drugs. Further, mutants detected in DAA-treated HCV-infected subjects can be evaluated for their contributions to resistance by inserting the identified mutant into an appropriate HCV replicon and subsequent in vitro examination [68]. Prediction of possible mutants that may emerge in the clinic is also determined by treating HCV replicon-containing cells with drug continuously for several weeks to months [69]. Often, cultures of replicon-bearing cells are treated at multiple or increasing concentrations of drug to identify resistance mutations that may emerge in the clinic under different treatment concentrations. HCV replicon clones that are susceptible to the drug are inhibited or degraded by cellular pathways, while replicons harboring mutations that confer resistance to the drug continue to replicate. Subsequent genomic sequencing analyses of the remaining replicons reveal the mutation(s) selected by the drug. These selected resistance mutations may have been preexisting or may have arisen via mutagenesis by the error-prone nature of the HCV polymerase. Often, individual mutations or genomic regions bearing mutations are engineered into laboratory replicon clones to explicitly define contribution of the mutation(s) to drug resistance and/or effect on replicon replication [66, 70–73].

3 HCV Therapies and Related Resistance: Host-Related Factors

3.1 Interferon-α and Ribavirin

Interferon-α and the purine-nucleotide analogue ribavirin have historically been part of the standard therapy for HCV and were responsible for improved cure rates of about 50 % by 2002 [2]. Interferon-α is administered in its pegylated form (PEG-IFN), thereby significantly enhancing its efficacy. The addition of a polyethylene-glycol side chain extends the bioavailability of the molecule and allows for a single rather than three injections per week. Currently available for HCV therapy are PEG-IFN-α-2a, PEG-IFN-α-2b, and PEG-IFN-β-1a. In some patients with extremely severe adverse side effects to IFN-α, therapy was successfully continued using an IFN-β-containing regimen [74]. Because IFN-α treatment is well known for its adverse side effects (anemia, fatigue, headaches, rashes, etc.), efforts are under way to eliminate this component from treatment regimens where possible, in particular for patients with poor tolerance to IFN. The recent approval of highly efficient second-generation DAAs opened new doors in this regard. For treatment-naïve patients infected with genotypes 2 and 3 treatment options now exist for the first time that lack IFN [75].

RBV remains to be an integral component of current treatment recommendations for all genotypes. Like IFN, RBV can cause significant side effects, including anemia, but its benefits to HCV therapy still outweigh its shortcomings. Despite extensive research efforts, the precise mechanisms by which RBV curtails chronic HCV infection are still not very well understood. It has been suggested that RBV potentiates the effect of IFN by promoting antiviral (type 1) cytokine expression [76] and by upregulating genes involved in IFN signaling [77, 78]. Other suggested mechanisms by which RBV acts on HCV infection include direct inhibition of the RNA-depending RNA polymerase by inducing premature chain termination, lethal mutagenesis caused by an increased error rate [79, 80], or competitive inhibition of inosine-5′-monophosphate dehydrogenase resulting in the depletion of intracellular pools of guanosine triphosphate [81]. The antiviral mechanism(s) by which RBV contributes to HCV clearance remains controversial and as such factors that influence RBV-related treatment response. Efforts to create RBV-resistant HCV replicon cell lines resulted in changes that mostly occurred in the host genome and caused defects in RBV uptake [82]. Consequently, host factors appear to play more of a role in RBV treatment response than viral factors. In

fact, it appears that neither IFN nor RBV exerts considerable viral pressure, meaning that treatment failure is not a result of the selection of viral resistance variants.

Induction of an antiviral state by the innate immune response is essential for combating viral infections and IFNs are an integral part of this first line of defense. Patients with intrinsic resistance to IFN have a much decreased chance of successfully completing antiviral treatment and often show little decline in HCV titer. Nonresponsivess and resistance to PEG-IFN/RBV therapy are not fully understood. Several host factors affect the clinical response, including age, gender, grade of liver fibrosis, race, serum viral load, and even insulin resistance. It was discovered in 2009 that host single-nucleotide polymorphisms around the interleukin-28B (*IL28B*) locus, a type III interferon, are strongly associated with response to IFN-α + RBV combination treatment and HCV clearance [83–85]. Type I (IFN-α and -β) and type III (IFN-λ) interferons are both induced upon HCV infection and activate the same downstream signaling events to restrict viral replication (Fig. 39.3) [86]. In the later phase of HCV infection the type II interferon IFN-γ induces a distinct, but partially overlapping, set of genes [87]. This phase leads in 30 % of patients to a successful clearance of the virus. In the remaining 70 % of individuals, the virus persists and may lead to severe clinical implications later in life, including liver cirrhosis and HCC. In chronically infected HCV patients, the response rate to

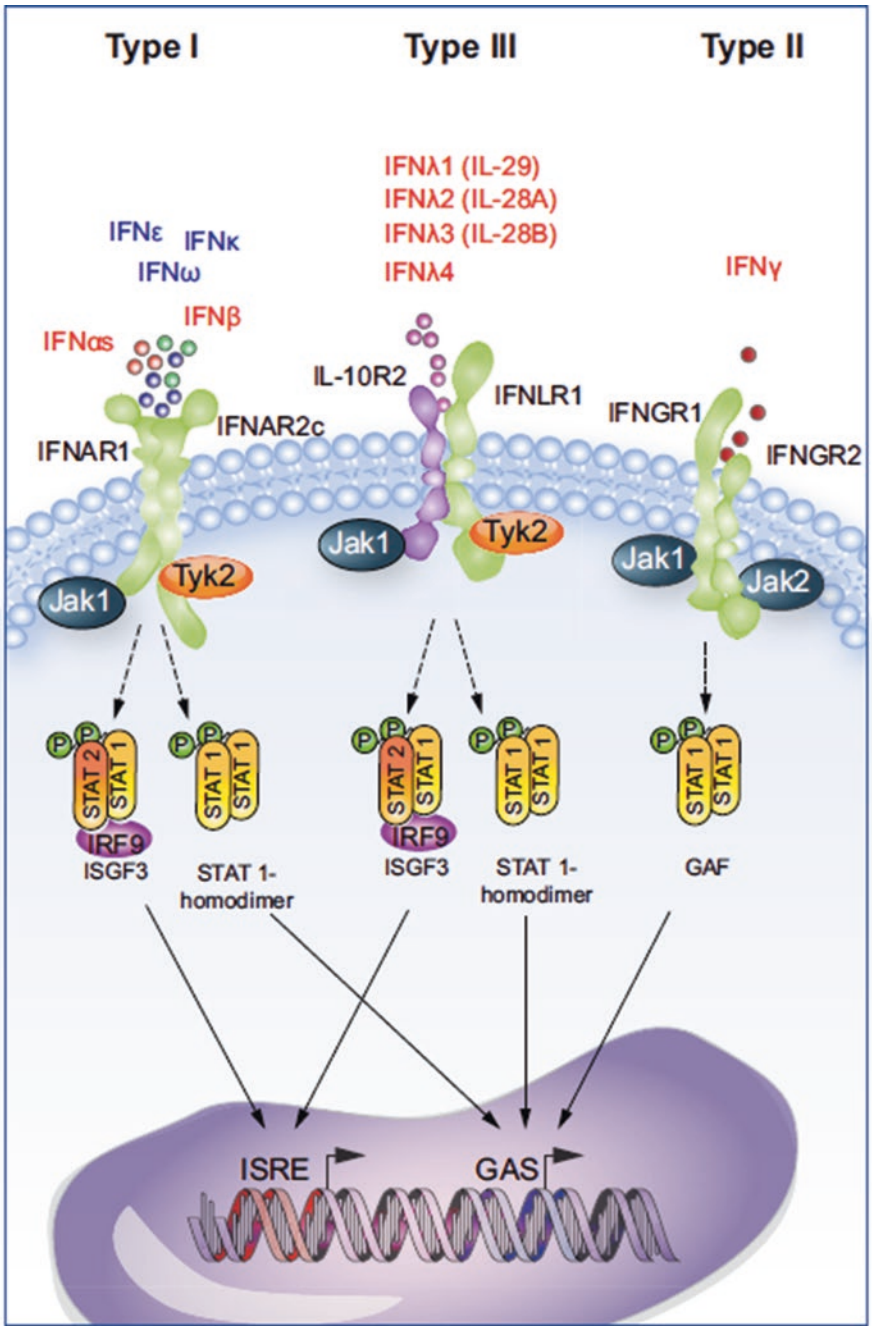


Fig. 39.3 IFN signaling through the Jak-STAT pathway. From Heim and Thimme, Journal of Hepatology (2013) 58:564–574

IFN-α+RBV treatment was shown to be innately tied to the viral load in that patients with high viral loads experience a significantly lower response rate to IFN/RBV antiviral therapy [88]. The underlying molecular mechanisms that influence HCV viral load, antiviral response, and resistance to PEG-IFN-α/RBV therapy in chronically infected HCV patients are still not well understood and are most likely governed by a complex combination of host and viral factors.

3.2 Allele-Specific Responses

The discovery of a correlation between polymorphisms around the human *IL28B* gene and virus clearance underscored the importance of type III interferons in HCV infection. The *IL28B* locus encodes IFN-λ-3, a type III interferon. The polymorphism rs12979860 located on chromosome 19 and 3.2 kb upstream of the *IL28B* gene was shown to strongly correlate with SVR in all patient groups analyzed, including European-Americans, African-Americans, and Hispanics [83]. Genotype 1-infected subjects carrying the T/C alleles were significantly less likely to achieve SVR than subjects carrying the C/C alleles, followed by subjects with the T/T alleles. The C/C allele was also associated with a higher rate of virus clearance in the acute phase of infection. Similar discoveries have been made with the polymorphism rs8099917 located 7.6 kb upstream of the *IL28B* locus, where a favorable response to PEG-IFN/RBV combination treatment is correlated with the T/T allele in genotype 1-infected patients. Why do type III interferons play such a crucial role in predicting response to PEG-IFN-α/RBV therapy as both type III (IFN-λ) and type I (IFN-α and -β) interferons activate almost an identical set of IFN-stimulated genes (ISGs)? The answer to this question may lie in the fact that the respective type I and type III IFNs bind to distinct receptor molecules. A recent study showed that cell surface expression of IFNAR1 (type I IFN receptor) is downregulated in HCV-infected cells, thereby interfering with type 1, but not type III IFN signaling [89]. Inhibition of IFNAR1 expression might be a direct result of ER stress and autophagy induced by HCV infection [90]. Moreover, the kinetics by which IFN-λ induces ISGs also differ from those of IFN-α [87]. Interestingly, the *IL28B* C/C genotype, while associated with a better response rate to IFN/RBV therapy, was also associated with higher viral loads in patients [83]. Higher viral loads generally predict a less favorable treatment response, suggesting that the molecular basis for the association of viral clearance with viral load and *IL28B* genotype differs. This finding also highlights the complex interplay of molecular mechanisms associated with intrinsic resistance against PEG-IFN-α/RBV therapy.

IL28B genotyping has become an integral part of evaluating the baseline characteristics of a patient entering an HCV clinical trial in the past few years. However, it can be expected that the predictability of the *IL28B* genotype will become markedly less profound with DAA-only combination regimens than for interferon-containing therapies. The benefit of ILB28B genotyping also remains controversial for patients infected with HCV genotypes 2 and 3 [91]. Nevertheless, in one study, an *IL28B* genotype-dependent early effect on viral kinetics was detected in an interferon-free regimen consisting of the nucleoside inhibitor mericitabine and the protease inhibitor danoprevir [92].

3.3 HCV Protein-Mediated and Genotype-Specific Susceptibility

HCV genotypes differ in their nucleotide sequence by about 30% and exhibit distinct geographical distribution [93]. While HCV diversifies further through subtypes and quasispecies variability, the genotype is the most predictive factor for IFN response even though the precise association between these two factors is still not completely understood. IFN resistance is particularly common in subjects infected with GT1, which is one reason why cure rates hovered around 50% before DAAs reached the market. In contrast, about 80% of GT2- or 3-infected individuals typically respond favorable to IFN/RBV combination therapy. GT2- and 3-infected patients also respond considerably better to IFN/RBV treatment than subjects infected with GT4. Several regions have been identified to be responsible for the observed genotypic differences in IFN response rates. First, an "IFN-sensitivity-determining region" (ISDR) was identified in the NS5A protein that appears to correlate with resistance to IFN. The ISDR was discovered because a clustering of amino acid changes was observed in this stretch of NS5A during IFN treatment [94]. It was found that the ISDR overlaps with the PKR-binding region and directly inhibits PKR activity, and that mutations in the ISDR lead to a loss of interaction of NS5A with PKR [95]. Inactivation of PKR is a common viral strategy to interfere with the innate immune response as PKR is induced by IFN and blocks viral replication. It has been suggested that the ISDR is under strong immune selection and exhibits limited degrees of freedom to mutate depending on the respective genotype, which could explain why genotypic differences are observed regarding IFN resistance [93]. Next to genotypic differences, HCV subtypes also differ in their ability to respond to IFN. The NS5A protein of GT1a, for example, is less efficient in blocking the antiviral effects of IFN than the NS5A protein of GT1b. Genetic diversity in the ISDR could very well account for these observations. However, while the correlation of ISDR and IFN response seemed to hold true for certain patient cohorts, some discrepancies surfaced when HCV isolates from European countries and the USA were ana-

lyzed [96]. Therefore, the general extent to which the ISDR can predict IFN response appears limited. Furthermore, a region in the viral E2 protein was also demonstrated to bind and inhibit PKR. Stretches of amino acids were shown to be homologous to the phosphorylation domain of PKR as well as to eIF2α [97]. The interaction of E2 with PKR-eIF2α prevents protein synthesis and thereby blocks the antiviral effects of IFN. It can be speculated that the greater resistance to IFN observed in GT1-infected subjects stems from a higher degree of homology of this region when compared to other genotypes, particularly genotypes 2 and 3. On a side note, the fact that HCV has several ways of inhibiting PKR, a tumor suppressor, may also account for the frequent development of HCCs in the advanced stages of the disease.

Another homology domain that was proposed to interfere with host signaling pathways was identified in NS5A from GT1b [98]. This domain was shown to interact with the Src-homology-3 (SH3) domain of growth factor receptor-binding protein 2 (Grb2). It facilitates activation of the Ras-MAP kinase–extracellular signal-regulated kinase (ERK) pathway and the induction of IFN gene expression [98]. Interestingly, the more IFN-sensitive GT2a and 2b also contain much weaker SH3 consensus-binding sequences.

The core region has also been implicated in conferring resistance to PEG-IFN/RBV treatment. Substitutions at amino acid positions 70 and 91 are significantly correlated with poor responses to therapy and also with increased risk of developing HCC in HCV patients infected with GT1b [99, 100]. In GT2a, amino acid position 4 was suggested to affect virologic response [101]. It was concluded that IFN-resistant core mutants attenuate the IFN response by upregulating the cellular interferon signal attenuator SOC3 (suppressor of cytokine signaling 3) [102]. It is difficult to comprehend the full picture of variables in host-virus interactions resulting from genotypic differences (and not all are listed here), but it can be presumed that each genotype interacts differently with its respective host cell and attains a certain degree of effectiveness in counteracting cell defenses.

3.4 Quasispecies Variability and Susceptibility

In addition to genotype and subtype differentiation, HCV diversifies further by existing as a quasispecies in the infected host. The entire HCV genome undergoes constant change and rapidly selects nonsynonymous resistance variants when exposed to antiviral drugs. Moreover, HCV contains hypervariable regions (HVRs) that experience nucleotide substitutions with much higher frequency compared to the rest of the genome and some of those have been associated with resistance to IFN treatment. For example, the E2 gene contains two HVRs that typically show high sequence complexity within a quasispecies. Interestingly, viruses isolated from individuals that showed responsiveness to IFN demonstrated only very little sequence diversity in those two HVRs [103]. It is possible that these individuals had the advantage of carrying quasispecies with low sequence variation in the E2 HVRs before treatment, thereby limiting the ability of the virus to escape host defenses. In fact, the E2 HVRs are thought to contain neutralizing epitopes so that an increased heterogeneity at these sites may increase the chance of the virus to hide from neutralizing antibodies. Moreover, two regions of variability exist in the carboxy-terminal region of the NS5A protein in which treatment-induced amino acid changes have been observed [104, 105]. Similar to the HVRs of the E2 gene, immune selection may also underlie a high degree of sequence variability in the NS5A HVRs, resulting in differences of how the virus evades intracellular antiviral defenses.

3.5 Innate Immune Response and Resistance to IFN/RBV Therapy

On a molecular level, all interferon signaling cascades converge on the Jak/STAT pathway to activate sets of interferon-stimulated genes (ISGs) in the nucleus that counteract a viral infection (Fig. 39.3) [86]. The number of genes induced by this cascade varies between cell types and can range between a couple of 100 to roughly 2000 genes. It is thought that this process essentially accelerates the host immune response; however, the interplay between all the IFN-induced proteins/pathways has not been completely mapped out. It has to be kept in mind that the effects of IFN also extend to noninfected cells, thereby providing enhanced protection from a pending viral infection to those cells.

Type 1 interferons (IFN-α family and IFN-β) signal through the IFNAR1/2 receptor. Type III interferons (IFN-λ family) bind to a distinct receptor (IL28Rα/IL10Rβ) (Fig. 39.3) [86]. Both pathways result in the phosphorylation of STAT1/STAT2 homo- and/or heterodimers, which are recruited to activated receptors through their SH2 domains. The type II interferon IFN-γ signals through the IFNGR1/IFNGR2 receptor, inducing its own distinct set of ISGs via the phosphorylation of STAT1 homodimers [86]. The expression profiles of ISGs have a direct impact on PEG-IFN/RBV responses and may account for variations in treatment efficiencies observed in the clinic. In a study that utilized gene expression profiling to compare PEG-IFN-α/RBV responders to nonresponders, 18 genes showed consistent differential expression between the two patient cohorts [106]. ISG15, for example, was found to be significantly upregulated before treatment in liver biopsies in patients who then did not respond to PEG-IFN/RBV therapy [107]. Polymorphisms in IFN-γ and IL-10 have also been associated with antiviral response rates [108, 109]. In addition,

mutations in other host genes such as MxA, OAS, and PKR have been suggested to be capable of influencing the response to IFN-based treatment [110, 111]. Certain regions in viral proteins specifically target cellular factors in the IFN signaling pathway that might be interrupted by RBV-induced mutations, resulting in an increased response rate. The disruption of core-STAT1 interactions following the accumulation of mutations in the N-terminal region of the core protein during treatment with IFN and RBV is thought to promote favorable treatment outcome and SVR [112]. A multitude of interactions between viral and host factors usually play a vital role in defining the success of any virus to take over the host cell and HCV is certainly not an exception.

3.6 p7 Inhibitors

The p7 protein is a viroporin protein that plays no known role in HCV genome replication, but is essential for assembly and release of infectious virus. Oligomers of the p7 protein form ion channels that allow transport of cations across the ER lumen membrane [113, 114]. Similar to other viroporins, rimantadine and amantadine interfere with p7 ion channel activity and reduce viral replication in cell culture; however, the adamantine inhibition of virus production was genotype specific and did not translate into sufficient efficacy of the clinic [115, 116]. The p7 L20F substitution has been linked to amantadine resistance in genotypes 1b and 2a, putatively due to a change in the p7 oligomer channel shape [116]. The most advanced inhibitor of p7 ion channel activity and HCV replication is BIT225, which has advanced to phase IIa trials in combination with IFN-α and RBV. Few sequence variants were detected in HCV isolates from BIT225-treated subjects; however, these substitutions were not linked to resistance to the drug. While additional candidate drugs (i.e., imino sugar derivatives) have been shown to inhibit p7 activity and HCV replication, no resistance analyses have been disclosed [114, 116].

3.7 Cyclophilin Inhibitors

A mostly neglected, but highly attractive class of anti-HCV host-targeting agents are the cyclophilin (Cyp) inhibitors. They represent viable options for combination therapy with DAAs as they are highly potent and exhibit pan-genotypic activity [117]. Moreover, targeting host factors creates naturally a much higher genetic barrier to resistance compared to viral targets that are transcribed by a profoundly error-prone RNA-dependent RNA polymerase.

The first Cyp inhibitor, cyclosporin A, was originally discovered as an immunosuppressant drug for preventing graft rejection in organ transplantation [118]. The discovery that also acts as an efficient inhibitor of viral replication came following testing for its hepatitis-associated anti-inflammatory effects in HCV-infected patients [119]. Second-generation cyclophilin inhibitors aimed at uncoupling immunosuppressive effects from antiviral activity and resulted in the development of several cyclosporin A derivatives, including NIM-811, alisporivir, and SCY-635. These compounds exhibit enhanced binding to their intracellular receptor, cyclophilin A (CypA), neutralizing its isomerase activity and also blocking the de novo formation of active viral replication complexes and double-membrane vesicles [120, 121]. In the absence of cyclophilin antagonists, such as cyclosporines, the receptor was suggested to bind directly to the NS5A protein of HCV (Fig. 39.4) [117]. This binding was shown to be dependent on the dose of the inhibitor, but independent of the genotype the NS5A protein was derived from [117, 122]. Consistent with the idea of a direct interaction, cyclosporine resistance mutations have been identified

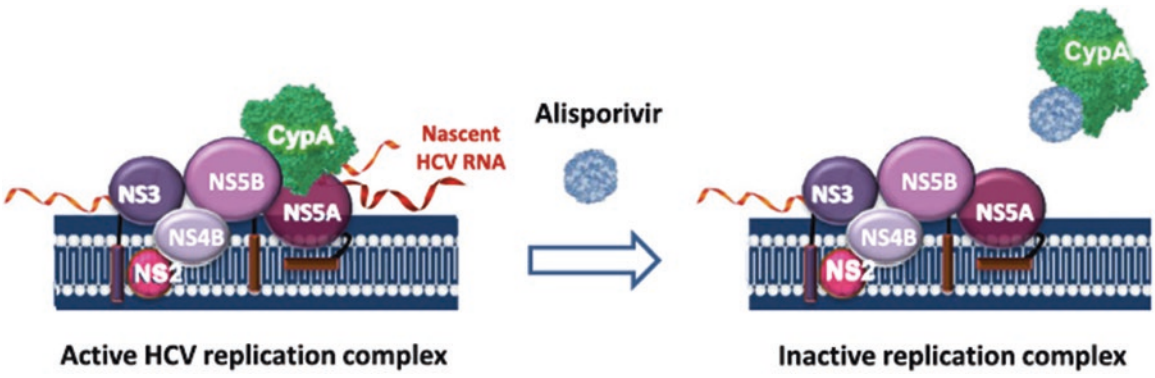


Fig. 39.4 Mechanism of action of cyclophilin inhibitors in HCV replication. Cyclophilin A (CypA) binds directly to the NS5A protein of HCV, a substrate of CypA PPIase, which is required for the formation of a functional viral replication complex. Cyclophilin inhibitors such as alisporivir inhibit CypA PPIase, blocking its interaction with NS5A, and thus inhibit viral replication. From Lin and Gallay (2013) Antiviral Research 99:68–77

in NS5A (i.e., D316E and Y317N) that can rescue the formation of double-membrane vesicles [120]. The amino acid change D320E in NS5A was identified to confer the majority of resistance to NIM-811, even though other minority resistance mutations were identified in the NS3 and NS5B genes [123]. Surprisingly, however, the D320E substitution does not appear to disrupt the ability of NS5A to bind cyclophilin A [122]. Then how exactly does this change interfere with the antiviral activity of Cyp inhibitors? It has been suggested that the D320E substitution may in some way bypass the HCV need for CypA by changing the mode of how NS5A interacts with NS5B [122]. Indeed, one study showed that the NS5A region Pro-306 to Glu-323 represents a common binding site for both NS5B and CypA and thus urges to be cautious about the interpretation of mutations in this specific region [124]. This is because observed effects of mutations in this stretch of amino acids could be due to modulations in NS5A-NS5B and/or NS5A-CypA interactions. Independent of these pitfalls, it has clearly been demonstrated that cyclophilin A binding to NS5A is correlated with isomerase activity. When a mutation is introduced into the enzymatic pocket of CypA, thereby abrogating isomerase activity, the protein fails to bind NS5A [122]. This underscores that NS5A binds directly to the isomerase pocket of CypA, and while challenges remain to understand their precise mechanism of action, Cyp inhibitors are in this aspect no different from many of the DAAs currently in clinical development.

To date, alisporivir (ALV) is the most advanced compound of the Cyp inhibitor class. It is currently tested in phase III clinical trials and has so far demonstrated higher efficacy in patients infected with HCV genotypes 2 and 3 than genotypes 1 and 4 [117]. As expected for a host-targeting antiviral, ALV exhibits pan-genotypic activity with EC_{50} values ranging between ~10 and 30 nM for genotypes 1–4 [125]. The compound has also shown promising results in combination with other DAAs, including NS3, NS5A, and NS5B inhibitors. For HCV GT1 and 4, additive effects were reported when ALV was combined with DAAs in vitro, while a specific and synergistic effect was observed on GT2 and 3 in combination with the NS5A inhibitor daclatasvir [125]. It was suggested that this effect may be due to the fact that both types of inhibitors target different domains of NS5A: ALV blocks interaction of NS5A with CypA by binding to domain II, while NS5A inhibitors interact with NS5A domain I. Importantly, no cross-resistance was detected between DAAs and ALV [122]. ALV remained fully active against DAA-resistant variants and, vice versa, ALV-resistant variants were fully susceptible to DAAs. These studies included the previously mentioned NS5A D320E mutation, which renders HCV more resistant to ALV and other Cyp inhibitors. Even the ALV-resistant double mutant (D320E/Y321N) remained fully susceptible to a range of DAAs such as boceprevir, telaprevir, sofosbuvir, and daclatasvir [122]. Likewise, ALV remained active against replicons carrying common DAA resistance variants in NS3 (R155Q, A156T), NS5A (L31V), and NS5B (S282T). Together with the fact that ALV has shown generally good tolerability, especially in IFN-free trials [117], these data support the idea that a combination of ALV with DAAs may afford a multigenotypic regimen. This is promising not only for specific patient subpopulations such as IFN nonresponders, but also for the HCV field altogether as it tries to move rapidly away from IFN-containing regimens.

4 HCV Therapies and Related Resistance: Direct-Acting Antivirals

The importance of resistance in the era of HCV direct-acting antivirals (DAAs) is a subject of intensive discussion between experts in the field. Amino acid substitutions that confer resistance to DAAs are observed in most patients with treatment failure, but the value of resistance testing in the clinic remains of debate. This is because it has become clear that the presence of DAA resistance polymorphisms prior to treatment is generally not predictive of clinical outcome. Poor interferon responsiveness, for example, has a comparable much higher predictive value of non-SVR than DAA baseline variants [126]. The question then arises whether it is worthwhile to routinely perform DAA resistance testing in the clinic when (1) baseline testing does not predict treatment outcome, (2) current DAA regimens achieve over 90 % cure rates in treatment-naive patients, and (3) we already know that virtually all patients with treatment failure do harbor resistance mutations. It has therefore been argued that it might be too high of a burden for clinicians to implement routine resistance testing as such procedures are cost and labor intensive. Possibly the most value in resistance testing lies with treatment failures prior to re-treatment to evaluate the persistence of previously selected variants. However, many research scientists feel that such an approach will lead to a loss of valuable data over time, and reflects an irresponsible strategy in the light of our incomplete knowledge of the HCV resistance landscape to date as well as in the future. Moreover, preexisting RAVs can shift overall SVR rates, either significantly or ever so slightly. Because DAAs have only very recently been introduced into the clinic, routine testing of the growing numbers of patients treated could prove crucial to gain a profound understanding of the long-term effects of DAA resistance in the broad HCV population.

4.1 Protease Inhibitors

4.1.1 The PI Landscape

The HCV NS3/4A serine protease inhibitors (PIs) were the first class of direct-acting antivirals (DAA) introduced in clinical practice. These compounds are either tetra-peptide α-ketoamide derivatives or have macrocyclic structures. Boceprevir (Merck & Co., Kenilworth, NJ) and telaprevir (Vertex Pharmaceuticals, Cambridge, MA), two linear PIs that covalently, though reversibly, bind the catalytic Ser139 residue of the protease, were introduced to the market in 2011. Both drugs were approved for the use against genotype 1 in combination with IFN/RBV and increased SVR rates by approximately 30 % in treatment-naive genotype 1 patients compared to dual therapy with IFN/RBV alone. It is difficult to achieve pan-genotypic activity with PIs, which is also true for the NS5A and non-nucleoside inhibitors. This is due to the sequence variation in the respective drug-binding sites between genotypes and subtypes [127]. Telaprevir showed relatively good activity in GT2, limited efficacy in GT4, and no activity in GT3 [128]. Boceprevir was demonstrated to exhibit some antiviral activity against genotypes 2 and 3. While HCV genotype 1 is the most common subtype in the USA and Europe, it is also the most difficult to treat. Only approximately half of the patients infected with HCV genotype 1 typically achieve SVR with IFN/RBV dual therapy. The introduction of boceprevir and telaprevir therefore dramatically changed the treatment landscape of patients infected with HCV GT1, increasing SVR rates to 70–80 % in treatment-naive patients [2]. Another advantage of the protease inhibitors was that treatment duration could be shortened from 48 weeks to 28 or even 24 weeks. Due to their similar mechanism of action, both first-wave PIs have overlapping resistance profiles. Amino acid substitutions conferring resistance to these classes of drugs emerge rapidly during monotherapy, and are readily observed in a majority of subjects who do not achieve SVR following triple therapy. Drug resistance with PIs is a complex interplay of molecular events whereby preexisting polymorphisms in the viral population are selected during the treatment process because they result in a decrease in inhibitory potency while retaining enzymatic activity of the NS3/4A protease and viral replication. It is important to understand that emergence of resistance is certainly a consequence of treatment failure, but not always the cause. To maximize viral response and to minimize resistance, combination regimens are a crucial strategy to limit the responsiveness of the virus to antiviral therapy. Interferon and ribavirin thus remained integral parts of HCV standard-of-care therapy for genotype 1 patients at the time.

Meanwhile, the development of additional classes of PIs pressed onward and resulted in the approval of a second-wave protease inhibitor in late 2013. Simeprevir (Janssen Therapeutics, Titusville, NJ) belongs to the class of macrocyclic protease inhibitors, which differ in their mode of action from the linear ketoamide compounds. Macrocyclic PIs act as non-covalent competitive inhibitors to the NS3 protein by mimicking a substrate derived from the NS5A/B cleavage site. Similar to boceprevir and telaprevir, simeprevir exhibits nanomolar potency against the wild-type enzyme with cure rates of about 80 % in treatment-naive GT1 patients after 12 weeks of treatment. In addition, it offers improved side effects, once-a-day dosing, and was approved in Europe and the USA for the use in GT1 as well as GT4 patients. The drug is not recommended to be used in GT2 and GT3 patients due to its poor in vitro and in vivo activity [129]. In the clinic, the first-wave PIs boceprevir and telaprevir have now practically been replaced with more advanced FDA-approved regimens that include simeprevir, the NS5A inhibitor ledipasvir, and the NS5B non-nucleoside inhibitor sofosbuvir [HCV 75]. Because simeprevir's profile exhibits a low genetic barrier for resistance and some cross-resistance to boceprevir and telaprevir, it has been suggested that the term "second-generation" PI is more applicable for upcoming investigational drugs with an improved resistance profile. Many such PIs are currently in late-stage development, some of which are expected to reach the market in the coming years, such as faldaprevir (BI 201335), asunaprevir (BMS-650032), danoprevir (RG7128), grazoprevir (MK-5172), and GS-9451. The most recent addition to the FDA-approved PI landscape has been paritaprevir (ABT-450/r) as part of Abbvie's three-DAA drug combination in December 2013.

4.1.2 Resistance to Macrocyclic PIs

Even though baseline polymorphisms are not directly predictive of clinical outcome, they can increase the overall risk of treatment failure, resulting in substantially reduced SVR rates. This became largely transparent with a single polymorphism (Q80K) in patients treated with simeprevir. The Q80K polymorphism is commonly found in GT1a patients (Fig. 39.5) [130] and is associated with an impaired response to simeprevir in vitro as well as in the clinic. In the QUEST studies, only 58 % of treatment-naive subjects with the Q80K polymorphism achieved SVR, which was comparable to the 55 % SVR rate of subjects in the placebo arm. In contrast, 84 % of patients without the Q80K polymorphism and treated with simeprevir achieved SVR [40]. The Q80K polymorphism is present, on average, in over one-third (~37 %) of GT1a-infected subjects at baseline compared to 0.8 % in subjects infected with GT1b [37]. Interestingly, the actual number varies depending on the geographic origin of the population in question. European patients infected with GT1a appear to carry the Q80K variant at a lower frequency compared to subjects born in the Americas. This is correlated with the observation that GT1a can be distinguished into two separate clades, clades 1 and 2, where clade 2 is more commonly found in Europe and is also associated with

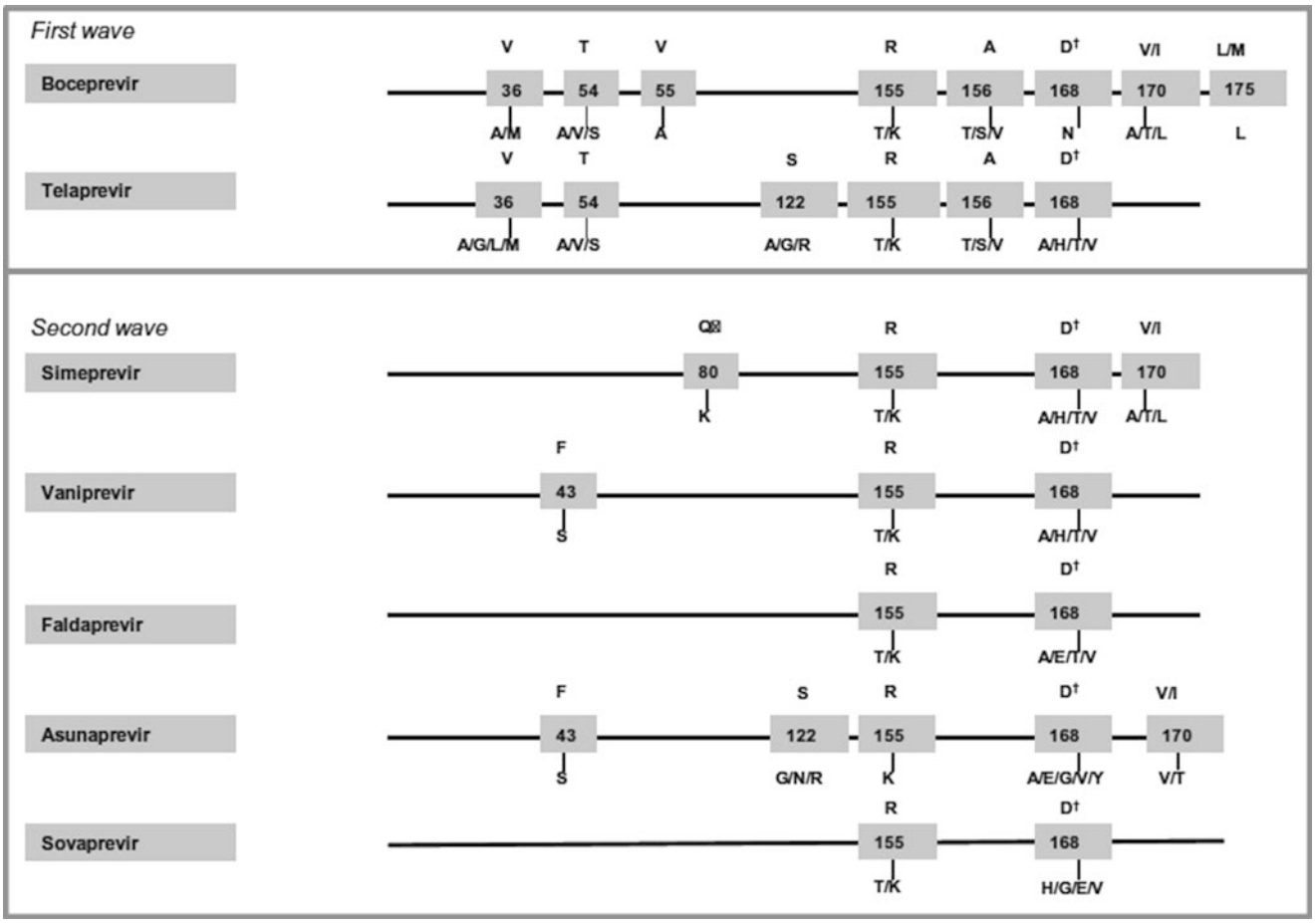


Fig. 39.5 Main resistance mutations associated with first and second wave of first generation of protease inhibitors. Q80K is a natural polymorphism found in 19–48 % of HCV genotype 1a and is associated with loss of susceptibility to simeprevir. D168Q is found in almost all HCV genotype 3 conferring natural resistance to most protease inhibitors. From Poveda et al., Antiviral Research (2014) 108:181–191

a lower Q80K frequency [131, 132]. The high prevalence of the Q80K polymorphism in the human population, together with its significant negative effect on treatment outcome, resulted in the FDA's decision to recommend Q80K testing for all GT1a patients receiving simeprevir-containing regimens. However, the effect of the Q80K substitution was shown to be less profound when simeprevir was used together with the nucleoside inhibitor sofosbuvir, a drug with a high genetic barrier to resistance [133].

The most common treatment-emergent RAVs observed in patients with treatment failure after exposure to simeprevir are R155K in GT1a and D168V in GT1b alone, or in combination with changes at position 122 or 80, including Q80R or Q80K (Fig. 39.5) [130, 134]. This is consistent with simeprevir's in vitro resistance profile, which was determined through the use of GT1a and GT1b replicon cell lines [135]. The clinical resistance patterns for other genotypes were shown to be similar to those identified in genotype 1. In a monotherapy study that tested the antiviral activity of simeprevir in genotypes 2–6, viral breakthrough in GTs 4–6 was associated with emergent variants, mainly Q80R, R155K, and/or D168E/V [129]. In those genotypes, changes at position 169 were also detected. For example, the substitution F169F/I occurred in one genotype 6 patient together with D168E. The combination of these two mutations was tested in a GT1b replicon assay and resulted in an increased resistance of 43- to 194-fold compared to D168E alone [129]. However, F169I alone had no effect on the efficacy of simeprevir. Overall, limited antiviral activity of simeprevir was observed in GTs 2 and 5, and no activity in GT3. A majority of patients infected with GT3 carry D168Q as a baseline variant, which was suggested to be the reason for simeprevir's, and most other PI's, lack of activity in this genotype [129]. This variant had also been shown to significantly reduce the in vitro activity of another macrocyclic protease inhibitor, ciluprevir (BILN-2061) [136]. The study of genotype-specific efficacies of PIs has been slow in general as the development of appropriate in vitro systems for GTs 3–6 has been lagging behind that of GTs 1 and 2. However,

significant advances have lately been made in this area and more attention has recently been given to study the efficacies of DAAs against all genotypes.

Macrocyclics currently in development aim at improving potency, pan-genotypic activity, and resistance profiles. Macrocyclization is a valuable strategy to enhance drug potency as these compounds have the inherent advantage of a constraint structure that limits the mixture of cis- and trans-rotamers seen with linear peptides. Macrocyclic structures are also less prone to proteolytic cleavage. The location of the macrocycle appears to play a role in avoiding drug resistance and novel P1–P3 (i.e., danoprevir) and P2–P4 (i.e., grazoprevir) constrained macrocyclic inhibitors are currently being evaluated in the clinic. Similar to simeprevir, danoprevir (RG7128), vaniprevir (MK-7009), and paritaprevir (ABT-450) are all highly susceptible to mutations at amino acid residues R155 and D168. For paritaprevir (ABT-450), a macrocyclic acrylsulfonamide inhibitor of the NS3 protease, a recent study showed that the most commonly selected variants following ABT-450 monotherapy were R155K and D168V in GT1a, and D168V in GT1b [137]. The substitution D168Y was demonstrated to exhibit the highest level of resistance in vitro in both 1a and 1b genotypes.

Grazoprevir (MK-5172) distinguishes itself from the first-generation PIs by retaining subnanomolar potency against substitutions at the 168 locus in GT1b replicon assays, though grazoprevir does show elevated susceptibility to the A156T and A156V variants [71, 138]. This is not surprising as NS3 position 156 has been associated with resistance to all PIs currently approved by the FDA (either in the clinic (boceprevir and telaprevir) or during in vitro studies (simeprevir)) and most if not all agents currently in development. Importantly, grazoprevir was shown to be effective against a range of HCV genotypes and subtypes (GTs 1, 1b, 2a, 2b, and 3a) [71]. Such second-generation PIs will provide important key components of future DAA-only combinations.

4.1.3 Resistance to Linear PIs

Compared to the common GT1a Q80K variant associated with simeprevir resistance, the baseline prevalence of variants associated with resistance against linear PIs, such as boceprevir and telaprevir, is relatively low in both GT1a and GT1b DAA-naive patients. Population sequencing of over 3000 subjects showed that lower level resistance variants (25 % increase of the IC_{50}) were present as the dominant species in only 0–3 % of patients [37]. High-level resistance variants (>25-fold increased IC_{50}) were not observed. For the two approved covalent linear inhibitors boceprevir and telaprevir, a consistent clinical resistance pattern was identified with primary treatment-emergent variants occurring at NS3 positions 36, 54, 155, 156, and 168 [Ann Forum Collab HIV 139]. The major amino acid substitutions at these loci were V36A/L/M, T54A/S, R155K/T, A156S/T/V, and D168N (Fig. 39.5) [130]. Additional boceprevir-resistant substitutions that exhibit no cross-resistance with telaprevir constitute V55A, V158I, and I/V170A/T. Naturally, the resistance profile is dependent on the genotype and subtype. Genotype 1a subjects mainly select variants at amino acid residues 36 and 155, while patients infected with genotype 1b experience changes at positions 54, 55, 156, and 170 [130]. Furthermore, the majority amino acid L175 in GT1a is a resistant variant in GT1b (M175L). Only a single-amino acid substitution was observed following telaprevir exposure that did not exhibit cross-resistance with boceprevir; the I132V substitution was detected in less than 10 % of treatment-failure GT1a patients; however, this variant did not shift the IC_{50} for telaprevir in vitro for GT1a, and V132 is the majority variant in subtype 1b [139]. The observed genotypic differences of selected variants often have their molecular basis in the number of nucleotide changes it takes to result in an amino acid change that confers resistance. The R155K resistance mutation, for example, requires only one nucleotide change in GT1a (AGG to AAG), but two nucleotide changes in GT1b (CGG to AAG). Moreover, the selection process is also influenced by genotypic differences in the viral fitness of drug-resistant viral populations. These aspects combined can explain why genotype 1b generally displays a higher genetic barrier to resistance compared to genotype 1a in patients treated with first-wave PIs.

Even when sequencing technologies with higher detection sensitivities are applied, most baseline NS3 RAVs do not appear to be present at significant frequencies. In one study, 33 individuals were subjected to NGS analysis, but baseline NS3 RAVs at residues 155, 156, and 168 remained undetectable even with a frequency cutoff at 0.25 % [30]. However, RAVs at those positions (R155K, R155T, D168N, and D168E) became detectable as early as 24 h after the first dose following monotherapy with investigational linear NS3 inhibitors (GS-9256 or GS-9451). Other investigational linear NS3 inhibitors currently in development exhibit, for the most part, fairly similar resistance profiles. Exposure to faldaprevir (BI 201335), a linear tripeptide that inhibits the NS3 protein through non-covalent interaction with the catalytic site, also results in treatment-emergent changes at amino acid residues R155, A156, and D168 in genotype 1 [140]. The R155K and D168V substitutions were the predominant selected variants following faldaprevir monotherapy in GT1a and GT1b, respectively. As discussed above, the D168V substitution is also commonly observed with the macrocyclic inhibitors in GT1b, while it is typically not seen with the covalent linear ketoamide agents. Also in contrast to boceprevir and telaprevir, substitutions at NS3 residues V36 and T54 were not associated with resistance against faldaprevir in vitro [141].

4.1.4 Persistence of NS3 RAVs

A comprehensive understanding of how long treatment-emergent variants associated with resistance against common NS3 inhibitors remain present in a viral population is important to provide the best re-treatment options for a patient. Even though preexisting RAVs are not highly predictive of clinical outcome, it has become clear that the presence of such RAVs can influence SVR rates and thus the risk of an individual patient to experience treatment failure. Long-term follow-up studies can provide information on the average persistence times of specific NS3 RAVs that may guide decisions on the best available re-treatment option as well as the general necessity of resistance testing. The ability of a specific RAV to persist in a viral population is strongly correlated with its viral fitness and replication capacity. The more an amino acid substitution inhibits viral replication and protein function, the faster it will be replaced by wild-type sequence in the absence of selection pressure. Nonetheless, such variants can persist at significant frequencies for a prolonged amount of time if they occur together with compensatory mutations that facilitate replicative fitness.

One study followed patients who had undergone treatment with boceprevir or telaprevir for an average of approximately 4 years to assess the persistence of common NS3 RAVs by clonal sequencing analysis. Variants at positions 36, 54, 55, 156, and 170, which commonly arise following exposure to boceprevir and telaprevir, had reverted to wild-type sequence in most patients infected with GT1 at long-term follow-up [142]. An initial inclination that RAVs not only occur more often but also persist considerably longer in GT1a compared to GT1b-infected patients was confirmed by a large study involving over 1700 subjects [27]. Resistance-associated variants following treatment with telaprevir were confirmed by population sequencing in 86 % of GT1a-infected patients, whereas only 56 % of subjects infected with GT1b presented with resistance by end of treatment. Furthermore, the study demonstrated that the medium time it took for an RAV to convert to wild-type sequence was 10.6 months in subtype 1a versus 0.9 months in subtype 1b [27]. This study confirmed that after removal of the drug selection pressure, wild-type virus outcompetes fitness-impaired virus populations either through outgrowth from a residual population of wild-type virus or by mutating back to wild-type sequence. Because the common 1a variants V36M and R155K have higher replication capability than the typical 1b variants, more time is required for those variants to be replaced with wild-type virus. It certainly has to be taken into account that sequencing methods with higher detection sensitivity may uncover mutations that persist for much longer periods of time at low frequencies. However, a small ultra-deep sequencing study performed with a 0.05 % frequency cutoff showed that the prevalence of telaprevir-resistant variants was not elevated compared to baseline after 4 years [31]. This suggests that even when highly sensitive sequencing methods are used, PI-induced RAVs rarely persist long-term. Comprehensive data on the persistence of RAVs following treatment with second-wave PIs is still scarce and it will take time before those data will be available. However, initial analyses are consistent with previous data based on studies with telaprevir or boceprevir, indicating that NS3 RAVs are generally not as stable as, for example, resistance mutations that occur in the NS5A gene. In a small cohort of patients co-treated with the investigational PI asunaprevir and daclatasvir, an NS5A inhibitor, NS3 RAVs generally decayed much faster over time compared to resistance variants in NS5A [143]. Paritaprevir-resistant variants were detected at 6 months in 50–60 % of patients following treatment cessation, but only in about 20 % of individuals by 12 months [144].

Even though resistance variants usually can be expected to exhibit impaired viral fitness, they can occur and persist naturally in the absence of drug exposure. For instance, while R155K substitutions are detected in less than 1 % of HCV-infected patients at the time, it is possible that such a variant remains present in the viral population for an extended period of time. In one patient such a naturally occurring R155K substitution was shown to coexist with wild-type variants for over 1 year and it was suggested that in some immunocompromised patients such RAVs may not display clinically significant reduced fitness [145, 146].

In general, RNA viruses that solely replicate in the cytoplasm of the infected cell have a lower chance to retain long-term persistence compared to other types of viruses as they do not have the capability to store their genomic material in the form of integrated DNA (retroviruses) or other long-lasting nuclear DNA forms (e.g., hepatitis B virus). Their entire replication cycle occurs in the cytoplasm and in the form of RNA intermediates. Without an archiving mechanism that provides access to long-term memory, it can be assumed that HCV virus populations will have to start from scratch to develop resistance once RAV frequencies have reverted to baseline levels.

4.2 NS4B Inhibitors

Numerous candidate drugs have been developed to abrogate the membranous web-forming function of the HCV NS4B protein. In doing so, the NS4B DAAs prevent the assembly of HCV nonstructural proteins into replication complexes, disrupting viral replication. Numerous chemical scaffolds have been demonstrated to specifically inhibit NS4B activity. Resistance analyses have demonstrated that inhibition of genotype 1 HCV replication by silibinin, pyrazolopyrimidines, piperazinones, indolpyridines, and imidazopyridines (such as GS-546288) can be overcome by the emergence of

variants at NS4B H94 (N/R), F98 (C/L), and/or V105 (L/M) [47, 147–149]. Accessory substitutions within NS4B (I22L, T45A, and F90L) have been demonstrated to enhance resistance conferred by H94 substitutions or improve the replication fitness of various NS4B mutations [47]. Furthermore, the activity of silibinin and these scaffolds is genotype specific, with low micromolar or better potency (as measured by 50 % effective concentration in vitro) against genotype 1 and often absent potency in genotype 2a. The predominant consensus NS4B L98 variant in genotype 2a is associated with most, but not all, of the resistance to NS4B inhibitors [148, 150–153]. While silibinin is not active against genotype 2a replicons in vitro as well, its resistance is unique compared to other NS4B inhibitor chemotypes. Silibinin-resistant variants include NS4B Q1914R in genotype 1b as well as F1809L and D1939N [154]. Derivatives of amiloride and clemizole are under investigation as potential selective NS4B inhibitors [47].

4.3 NS5A Inhibitors

4.3.1 The NS5A Inhibitor Landscape

The nonstructural 5A protein (NS5A) is an essential component of the HCV replication complex. While it does not exhibit any known enzymatic activities, it is thought to be a multifunctional protein with roles in ER membrane localization, cell signaling, genomic RNA replication, and viral assembly [155–157]. The NS5A protein has been of major interest as an antiviral target for the management of HCV infections as inhibitors of this protein show picomolar in vitro potency, which translates into a rapid virological response in patients, and broad genotypic coverage. Daclatasvir (formerly BMS-790052) was the first-in-class inhibitor developed by Bristol-Myers Squibb and approved in Europe in August 2014. Like the protease inhibitors, NS5A inhibitors exhibit a low genetic barrier to resistance so that they are most effective in combination with other DAAs. Daclatasvir in combination with the PI asunaprevir constituted the first all-oral interferon- and ribavirin-free regimen for the treatment of HCV in Japan. This regimen achieved similar SVR rates in GT1b infection when compared to other approved therapies that contained interferon and ribavirin, but was not considered optimal for GT1a-infected subjects [158].

In October 2014, the FDA approved a fixed-dose combination of the NS5A inhibitor ledipasvir together with the polymerase inhibitor sofosbuvir (Gilead Sciences). This combination treatment (Harvoni™) significantly contributed to the advancement of the HCV treatment landscape for patients infected with genotype 1 by further limiting the need for ribavirin and interferon as well as reducing treatment duration. Individual treatment regimens and times depend on prior treatment history, cirrhosis status, and baseline viral load, but impressive SVR rates of above 90 % are now considered to be the norm with the recently approved treatment regimens for GT1. Similarly to Harvoni™, a combination regimen of daclatasvir with sofosbuvir with and without ribavirin for 12 or 24 weeks also proved highly potent. In an open-label study, 98 % of patients infected with GT1 achieved SVR independent on whether they were treatment naive or experienced [159]. For patients infected with GT2 or GT3, SVR rates were significantly lower with 92 % for patients with genotype 2 infection and 89 % for patients with genotype 3 infection [159]. It appears that all first-generation NS5A inhibitors exhibit in general lower antiviral activity in GTs 2 and 3 compared to GT1 in the clinic. This is also consistent with data observed from in vitro studies. For example, the EC_{50} values measured in full-length HCV replicon assays for ledipasvir were 31 pM and 4 pM for GTs 1a and 1b, respectively, while in chimeric replicon assays encoding NS5A sequences from clinical GT2a, GT2b, and GT3a isolates, much larger values of 21–249 nM (GT2a), 16–530 nM (GT2b), and 168 nM (GT3a) were measured [160]. Similarly, daclatasvir also shows less potency in GTs 2 and 3 in vitro compared to GTs 1a and 1b [161]. It is important to note that with increasingly effective treatments available for GT1, other genotypes (i.e., GT3) face more and more of a challenge to keep up with the much improved treatment standards.

In December 2014, the FDA approved Abbvie's three-DAA drug combination for the treatment of patients with HCV genotype 1 infection, including those with compensated cirrhosis. The NS5A inhibitor ombitasvir (ABT-267) is an integral part of this DAA regimen that also comprises the protease inhibitor paritaprevir (ABT-450) and the non-nucleoside inhibitor dasabuvir (ABT-333). This drug combo delivered compelling functional cure rates in the mid-90 % range during clinical trials and is expected to put significant pressure on the existing HCV price market. Ombitasvir, structurally related to both daclatasvir and ledipasvir, exhibits EC_{50} values of 14 pM and 5 pM in GTs 1a and 1b HCV full-length replicon assays, respectively, but most impressively maintains its picomolar activity also against GT2 and 3 in vitro [144].

4.3.2 Resistance to NS5A Inhibitors

All currently approved first-generation NS5A replication complex inhibitors, including ledipasvir, daclatasvir, and ombitasvir, share a similar resistance profile. Because they exhibit a low genetic barrier to resistance, mutations can arise rapidly during monotherapy. Even though the precise function(s) of the NS5A protein remains somewhat unclear, resistance was mapped consistently and exclusively to the N-terminal region—specifically the first 100 amino acids—of the NS5A protein. This indicates that the currently approved NS5A inhibitors directly target the NS5A protein and act by comparable mechanisms of action. Rather than

affecting protein stability of dimerization, the current NS5A inhibitors appear to interfere with the formation of the membranous web [162]. This idea is supported by the close positioning of the major resistance variants to the membrane-proximal surface of the dimer interface.

Similar to what has been observed with PIs, resistance patterns differ depending on the genotype they arise in, and resistance is also more easily induced in patients infected with GT1a than GT1b [26, 163]. For daclatasvir, the best studied compound of this class of inhibitors, the primary substitutions of resistance are M28T, Q30E/H/R, L31M/V, P32L, and Y93C/H/N for GT1a, and L31F/V, P32L, and Y93H/N for GT1b [26, 28, 143, 163]. L23F, R30Q, and P58S represent secondary resistance variants for genotype 1b. These substitutions do not confer resistance to other classes of DAAs such as the protease or NS5B inhibitors. Y93N was shown to confer the highest resistance in the GT1a replicon system, followed by the Q30E substitution. Both changes shift the EC_{50} values >25,000-fold into the nanomolar range [28]. In contrast, the compound retained sub-nanomolar potency against all single-amino acid substitutions in the GT1b replicon system. However, a deletion (P32del) has been detected to arise in clinical trials in patients infected with genotype 1b that confers extremely high resistance to daclatasvir (>390,000-fold resistance) in vitro [28]. For ledipasvir, the substitution Y93H was found to be the major resistance mutation for both GT1a and GT1b in the HCV cell replicon system. In addition, the substitution Q30E emerged in GT1a replicons [160]. The amino acid changes at those positions conferred high levels of resistance to ledipasvir with >1000-fold increased EC_{50} values. Compared to daclatasvir, ledipasvir is less active against the Y93H mutation (0.05 nM vs. 5 nM) in GT1b, but more active against the M28T and Q30H variants in GT1a [164]. Ledipasvir's clinical resistance profile is also consistent with the obtained in vitro data and demonstrated resistance at the NS5A loci 28, 30, 31, and 93 [165]. In vitro resistance selection with Abbvie's ombitasvir also identified amino acid positions 28, 30, 31, 58, and 93 as the major resistance-associated loci for GTs 1–6 [166]. The substitutions M28T, Q30R, and Y93C/H conferred over 800-fold levels of resistance, while M28V increased EC_{50} values by 58-fold in the GT1a replicon system. Similar to what was observed with daclatasvir, the major resistance mutations in the GT1b replicon system showed a more moderate increase in levels of resistance. The Y93H substitution demonstrated a 77-fold increase in the EC_{50} value, but single-amino acid variants at positions 28, 30, and 31 conferred <10-fold resistance [166, 167].

Resistance in HCV genotype 2 has been an interesting story for the first generation of NS5A inhibitors. The amino acid position 31 is polymorphic in both GT2a and GT2b with the majority of individuals carrying a methionine at this position and the second most common variant being leucine [166, 168]. NS5A inhibitors of the first generation often show minimal activity against the M31 baseline polymorphisms in patients infected with GT2 [168]. Daclatasvir demonstrated the lowest resistance barrier for GT2a-M31 in vitro relative to all other genotypes [169]. Importantly, it appears that when daclatasvir is used in combination with an NS3 or NS5B inhibitor in GT2 patients, the negative effect of the M31 polymorphism is largely attenuated in the clinic [159, 169]. Moreover, L31M is the consensus sequence in genotype 4 and this does not appear to negatively influence antiviral activity of first-generation NS5A inhibitors [168]. For ombitasvir, the predominant GT2-selected variants did not include M31, but were T24A and F28S for GT2a and L31V and Y93H for GT2b [166]. The behavior of these mutants was assessed in the L31 as well as the M31 background, but no difference was detected in the respective levels of resistance for GT2a. For GT2b, the L28F variant showed higher resistance levels in the M31 background, but not as high as the 31V variant alone [166].

Compared to genotypes 1a and 1b, much less is known about clinical resistance regarding NS5A inhibitors in genotypes 3 and 4. The substitution A30K may constitute a relevant baseline polymorphism in genotype 3 in vivo as a clinical trial patient with this baseline variant experienced virologic failure following treatment with daclatasvir and sofosbuvir in the absence of any other treatment-emergent RAV [159]. While the A30K substitution was shown to result in over a 60-fold increase in resistance levels, the substitutions L31F (>320-fold increase) and Y93H (>2750-fold increase) were the two major RAVs that emerged during in vitro selection experiments in genotype 3a replicon assays [170]. In vitro experiments with daclatasvir in genotype 4a hybrid cell lines implicated the amino acid position 30 in resistance [171].

A significant body of evidence has emerged over the past few years that preexisting RAVs in the NS5A gene can significantly affect clinical outcome [164, 168, 172]. Like NS3 baseline polymorphisms, they are not predictive of treatment success; however, they have the potential to negatively influence SVR rates and with that the probability of an individual to experience virologic failure. It appears that with the exception of the NS3 Q80K polymorphism in GT1a, NS5A baseline RAVs have a greater impact on treatment outcome than NS3 polymorphisms [173]. In addition, NS5A RAVs have a higher prevalence in the general population compared to NS3 preexisting resistance-associated polymorphisms [37, 174, 175]. A Japanese study showed that baseline NS5A RAVs, especially Y93H, are highly prevalent in subjects infected with GT1b with 2.2 % harboring L31I/M and 19 % harboring Y93H polymorphisms [175]. Interestingly, Y93H substitutions at baseline were correlated with lower platelet counts, higher viral loads, and higher prevalence of the IL28B T/T genotype. In contrast, only 7.9 % of GT1b sub-

jects investigated presented with NS3 baseline RAVs, including V36L, T54S, Q80K/R, A156S, and D168E/T, and RAVs occurring in both genes in a single subject were rare [175]. Another study performed in Japan showed similar results with over 20 % prevalence of the Y93H substitution [173]. Yet in another Japanese cohort of 307 GT1b-infected subjects, NS3 and NS5A baseline RAVs were identified in 4.9 % and 11.2 % of individuals, respectively [174]. Again, dual resistance in both genes was rare. The overall number of NS5A baseline RAVs, particularly the Y93H substitution, seems significantly higher in the Japanese population compared to the USA or Europe. Deep sequencing analysis of 110 Japanese GT1b samples was also consistent with this observation, discovering L31M/V/F in 11.8 % and the Y93H variant in 30.9 % of patients [34]. The Y93H mutation was found significantly more often in subjects who carried the T/T allele at the IL28B locus. This suggests that many subjects carrying the T/T allele not only may show higher resistance to interferon, but are also more likely to develop resistance to first-generation NS5A inhibitors such as daclatasvir. In a large European study consisting of 239 subjects infected with genotype 1b, lower numbers were seen regarding preexisting NS5A RAVs with 3.8 and 6.3 % harboring the Y93H and L31M variants [37]. This is much more consistent with data obtained from the Los Alamos database, which estimated the prevalence for the Y93H substitution at around 4 % [176]. In general, NS5A resistance-associated baseline polymorphisms were seen to occur in all genotypes [177] and while drug resistance does play a considerable role in the world of NS5A inhibitors, their efficacy cannot be underestimated as integral part of HCV combination regimens. Moreover, second-generation NS5A inhibitors currently in development, such as GS-5816, ACH-3102, and elbasvir (MK-8742), exhibit improved resistance barriers while maintaining potency.

4.3.3 Persistence of NS5A Treatment-Emergent RAVs

Treatment-emergent NS5A RAVs have been observed to persist for extended periods of time (>1 year) following cessation of treatment. Selected ledipasvir-resistant variants were still present at 48 weeks in 50 % of individuals infected with genotypes 1a and 1b following 3 days of drug exposure [163]. More in-depth information on the persistence of ledipasvir-associated RAVs is not available to date [160]. Persistence data on other NS5A inhibitors remain scarce, but the emerging information appears consistent in that NS5A variants selected in response to the first-generation replication complex inhibitors often persist for over a year, if not years. This is in stark contrast to what has been observed for NS3, where treatment-emergent substitutions generally revert to wild-type sequence within a year. Following treatment with Abbvie's ombitasvir, treatment-emergent resistance variants were still detected up to 1 year post-end of treatment in all subjects for which data were available [144]. It has to be considered that persistence times of NS5A RAVs, similar to NS3 RAVs, vary depending on their genetic environment (i.e., the genotype they arise in); however, very little is known about such variables for the NS5A inhibitors. Decay kinetics most certainly also differ between specific variants within the same genotype or depend on how variants are linked to each other. For example, substitutions at NS5A residue 31 were relatively more stable compared to changes at position 93 in patients following exposure to daclatasvir (though the follow-up analysis was conducted for only up to a maximum of 6 months) [28], and for the PI telaprevir, it was shown that resistant variants analyzed by population sequencing were demonstrated to persist significantly longer when detected together in a patient [27]. The NS3 double-mutation V36M + R155K persisted significantly longer in genotype 1a subjects than in patients carrying V36M or R155K alone. Lastly, the lack of detection of a specific RAV in a viral population does not necessarily mean that the variant is completely absent. RAVs may be present at subdetectable levels—and with the data we currently have available on this subject, the long-term clinical impact of viruses carrying treatment-emergent substitutions is unknown.

4.4 Nucleoside Inhibitors

Nucleoside inhibitors of the HCV polymerase are modified derivatives of endogenous nucleosides that are phosphorylated to their active triphosphate, which terminates nascent HCV RNA strand synthesis [178–180]. The triphosphates of these nucleoside inhibitor prodrugs are competitive substrates with endogenous nucleotides [181, 182]. Modifications of the base and sugar moieties, often combined with monophosphate prodrugs at the sugar 5′, have been examined for antiviral activity in vitro and in clinical trials, leading to regulatory approval of a nucleotide prodrug with a >99 % cure rate. HCV nucleoside inhibitors are active against all HCV genotypes (pan-genotypic) and have a high barrier to resistance [130, 182–184]. Variants less susceptible to inhibition by the nucleoside inhibitors in vitro are not detected as pre-emergent mutations in the treatment-naive HCV patient population [41, 185–187]. The emergence of resistance-associated variants to nucleoside inhibitors in vitro is significantly delayed compared to that of the non-nucleoside, protease, and NS5A inhibitors of HCV replication. RAVs are detected within days of selection with these latter inhibitors, while for nucleoside inhibitors, RAVs may not be observed by population sequencing until 29 to >100 days of drug treatment [70, 188]. This successful profile for the nucleoside prodrug inhibitors was an evolved process; however, they are viewed as the cornerstone of modern HCV therapy [189].

The initial series of nucleoside inhibitors of HCV to be evaluated in clinical trials included the 2′-C-methyl derivatives MK-0608 (an adenosine analog) and the modified cytidines NM107, PSI-6130, and R1479 [181, 190, 191]. Treatment of HCV replicon-bearing cells with MK-0608, NM107, PSI-6130, or the later 2′-cyclopropyl cytidine analog TMC647078 selected for the NS5B S282T substitution, the hallmark variant for most of the 2′-modified nucleosides [181, 190, 191]. Replicons and purified enzymes bearing the S282T mutation were 20–100-fold less susceptible to these nucleos(t)ide inhibitors [188, 192]. Conversely, the polymerase S96T and N142T substitutions were individually or co-selected following treatment with the 4′-azido-modified R1479 drug, leading to a very mild (three- to fourfold) loss in susceptibility to R1479. The 2′-C-methylnucleosides are not cross-resistant to R1479 and each of their selected RAVs (S282T or S96T/N142T) were significantly replication deficient compared to the wild-type replicon [188, 193, 194]. The S282T substitution significantly impairs the HCV polymerase catalytic efficiency and reduces the replication capacity of HCV replicons to 3–30 % of wild type in hepatic cell lines [130, 195]. NM283 and R7128 are modified simple prodrugs of the NM107 and PSI-6130 nucleoside inhibitors, respectively, and retain the resistance profile of their parent nucleosides [188].

The second-generation nucleotide prodrug inhibitors of HCV polymerase incorporated chemical moieties that were more selectively and efficiently metabolized by liver enzymes to yield significantly higher levels of the active triphosphate species than earlier nucleoside inhibitors [196–203]. Like most 2′-C-methylnucleoside derivatives, sofosbuvir (GS-7977), a 2′-Me, 2′-F-uridine derivative prodrug, was less effective against the S282T polymerase mutant and selected for S282T in cellular replicon studies. The extent of S282T resistance to sofosbuvir in cell culture was genotype specific. While S282T in the context of genotype 1a and 1b replicons was modestly resistant to sofosbuvir, this substitution was not significantly sofosbuvir resistant when cloned into a genotype 2a replicon [69]. Rather, resistance to sofosbuvir was only achieved in genotype 2a when the T279A, M289L, and I293L substitutions were co-selected with S282T and the fitness-adaptive NS5B substitutions M434T and H479P [69]. The S282T substitution was also less susceptible to other 2′-C-methlynucleoside-based prodrugs, conferring greater than threefold resistance to IDX184, IDX19368, IDX21437; AL-516; and GS-6620, ACH-3422, and INX-08189 (BMS-986094) [182, 196, 198, 199, 203–205]. ALS-2200, the mixed diastereoisomer uridine prodrug of VX-135 is 89-fold less effective against the S282T substitution, with an additional mild resistance observed against replicons bearing the L159F/I262V/L320F triple mutant [180]. Contrary to all other 2′-C-methylnucleoside or nucleotide prodrug inhibitors, PSI-352938 (GS-938) and PSI-353661, both 2′-F, 2′-Me-guanosine prodrugs, did not select for and retained full activity against the S282T and/or S96T/N142T substitutions [197, 200, 206]. Long-term treatment of genotype 1a or 1b replicon-bearing hepatocytes with PSI-352938 or PSI-353661 failed to select for resistant mutants; however, the S15G/C223H/V321I triple mutant was selected by these drugs individually in genotype 2a replicon-bearing cells. Phenotypic analyses demonstrated that G15/H223/I321-bearing genotype 1b or 2a replicons were 5–16.5-fold resistant to either PSI-352938 or PSI-353661 [207]. PSI-352938 was not cross-resistant to any HCV direct-acting antiviral resistance-associated variant examined; however, neither it nor PSI-353661 progressed through clinical trials [207].

Emergence of resistance-associated variants to HCV nucleoside and nucleotide prodrug inhibitors is rarely observed in clinical trials [208–212]. The S282T substitution was detected in a single patient with viral breakthrough following 14 weeks of treatment with NM283 [213]. S282T also emerged in two genotype 1a subjects after extended treatment duration with R7128 (mericitabine) and a protease inhibitor [214]. Treatment with mericitabine, IFN, and ribavirin co-selected the L159F and L320F substitutions in a single genotype 1b subject. The L159F/L320F substitutions conferred 3.1–5.5-fold resistance to mericitabine in cellular replicon assays [215, 216]. Treatment-emergent resistance mutations were not detected for the 4′-azido nucleoside R1479 nor the 2′-methylguanosine prodrug IDX184 in clinical trials [202, 213]. Among the thousands of HCV patients treated with a sofosbuvir-containing regimen, the S282T substitution has emerged in three clinical trial subjects and succumbed to continued treatment [130, 217–219]. Aside from substitutions at S282, the L159F, L320F, C316N, and V321A substitutions were associated with resistance to sofosbuvir in <4.4 % of subjects with virologic failure [220]. The S282T substitution emerged in three genotype 1a subjects treated with a combination of the uridine nucleotide prodrug VX-135 and the NS5A replication inhibitor daclatasvir [221]. Interestingly, S282T did not emerge in subjects treated with VX-135 and ribavirin [210, 222]. The infrequent emergence of the S282T, L159F/L320F, or S96T/N142T substitutions in patients treated with nucleoside inhibitors may be explained by their absence among the viral quasispecies, potentially due to their poor replicative capacity [220, 223–225].

4.5 Non-nucleoside Inhibitors

The non-nucleoside inhibitors (NNIs) are chemically diverse allosteric binders of the HCV NS5B RNA-dependent RNA polymerase. Upon binding to the HCV polymerase, the NNI inhibitors induce conformational changes in the enzyme, disrupting replication activity in a reversible manner that is non-competitive with the polymerase's NTP substrate [226]. The

structure of the HCV polymerase is similar to that of other polymerases, having thumb, finger, and palm tertiary structures surrounding an active site that polymerizes nascent strand synthesis via coordination with two divalent cations (Mg^{2+} for the HCV polymerase) (Fig. 39.6) [227]. The thumb and palm domains can be structurally differentiated further into two subdomains each, with specific chemotypes of NNIs binding each site individually. Numerous NNIs for HCV have been in development since the early 1990s; however, only one has been approved as a component of a combination therapy with other direct-acting HCV antivirals [144]. The in vitro potency of the HCV NNIs is typically in the low nanomolar EC_{50} range in cellular replicon assays, though this activity is limited to genotype 1-derived sequences with few exceptions. A significant loss in antiviral activity is observed for genotypes 2–6 in biochemical enzyme and intergenotypic replicon assays, which translates into poor activity against these genotypes in clinical trials. Each class of HCV NNI has a relatively unique pattern of resistance mutations that emerge rapidly in cellular replicon selection studies. Figure 39.7 summarizes the resistance patterns for the nonnucleoside as well as the nucleoside inhibitors of HCV polymerase. Since the binding sites of the NNIs to the HCV polymerase are largely chemotype specific, NNIs that bind to different domains are typically not cross-resistant. The mutants that confer resistance to an NNI in cell culture are frequently, but not always, observed as resistance-associated variants following viral breakthrough to the NNI in the clinic. The frequency and rapid emergence of resistance-associated variants observed in the clinic can be attributed to their preexistence within the HCV quasispecies population detected in HCV subjects [223]. The emergence of resistance against one NNI does not preclude treatment with another NNI having a different resistance phenotype. Further, the use of multiple DAAs from the PI, NS5A, and nucleoside classes of HCV inhibitors has been shown to diminish or prevent the emergence of NNI-associated variants.

4.5.1 Finger-Loop and Thumb 1 Site Binders

The benzimidazole- and indole-based compounds bind to the HCV polymerase allosteric thumb 1 site (NNI-1) and disrupt its interaction with the finger-loop subdomain. In doing so, the polymerase is locked in an inactive open state, preventing initiation of nascent strand HCV genome synthesis [227–230]. Mutations at residues T389 (T389A/S), P495 (P495A/L/S), P496 (P496A/S), or V499 (to alanine) decrease the potency of the benzimidazoles by altering the conformation of the finger-loop/thumb 1 domains and abrogating compound interactions [230]. The structurally diverse indole-based NNIs, such as BILB 1941, BI 207524, BMS-791325, MK-3281, and TMC647055, also bind the thumb 1 site and are less effective against the resistance-associated variants at residue 495 [73, 231–234]. Resistance to certain indoles has also been associated with additional variants at L392, A421V, M426L, and V494 in in vitro selection studies and viral isolates from clinical trial breakthroughs; however, this class of thumb-binders has demonstrated a broader activity against genotype 3a, 4a, 5a, and 6a NS5B intergenotypic replicons [235, 236]. A significant loss in activity remains against genotype 2 NS5B-bearing replicons, consistent with reduced affinity of the compounds to NS5B as demonstrated with BMS-791325 and TMC647055 [73, 232, 235].

4.5.2 Thumb Site 2 Binders

The thumb site 2 binders are distinguishable from thumb 1 site binders in their chemical composition and resistance patterns. The hallmark resistance-associated variants that emerge upon treatment with thumb site 2 binders are localized to the M423

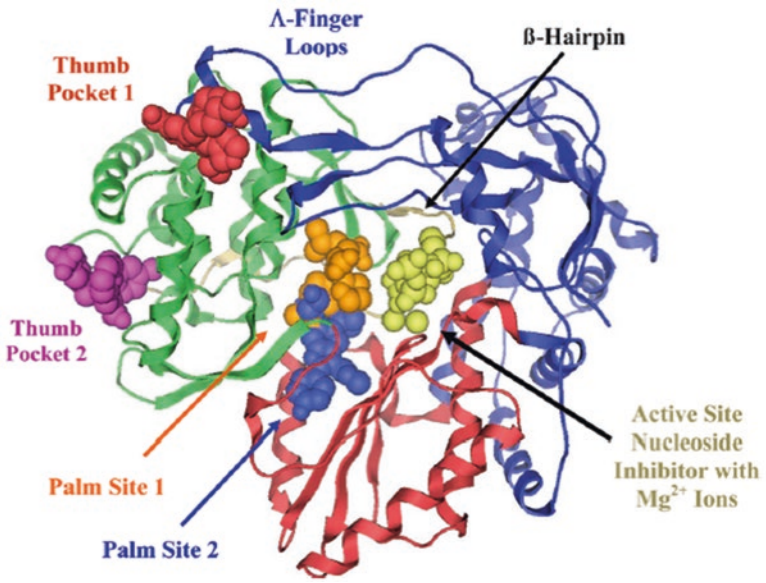


Fig. 39.6 Ribbon structure of the HCV NS5B RNA-dependent RNA polymerase. From Beaulieuet al., 2012 J. Med Chem

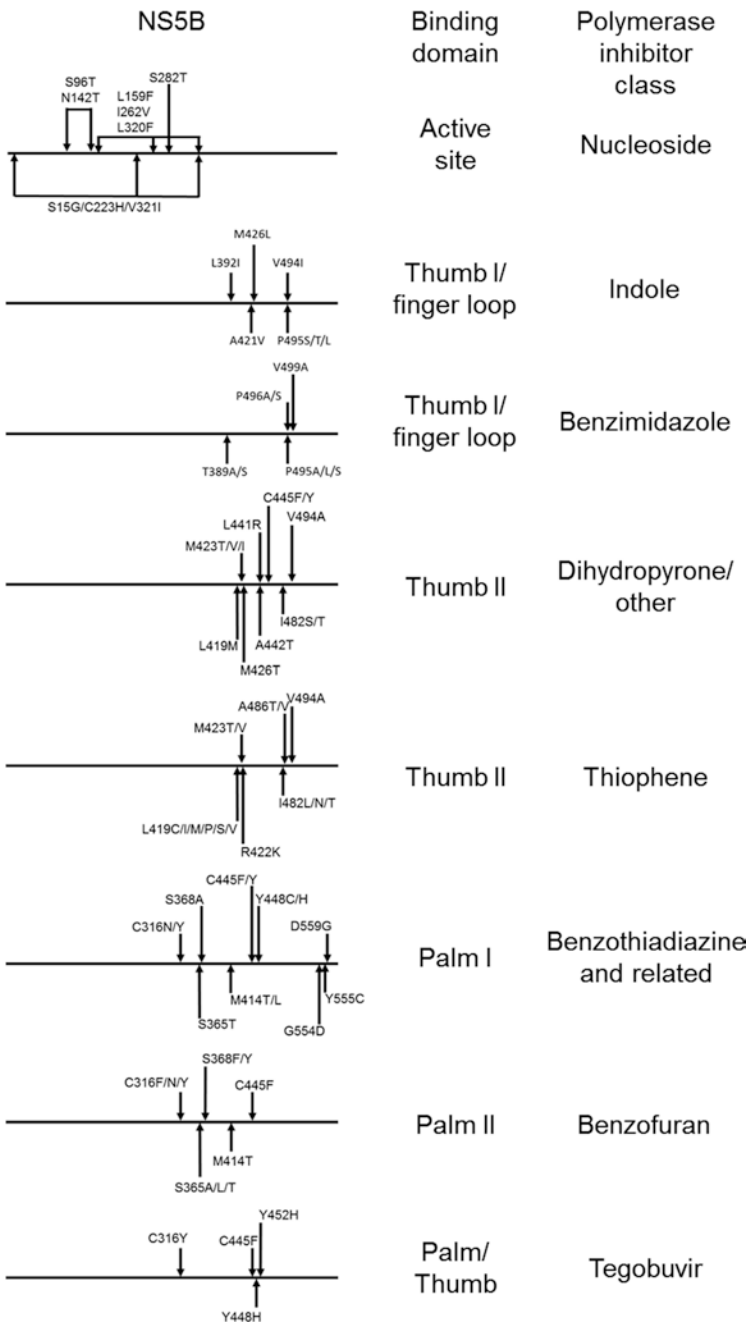


Fig. 39.7 Summary of common resistance-associated variants selected by inhibitors of HCV polymerase

locus, predominantly M423T, with valine and isoleucine substitutions also prevalent. The dihydropyrone-based inhibitors, such as AG-021541, have reduced activity against M423 variants as well as the M426T, I482S/T, and V494A substitutions [237]. The resistance phenotype of a close derivative to AG-021541, filibuvir (PF-868554), shares a similar resistance pattern. The loss in susceptibility for filibuvir has been attributed to conformational changes in the polymerase structure leading to decreased binding affinity (decreased van der Waals interactions) and binding site engagement of filibuvir to the mutant polymerase at M423 and V494 [238]. Substitutions of the HCV polymerase at M423 and M426 are also less susceptible to pyranoindole-based inhibitors, as are the L419M, L441R, A442T, and C445F/Y variants that encompass the region near the binding site of the compounds. Variants at distal residues from the pyranoindole binding site emerged at the T19 and M71 within the finger domain and A338 of the polymerase palm, but their contributions to resistance toward these compounds may be adaptive, providing a replication advantage toward co-selected variants [239]. The N-phenylbenzenesulfonamide class of thumb 2 binders interacts with both the L419 and M423 residues, as evidenced by

reduced susceptibility of variants at these loci and confirmed by co-crystallization studies [240]. The thiophene-based HCV NNIs, including VX-222, GS-9669, and VCH-759, have the most extensive resistance pattern of the thumb site 2 binders [68, 241–244]. Resistance to thiophenes is associated with substitutions at several or all of the following loci: L419 (C, I, M, P, S and V), R422 (K), M423 (T and V), I482 (L, N and T), A486 (T and V), and V494 (A) [68, 241–244]. As with other thumb site 2 inhibitors, resistance to the thiophenes is due to their decreased binding affinity and interaction with polymerase variants selected by drug treatment [245, 246]. The thumb site 2 inhibitors are not cross-resistant to protease, NS5A, or nucleoside inhibitors, or non-nucleoside inhibitors that bind the palm sites [242]. Even more intriguing is that the dihydropyrones retain full activity against resistance-associated variants that emerge from neighboring thumb site 1 binders [237, 242].

4.5.3 Palm-Site Binders

The palm domain of the HCV polymerase bears two distinct allosteric binding sites for non-nucleoside inhibitors. The palm I site is more central in the polymerase core and close to the active site. The palm II allosteric binding site overlaps a portion of the palm I site, but extends lower into the base of the palm of the polymerase. Due to the partial overlap between the two palm allosteric inhibitor-binding sites, the resistance patterns for the non-nucleoside inhibitors that bind these sites of the polymerase are less distinctive than those of active-site and thumb-binding inhibitors. The majority of clinical candidate non-nucleoside inhibitors have been based on a benzothiadiazine core that directly interacts with the palm I site of the HCV polymerase to inhibit initiation of RNA synthesis [247]. The resistance profile of benzothiadiazine-based palm I site inhibitors is composed of variants that emerge primarily at the C316, M414, and Y448 loci, with substitutions prevalent at the H95, C451, G554, S556, G558, and D559 loci as well in genotype 1b [248, 249]. Among the substitutions selected by treatment with benzothiadiazines, the most prevalent are C316Y, M414T, Y448H, and G554D. The side chain of the M414T substitution projects into the active site of the polymerase from the thumb domain and has been demonstrated to abrogate the interaction of the mutant polymerase and the benzothiadiazine inhibitor [249]. The individual contributions of less prevalent substitutions are varied, with some conserving the binding of the compound to the mutant polymerase [249]. They have been shown to increase the catalytic efficiency of the mutant polymerase, increasing the replication competency of the mutant virus/replicon and potentially providing an adaptive advantage to co-selected substitutions with replication deficiencies [250]. Indeed, co-selected polymerase substitutions have been demonstrated to increase the replication competency of the M414T [251]. Dasabuvir (ABT-333) selects for resistance-associated variants at the C316, M414, and Y448 loci, as well as less frequent substitutions at S368, N411, C445, A553, S556, and D559 [252]. When tested as individual substitutions in cellular replicon assays, these variants confer a 10- to >1000-fold reduction in susceptibility to dasabuvir compared to the wild-type replicon [247]. The S368A, Y448H, G554D, Y555C, and D559G substitutions emerge upon treatment of genotype 1b-replicon-bearing hepatocyte cultures with A-837093; however, variants are not selected at the M414 locus, differentiating A-837093 from the other palm 1-site benzothiadiazine allosteric NS5B inhibitors [253]. Resistance to the benzothiadiazine palm I-site binder ANA598 is attributed to substitutions at fewer loci, including M414T/L, G554D, and D559G [246, 254]. Although the particular binding pocket of IDX375 was not defined, replicons with the C316Y, C445F/Y, S365T, M414T, and Y448H substitutions were less susceptible to IDX375. While A442T was selected during long-term treatment of genotype 1b replicons in cell culture, this substitution was equally susceptible to IDX375. Interestingly, while replicons bearing the C316Y mutation are highly resistant to IDX375, this mutation was not selected in vitro [255]. The palm I-site binder, JTK-853, has a resistance pattern indicative of the benzothiadiazines, with resistance at M414T and Y448C/H, but is also less active against variants at the C445 locus, similar to IDX375. JTK-853 is relatively unique among the NNIs because it has a significant loss in activity against genotype 1a (approximately 20-fold higher EC_{50}) and variants were also selected by JTK-853 treatment at the L466 locus (L466F in patients and L466V selected in vitro) [46, 256].

Allosteric non-nucleoside inhibitors that bind to the palm II site of the HCV polymerase are derivatives of the benzofuran chemotype. The most advanced palm II-site inhibitors in the clinic were HCV-796 and GSK2485852. Susceptibility to these benzofurans is predominantly decreased by substitutions at the C316 and S365 loci, specifically C316F/N/Y and S365A/T. These loci are directly involved in the binding of the HCV polymerase to the benzofuran backbone and substitutions at C316 and S365 decrease the binding affinity of the drugs by altering the structure of their binding pockets or impairing hydrogen bonding between the compounds and their binding residues [257–259]. Treatment with HCV-796 or GSK2485852 can also select for the M414T or C445F substitutions, respectively; however, their contributions to resistance appear to be compensatory, providing a replicative advantage to co-selected variants with a reduced fitness [258, 260].

Complex differential patterns of resistance toward HCV-796 and GSK2485852 were observed when these compounds were tested against a panel of site-directed NS5B mutants in vitro; however, resistant substitutions common to both compounds included C316F/Y, S365L, and S368F/Y [258].

Tegobuvir (GS-9190) is distinctive from the other NNIs in that it binds both the palm I and thumb sites and requires

metabolic activation to inhibit HCV polymerase activity. While tegobuvir inhibits the HCV replicon in cellular assays, it does not inhibit the enzymatic activity of HCV polymerase in in vitro biochemical assays. Tegobuvir is an imidazopyridine prodrug bearing multiple aromatic fluorine atoms that is oxidized by cellular cytochrome P450 and further modified in a glutathione-dependent manner to an active metabolite. The antiviral activity of the tegobuvir metabolite is suppressed by the emergence of the polymerase C316Y, C445F, Y448H, and Y452H substitutions [261–263].

4.5.4 Active-Site Non-nucleoside Inhibitors

Distinct from the inhibitors that bind allosteric sites on the HCV polymerase, derivatives of α, γ-diketoacid (DKA) and dihydroxypyrimidine bind to the active site. The DKAs and dihydroxypyrimidines chelate at least one of the magnesium cations in the polymerase active site, resulting in the inhibition of nascent RNA strand elongation by preventing nucleotide incorporation as well as pyrophosphorylysis (the removal of incorporated nucleosides by pyrophosphate-mediated excision). Based on this mechanism of action, these drugs are classified as pyrophosphate (PPi) mimetics. The substituted pyrimidine-like moiety of the dihydroxypyrimidines likely interacts with the magnesium ions associated with the active site of the HCV polymerase, while the remainder of the compound (regarded as the specificity domain) is responsible for direct interactions with other areas of the polymerase [264–268]. The G152E and P156L substitutions of the polymerase result in a mild resistance to dihydroxypyrimidines (four- to fivefold). These substitutions alter interactions between the specificity domains of the dihydroxypyrimidines and the polymerase, excluding the compounds from associating with their binding residues, which decreases their affinity to the polymerase [267, 268]. Neither substitution impairs the pyrophosphorolytic efficiency of the mutant polymerase enzyme. Although the DKAs and dihydroxypyrimidines can compete with nucleosides for binding to the active site, these compounds inhibit the pyrophosphorylysis (excision) of strand-terminating nucleosides once they are incorporated into the nascent HCV RNA. Whether this class of NNIs is antagonistic or beneficial to the efficacy of DAA nucleoside analogs for the treatment of HCV is unclear [264–268].

5 Summary

The treatment of chronic HCV infection has advanced from interferon immunostimulation to regimens composed entirely of direct-acting antivirals. The advent of interferon- and ribavirin-sparing therapies for HCV has greatly improved the cure rates and on-treatment quality of life for patients while reducing the duration of treatment. Clinical studies and supporting in vitro resistance analyses have demonstrated that the prevention of resistance-associated variant emergence can be achieved by a combination of multiple direct-acting HCV antivirals. While clinical studies demonstrate pan-genotypic activity and infrequent emergence of resistance following treatment with a second-generation HCV nucleotide prodrug, combining these drugs with at least one other HCV direct-acting antiviral may completely prevent resistance emergence given the optimal dual combination. Therapeutic regimens absent a nucleoside inhibitor appear to require additional drugs (up to four) with nonoverlapping mechanisms of action [189]. Resistance variants have emerged in patients prescribed the current approved HCV combination regimens, thus necessitating continued vigilance and potential identification of additional therapies. The successes achieved in the evolution of HCV treatments absent of resistance may prove as invaluable guidance for the development of improved therapies for viruses with a still unmet medical need.

References

1. Schvarcz R, Weiland O, Wejstal R, Norkrans G, Fryden A, Foberg U. A randomized controlled open study of interferon alpha-2b treatment of chronic non-A, non-B posttransfusion hepatitis: no correlation of outcome to presence of hepatitis C virus antibodies. Scand J Infect Dis. 1989;21(6):617–25.
2. Rice CM, Saeed M. Hepatitis C: treatment triumphs. Nature. 2014;510(7503):43–4.
3. Ansaldi F, Orsi A, Sticchi L, Bruzzone B, Icardi G. Hepatitis C virus in the new era: perspectives in epidemiology, prevention, diagnostics and predictors of response to therapy. World J Gastroenterol. 2014;20(29):9633–52.
4. Boesecke CaW J-C. Hepatitis C. In: Mauss S et al., editors. Hepatology: a clinical textbook. Germany: Flying Publisher; 2014.
5. Kohli A, Shaffer A, Sherman A, Kottilil S. Treatment of hepatitis C: a systematic review. JAMA. 2014;312(6):631–40.
6. Chak E, Talal AH, Sherman KE, Schiff ER, Saab S. Hepatitis C virus infection in USA: an estimate of true prevalence. Liver Int. 2011;31(8):1090–101.
7. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology. 2013;57(4):1333–42.
8. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis. 2000;20(1):1–16.
9. Lavanchy D. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect. 2011;17(2):107–15.
10. Razavi H, Gower E, Hindman S, Estes C. Global distribution of HCV genotypes. In: The 64th annual meeting of the AASLD, Washington, DC; 1–5 Nov 2013.
11. http://www.cdc.gov/hepatitis/hcv/hcvfaq.htm
12. Buhler S, Bartenschlager R. New targets for antiviral therapy of chronic hepatitis C. Liver Int. 2012;32 Suppl 1:9–16.
13. Giannini C, Brechot C. Hepatitis C virus biology. Cell Death Differ. 2003;10 Suppl 1:S27–38.
14. Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. Hepatology. 2004;39(1):5–19.
15. Jones CT, Murray CL, Eastman DK, Tassello J, Rice CM. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J Virol. 2007;81(16):8374–83.

16. StGelais C, Foster TL, Verow M, Atkins E, Fishwick CW, Rowlands D, Harris M, Griffin S. Determinants of hepatitis C virus p7 ion channel function and drug sensitivity identified *in vitro*. J Virol. 2009;83(16):7970–81.
17. Zeisel MB, Barth H, Schuster C, Baumert TF. Hepatitis C virus entry: molecular mechanisms and targets for antiviral therapy. Front Biosci (Landmark Ed). 2009;14:3274–85.
18. OuYang B, Xie S, Berardi MJ, Zhao X, Dev J, Yu W, Sun B, Chou JJ. Unusual architecture of the p7 channel from hepatitis C virus. Nature. 2013;498(7455):521–5.
19. Nayak A, Pattabiraman N, Fadra N, Goldman R, Kosakovsky Pond SL, Mazumder R. Structure-function analysis of hepatitis C virus envelope glycoproteins E1 and E2. J Biomol Struct Dyn. 2015;33(8):1682–94.
20. Murray CL, Jones CT, Tassello J, Rice CM. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J Virol. 2007;81(19):10220–31.
21. Pieroni L, Santolini E, Fipaldini C, Pacini L, Migliaccio G, La Monica N. *In vitro* study of the NS2-3 protease of hepatitis C virus. J Virol. 1997;71(9):6373–80.
22. Shaw J, Fishwick CW, Harris M. Epoxide based inhibitors of the hepatitis C virus non-structural 2 autoprotease. Antiviral Res. 2015;117:20–6.
23. Martell M, Esteban JI, Quer J, Genescà J, Weiner A, Esteban R, Guardia J, Gómez J. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J Virol. 1992;66(5):3225–9.
24. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-alpha therapy. Science. 1998;282(5386):103–7.
25. Lange CM, Sarrazin C, Zeuzem S. Review article: specifically targeted anti-viral therapy for hepatitis C—a new era in therapy. Aliment Pharmacol Ther. 2010;32(1):14–28.
26. Fridell RA, Wang C, Sun JH, O'Boyle DR, Nower P, Valera L, Qiu D, Roberts S, Huang X, Kienzle B, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: *in vitro* and *in vivo* correlations. Hepatology. 2011;54(6):1924–35.
27. Sullivan JC, De Meyer S, Bartels DJ, Dierynck I, Zhang EZ, Spanks J, Tigges AM, Ghys A, Dorrian J, Adda N, et al. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. Clin Infect Dis. 2013;57(2):221–9.
28. Wang C, Sun JH, O'Boyle DR, Nower P, Valera L, Roberts S, Fridell RA, Gao M. Persistence of resistant variants in hepatitis C virus-infected patients treated with the NS5A replication complex inhibitor daclatasvir. Antimicrob Agents Chemother. 2013;57(5):2054–65.
29. Howorka S, Cheley S, Bayley H. Sequence-specific detection of individual DNA strands using engineered nanopores. Nat Biotechnol. 2001;19(7):636–9.
30. Svarovskaia ES, Martin R, McHutchison JG, Miller MD, Mo H. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. J Clin Microbiol. 2012;50(10):3267–74.
31. Thomas XV, de Bruijne J, Sullivan JC, Kieffer TL, Ho CK, Rebers SP, de Vries M, Reesink HW, Weegink CJ, Molenkamp R, et al. Evaluation of persistence of resistant variants with ultra-deep pyrosequencing in chronic hepatitis C patients treated with telaprevir. PLoS One. 2012;7(7):e41191.
32. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Saitoh S, Ikeda K, Kumada H. Evolution of simeprevir-resistant variants over time by ultra-deep sequencing in HCV genotype 1b. J Med Virol. 2014;86(8):1314–22.
33. Kosaka K, Imamura M, Hayes CN, Abe H, Hiraga N, Yoshimi S, Murakami E, Kawaoka T, Tsuge M, Aikata H, et al. Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1. J Viral Hepat. 2014;22:158–65.
34. Miura M, Maekawa S, Sato M, Komatsu N, Tatsumi A, Takano S, Amemiya F, Nakayama Y, Inoue T, Sakamoto M, et al. Deep sequencing analysis of variants resistant to the non-structural 5A inhibitor daclatasvir in patients with genotype 1b hepatitis C virus infection. Hepatol Res. 2014;44:E360–7.
35. Bartolini B, Giombini E, Zaccaro P, Selleri M, Rozera G, Abbate I, Comandini UV, Ippolito G, Solmone M, Capobianchi MR. Extent of HCV NS3 protease variability and resistance-associated mutations assessed by next generation sequencing in HCV monoinfected and HIV/HCV coinfected patients. Virus Res. 2013;177(2):205–8.
36. Paolucci S, Fiorina L, Piralla A, Gulminetti R, Novati S, Barbarini G, Sacchi P, Gatti M, Dossena L, Baldanti F. Naturally occurring mutations to HCV protease inhibitors in treatment-naïve patients. Virol J. 2012;9:245.
37. Bartels DJ, Sullivan JC, Zhang EZ, Tigges AM, Dorrian JL, De Meyer S, Takemoto D, Dondero E, Kwong AD, Picchio G, et al. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J Virol. 2013;87(3):1544–53.
38. De Meyer S, Dierynck I, Ghys A, Beumont M, Daems B, Van Baelen B, Sullivan JC, Bartels DJ, Kieffer TL, Zeuzem S, et al. Characterization of telaprevir treatment outcomes and resistance in patients with prior treatment failure: results from the REALIZE trial. Hepatology. 2012;56(6):2106–15.
39. Forns X, Lawitz E, Zeuzem S, Gane E, Bronowicki JP, Andreone P, Horban A, Brown A, Peeters M, Lenz O, et al. Simeprevir with peginterferon and ribavirin leads to high rates of SVR in patients with HCV genotype 1 who relapsed after previous therapy: a phase 3 trial. Gastroenterology. 2014;146(7):1669–79. e3.
40. Jacobson IM, Gordon SC, Kowdley KV, Yoshida EM, Rodriguez-Torres M, Sulkowski MS, Shiffman ML, Lawitz E, Everson G, Bennett M, et al. Sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options. N Engl J Med. 2013;368(20):1867–77.
41. Di Maio VC, Cento V, Mirabelli C, Artese A, Costa G, Alcaro S, Perno CF, Ceccherini-Silberstein F. Hepatitis C virus genetic variability and the presence of NS5B resistance-associated mutations as natural polymorphisms in selected genotypes could affect the response to NS5B inhibitors. Antimicrob Agents Chemother. 2014;58(5):2781–97.
42. Kwong AD, Najera I, Bechtel J, Bowden S, Fitzgibbon J, Harrington P, Kempf D, Kieffer TL, Koletzki D, Kukolj G, et al. Sequence and phenotypic analysis for resistance monitoring in hepatitis C virus drug development: recommendations from the HCV DRAG. Gastroenterology. 2011;140(3):755–60.
43. Middleton T, He Y, Pilot-Matias T, Tripathi R, Lim BH, Roth A, Chen CM, Koev G, Ng TI, Krishnan P, et al. A replicon-based shuttle vector system for assessing the phenotype of HCV NS5B polymerase genes isolated from patient populations. J Virol Methods. 2007;145(2):137–45.
44. Tripathi RL, Krishnan P, He Y, Middleton T, Pilot-Matias T, Chen CM, Lau DT, Lemon SM, Mo H, Kati W, et al. Replication efficiency of chimeric replicon containing NS5A-5B genes derived from HCV-infected patient sera. Antiviral Res. 2007;73(1):40–9.
45. Sheaffer AK, Lee MS, Hernandez D, Chaniewski S, Yu F, Falk P, Friborg J, Zhai G, McPhee F. Development of a chimeric replicon system for phenotypic analysis of NS3 protease sequences from HCV clinical isolates. Antivir Ther. 2011;16(5):705–18.
46. Ogura N, Toyonaga Y, Ando I, Hirahara K, Shibata T, Turcanu G, Pai S, Yee K, Gerhardt B, Rodriguez-Torres M, et al. Genotypic and phenotypic analyses of hepatitis C virus from patients treated with JTK-853 in a three-day monotherapy. Antimicrob Agents Chemother. 2013;57(1):436–44.

47. Rajyaguru S, Yang H, Martin R, Miller MD, Mo H. Development and characterization of a replicon-based phenotypic assay for assessing HCV NS4B from clinical isolates. Antiviral Res. 2013;100(2):328–36.
48. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science. 1999;285(5424):110–3.
49. Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. J Virol. 2001;75(18):8516–23.
50. Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol. 2003;77(5):3181–90.
51. Gu B, Gates AT, Isken O, Behrens SE, Sarisky RT. Replication studies using genotype 1a subgenomic hepatitis C virus replicons. J Virol. 2003;77(9):5352–9.
52. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology. 2003;125(6):1808–17.
53. Bartenschlager R, Sparacio S. Hepatitis C virus molecular clones and their replication capacity *in vivo* and in cell culture. Virus Res. 2007;127(2):195–207.
54. Saeed M, Scheel TK, Gottwein JM, Marukian S, Dustin LB, Bukh J, Rice CM. Efficient replication of genotype 3a and 4a hepatitis C virus replicons in human hepatoma cells. Antimicrob Agents Chemother. 2012;56(10):5365–73.
55. Wose Kinge CN, Espiritu C, Prabdial-Sing N, Sithebe NP, Saeed M, Rice CM. Hepatitis C virus genotype 5a subgenomic replicons for evaluation of direct-acting antiviral agents. Antimicrob Agents Chemother. 2014;58(9):5386–94.
56. Lanford RE, Guerra B, Lee H. Hepatitis C virus genotype 1b chimeric replicon containing genotype 3 NS5A domain. Virology. 2006;355(2):192–202.
57. Herlihy KJ, Graham JP, Kumpf R, Patick AK, Duggal R, Shi ST. Development of intergenotypic chimeric replicons to determine the broad-spectrum antiviral activities of hepatitis C virus polymerase inhibitors. Antimicrob Agents Chemother. 2008; 52(10):3523–31.
58. Imhof I, Simmonds P. Development of an intergenotypic hepatitis C virus (HCV) cell culture method to assess antiviral susceptibilities and resistance development of HCV NS3 protease genes from HCV genotypes 1 to 6. J Virol. 2010;84(9):4597–610.
59. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med. 2005;11(7):791–6.
60. Ait-Goughoulte M, Kanda T, Meyer K, Ryerse JS, Ray RB, Ray R. Hepatitis C virus genotype 1a growth and induction of autophagy. J Virol. 2008;82(5):2241–9.
61. Saeed M, Gondeau C, Hmwe S, Yokokawa H, Date T, Suzuki T, Kato T, Maurel P, Wakita T. Replication of hepatitis C virus genotype 3a in cultured cells. Gastroenterology. 2013;144(1):56–8. e57.
62. Ma Y, Shimakami T, Luo H, Hayashi N, Murakami S. Mutational analysis of hepatitis C virus NS5B in the subgenomic replicon cell culture. J Biol Chem. 2004;279(24):25474–82.
63. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc Natl Acad Sci U S A. 2006;103(7):2310–5.
64. Li YP, Ramirez S, Jensen SB, Purcell RH, Gottwein JM, Bukh J. Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. Proc Natl Acad Sci U S A. 2012;109(48):19757–62.
65. Yu M, Corsa AC, Xu S, Peng B, Gong R, Lee YJ, Chan K, Mo H, Delaney W, Cheng G. *In vitro* efficacy of approved and experimental antivirals against novel genotype 3 hepatitis C virus subgenomic replicons. Antiviral Res. 2013;100(2):439–45.
66. Bilello JP, Lallos LB, McCarville JF, La Colla M, Serra I, Chapron C, Gillum JM, Pierra C, Standring DN, Seifer M. *In vitro* activity and resistance profile of samatasvir, a novel NS5A replication inhibitor of hepatitis C virus. Antimicrob Agents Chemother. 2014;58(8):4431–42.
67. Yang H, Robinson M, Corsa AC, Peng B, Cheng G, Tian Y, Wang Y, Pakdaman R, Shen M, Qi X, et al. Preclinical characterization of the novel hepatitis C virus NS3 protease inhibitor GS-9451. Antimicrob Agents Chemother. 2014;58(2):647–53.
68. Jiang M, Zhang EZ, Ardzinski A, Tigges A, Davis A, Sullivan JC, Nelson M, Spanks J, Dorrian J, Nicolas O, et al. Genotypic and phenotypic analyses of hepatitis C virus variants observed in clinical studies of VX-222, a nonnucleoside NS5B polymerase inhibitor. Antimicrob Agents Chemother. 2014;58(9):5456–65.
69. Lam AM, Espiritu C, Bansal S, Micolochick Steuer HM, Niu C, Zennou V, Keilman M, Zhu Y, Lan S, Otto MJ, et al. Genotype and subtype profiling of PSI-7977 as a nucleotide inhibitor of hepatitis C virus. Antimicrob Agents Chemother. 2012;56(6):3359–68.
70. McCown MF, Rajyaguru S, Le Pogam S, Ali S, Jiang WR, Kang H, Symons J, Cammack N, Najera I. The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogs than to nonnucleoside polymerase or protease inhibitors. Antimicrob Agents Chemother. 2008;52(5):1604–12.
71. Summa V, Ludmerer SW, McCauley JA, Fandozzi C, Burlein C, Claudio G, Coleman PJ, Dimuzio JM, Ferrara M, Di Filippo M, et al. MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants. Antimicrob Agents Chemother. 2012;56(8):4161–7.
72. Kieffer TL, George S. Resistance to hepatitis C virus protease inhibitors. Curr Opin Virol. 2014;8C:16–21.
73. Lemm JA, Liu M, Gentles RG, Ding M, Voss S, Pelosi LA, Wang YK, Rigat KL, Mosure KW, Bender JA, et al. Preclinical characterization of BMS-791325, an allosteric inhibitor of hepatitis C Virus NS5B polymerase. Antimicrob Agents Chemother. 2014;58(6):3485–95.
74. Sato A, Yamauchi M, Yamada T, Kumano R, Adachi K, Ishii T, Hayashi M, Kumon D. Successful natural interferon-β plus ribavirin therapy in a chronic hepatitis C patient after discontinuation of interferon-α treatment due to arrhythmia and interstitial pneumonia. Clin J Gastroenterol. 2014;7:355–60.
75. AASLD/IDSA/IAS-USA: Recommendations for testing, managing, and treating hepatitis C. 2014. http://www.hcvguidelines.org/fullreport.
76. Tam RC, Pai B, Bard J, Lim C, Averett DR, Phan UT, Milovanovic T. Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile. J Hepatol. 1999;30(3):376–82.
77. Thomas E, Feld JJ, Li Q, Hu Z, Fried MW, Liang TJ. Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. Hepatology. 2011;53(1):32–41.
78. Liu WL, Yang HC, Su WC, Wang CC, Chen HL, Wang HY, Huang WH, Chen DS, Lai MY. Ribavirin enhances the action of interferon-α against hepatitis C virus by promoting the p53 activity through the ERK1/2 pathway. PLoS One. 2012;7(9):e43824.
79. Hofmann WP, Polta A, Herrmann E, Mihm U, Kronenberger B, Sonntag T, Lohmann V, Schönberger B, Zeuzem S, Sarrazin C. Mutagenic effect of ribavirin on hepatitis C nonstructural 5B quasispecies *in vitro* and during antiviral therapy. Gastroenterology. 2007;132(3):921–30.
80. Lutchman G, Danehower S, Song BC, Liang TJ, Hoofnagle JH, Thomson M, Ghany MG. Mutation rate of the hepatitis C virus NS5B in patients undergoing treatment with ribavirin monotherapy. Gastroenterology. 2007;132(5):1757–66.

81. Zhou S, Liu R, Baroudy BM, Malcolm BA, Reyes GR. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. Virology. 2003;310(2):333–42.
82. Pfeiffer JK, Kirkegaard K. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. J Virol. 2005;79(4):2346–55.
83. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461(7262):399–401.
84. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41(10):1100–4.
85. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41(10):1105–9.
86. Heim MH, Thimme R. Innate and adaptive immune responses in HCV infections. J Hepatol. 2014;61(1 Suppl):S14–25.
87. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, Rice CM. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology. 2006;131(6):1887–98.
88. Reddy KR, Shiffman ML, Rodriguez-Torres M, Cheinquer H, Abdurakhmanov D, Bakulin I, Morozov V, Silva GF, Geyvandova N, Stanciu C, et al. Induction pegylated interferon alfa-2a and high dose ribavirin do not increase SVR in heavy patients with HCV genotype 1 and high viral loads. Gastroenterology. 2010;139(6):1972–83.
89. Chandra PK, Bao L, Song K, Aboulnasr FM, Baker DP, Shores N, Wimley WC, Liu S, Hagedorn CH, Fuchs SY, et al. HCV infection selectively impairs type I but not type III IFN signaling. Am J Pathol. 2014;184(1):214–29.
90. Liu J, HuangFu WC, Kumar KG, Qian J, Casey JP, Hamanaka RB, Grigoriadou C, Aldabe R, Diehl JA, Fuchs SY. Virus-induced unfolded protein response attenuates antiviral defenses via phosphorylation-dependent degradation of the type I interferon receptor. Cell Host Microbe. 2009;5(1):72–83.
91. Mangia A, Mottola L, Santoro R. Interleukin 28B polymorphisms as predictor of response in hepatitis C virus genotype 2 and 3 infected patients. World J Gastroenterol. 2013;19(47):8924–8.
92. Chu TW, Kulkarni R, Gane EJ, Roberts SK, Stedman C, Angus PW, Ritchie B, Lu XY, Ipe D, Lopatin U, et al. Effect of IL28B genotype on early viral kinetics during interferon-free treatment of patients with chronic hepatitis C. Gastroenterology. 2012;142(4):790–5.
93. Simmonds P. Genetic diversity and evolution of hepatitis C virus—15 years on. J Gen Virol. 2004;85(Pt 11):3173–88.
94. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest. 1995;96(1):224–30.
95. Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology. 1997;230(2):217–27.
96. Taylor DR, Shi ST, Lai MM. Hepatitis C virus and interferon resistance. Microbes Infect. 2000;2(14):1743–56.
97. Pavio N, Taylor DR, Lai MM. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J Virol. 2002;76(3):1265–72.
98. Tan SL, Nakao H, He Y, Vijaysri S, Neddermann P, Jacobs BL, Mayer BJ, Katze MG. NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling. Proc Natl Acad Sci U S A. 1999;96(10):5533–8.
99. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology. 2005;48(6):372–80.
100. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Arase Y, Ikeda K, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology. 2007;46(5):1357–64.
101. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Saitoh S, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 2a high viral load and virological response to interferon-ribavirin combination therapy. Intervirology. 2009;52(6):301–9.
102. Funaoka Y, Sakamoto N, Suda G, Itsui Y, Nakagawa M, Kakinuma S, Watanabe T, Mishima K, Ueyama M, Onozuka I, et al. Analysis of interferon signaling by infectious hepatitis C virus clones with substitutions of core amino acids 70 and 91. J Virol. 2011; 85(12):5986–94.
103. Okada S, Akahane Y, Suzuki H, Okamoto H, Mishiro S. The degree of variability in the amino terminal region of the E2/NS1 protein of hepatitis C virus correlates with responsiveness to interferon therapy in viremic patients. Hepatology. 1992;16(3):619–24.
104. Nousbaum J, Polyak SJ, Ray SC, Sullivan DG, Larson AM, Carithers RL, Gretch DR. Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy. J Virol. 2000;74(19):9028–38.
105. Sarrazin C, Herrmann E, Bruch K, Zeuzem S. Hepatitis C virus nonstructural 5A protein and interferon resistance: a new model for testing the reliability of mutational analyses. J Virol. 2002;76(21):11079–90.
106. Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, Heathcote J, Edwards AM, McGilvray ID. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology. 2005;128(5):1437–44.
107. Chen L, Borozan I, Sun J, Guindi M, Fischer S, Feld J, Anand N, Heathcote J, Edwards AM, McGilvray ID. Cell-type specific gene expression signature in liver underlies response to interferon therapy in chronic hepatitis C infection. Gastroenterology 2010;138(3):1123–33. e1121–3.
108. Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. Hepatology. 2001;33(3):708–12.
109. Huang Y, Yang H, Borg BB, Su X, Rhodes SL, Yang K, Tong X, Tang G, Howell CD, Rosen HR, et al. A functional SNP of interferon-gamma gene is important for interferon-alpha-induced and spontaneous recovery from hepatitis C virus infection. Proc Natl Acad Sci U S A. 2007;104(3):985–90.
110. Knapp S, Yee LJ, Frodsham AJ, Hennig BJ, Hellier S, Zhang L, Wright M, Chiaramonte M, Graves M, Thomas HC, et al. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. Genes Immun. 2003;4(6):411–9.
111. Suzuki F, Arase Y, Suzuki Y, Tsubota A, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, et al. Single nucleotide polymorphism of the MxA gene promoter influences the

response to interferon monotherapy in patients with hepatitis C viral infection. J Viral Hepat. 2004;11(3):271–6.
112. Anjum S, Afzal MS, Ahmad T, Aslam B, Waheed Y, Shafi T, Qadri I. Mutations in the STAT1-interacting domain of the hepatitis C virus core protein modulate the response to antiviral therapy. Mol Med Rep. 2013;8(2):487–92.
113. Khaliq S, Jahan S, Hassan S. Hepatitis C virus p7: molecular function and importance in hepatitis C virus life cycle and potential antiviral target. Liver Int. 2011;31(5):606–17.
114. Atoom AM, Taylor NG, Russell RS. The elusive function of the hepatitis C virus p7 protein. Virology. 2014;462–463:377–87.
115. Griffin S, Stgelais C, Owsianka AM, Patel AH, Rowlands D, Harris M. Genotype-dependent sensitivity of hepatitis C virus to inhibitors of the p7 ion channel. Hepatology. 2008;48(6): 1779–90.
116. Foster TL, Verow M, Wozniak AL, Bentham MJ, Thompson J, Atkins E, Weinman SA, Fishwick C, Foster R, Harris M, et al. Resistance mutations define specific antiviral effects for inhibitors of the hepatitis C virus p7 ion channel. Hepatology. 2011;54(1):79–90.
117. Gallay PA, Lin K. Profile of alisporivir and its potential in the treatment of hepatitis C. Drug Des Devel Ther. 2013;7:105–15.
118. Lin K, Gallay P. Curing a viral infection by targeting the host: the example of cyclophilin inhibitors. Antiviral Res. 2013;99(1):68–77.
119. Inoue K, Sekiyama K, Yamada M, Watanabe T, Yasuda H, Yoshiba M. Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. J Gastroenterol. 2003;38(6):567–72.
120. Madan V, Paul D, Lohmann V, Bartenschlager R. Inhibition of HCV replication by cyclophilin antagonists is linked to replication fitness and occurs by inhibition of membranous web formation. Gastroenterology 2014;146(5):1361–72. e1361–9.
121. Chatterji U, Bobardt M, Tai A, Wood M, Gallay PA. Cyclophilin and NS5A inhibitors, but not other anti-hepatitis C virus (HCV) agents, preclude HCV-mediated formation of double-membrane-vesicle viral factories. Antimicrob Agents Chemother. 2015;59(5):2496–507.
122. Chatterji U, Lim P, Bobardt MD, Wieland S, Cordek DG, Vuagniaux G, Chisari F, Cameron CE, Targett-Adams P, Parkinson T, et al. HCV resistance to cyclosporin A does not correlate with a resistance of the NS5A-cyclophilin A interaction to cyclophilin inhibitors. J Hepatol. 2010;53(1):50–6.
123. Puyang X, Poulin DL, Mathy JE, Anderson LJ, Ma S, Fang Z, Zhu S, Lin K, Fujimoto R, Compton T, et al. Mechanism of resistance of hepatitis C virus replicons to structurally distinct cyclophilin inhibitors. Antimicrob Agents Chemother. 2010;54(5):1981–7.
124. Rosnoblet C, Fritzinger B, Legrand D, Launay H, Wieruszeski JM, Lippens G, Hanoulle X. Hepatitis C virus NS5B and host cyclophilin A share a common binding site on NS5A. J Biol Chem. 2012;287(53):44249–60.
125. Chatterji U, Garcia-Rivera JA, Baugh J, Gawlik K, Wong KA, Zhong W, Brass CA, Naoumov NV, Gallay PA. The combination of alisporivir plus an NS5A inhibitor provides additive to synergistic anti-hepatitis C virus activity without detectable cross-resistance. Antimicrob Agents Chemother. 2014;58(6):3327–34.
126. Barnard RJ, Howe JA, Ogert RA, Zeuzem S, Poordad F, Gordon SC, Ralston R, Tong X, Sniukiene V, Strizki J, et al. Analysis of boceprevir resistance associated amino acid variants (RAVs) in two phase 3 boceprevir clinical studies. Virology. 2013;444(1–2):329–36.
127. López-Labrador FX, Moya A, Gonzàlez-Candelas F. Mapping natural polymorphisms of hepatitis C virus NS3/4A protease and antiviral resistance to inhibitors in worldwide isolates. Antivir Ther. 2008;13(4):481–94.
128. Foster GR, Hézode C, Bronowicki JP, Carosi G, Weiland O, Verlinden L, van Heeswijk R, van Baelen B, Picchio G, Beumont M. Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. Gastroenterology 2011;141(3):881–9. e881.
129. Lenz O, Vijgen L, Berke JM, Cummings MD, Fevery B, Peeters M, De Smedt G, Moreno C, Picchio G. Virologic response and characterisation of HCV genotype 2–6 in patients receiving TMC435 monotherapy (study TMC435-C202). J Hepatol. 2013;58(3):445–51.
130. Poveda E, Wyles DL, Mena A, Pedreira JD, Castro-Iglesias A, Cachay E. Update on hepatitis C virus resistance to direct-acting antiviral agents. Antiviral Res. 2014;108C:181–91.
131. Pickett BE, Striker R, Lefkowitz EJ. Evidence for separation of HCV subtype 1a into two distinct clades. J Viral Hepat. 2011;18(9):608–18.
132. De Luca A, Bianco C, Rossetti B. Treatment of HCV infection with the novel NS3/4A protease inhibitors. Curr Opin Pharmacol. 2014;18C:9–17.
133. Lawitz E, Sulkowski MS, Ghalib R, Rodriguez-Torres M, Younossi ZM, Corregidor A, DeJesus E, Pearlman B, Rabinovitz M, Gitlin N, et al. Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study. Lancet. 2014;384(9956):1756–65.
134. Zeuzem S, Berg T, Gane E, Ferenci P, Foster GR, Fried MW, Hezode C, Hirschfield GM, Jacobson I, Nikitin I, et al. Simeprevir increases rate of sustained virologic response among treatment-experienced patients with HCV genotype-1 infection: a phase IIb trial. Gastroenterology 2014;146(2):430–41. e436.
135. Lenz O, Verbinnen T, Lin TI, Vijgen L, Cummings MD, Lindberg J, Berke JM, Dehertogh P, Fransen E, Scholliers A, et al. *In vitro* resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. Antimicrob Agents Chemother. 2010;54(5): 1878–87.
136. Thibeault D, Bousquet C, Gingras R, Lagacé L, Maurice R, White PW, Lamarre D. Sensitivity of NS3 serine proteases from hepatitis C virus genotypes 2 and 3 to the inhibitor BILN 2061. J Virol. 2004;78(14):7352–9.
137. Pilot-Matias T, Tripathi R, Cohen D, Gaultier I, Dekhtyar T, Lu L, Reisch T, Irvin M, Hopkins T, Pithawalla R, et al. *In vitro* and *in vivo* antiviral activity and resistance profile of the hepatitis C virus NS3/4A protease inhibitor ABT-450. Antimicrob Agents Chemother. 2014;59:988–97.
138. Ali A, Aydin C, Gildemeister R, Romano KP, Cao H, Ozen A, Soumana D, Newton A, Petropoulos CJ, Huang W, et al. Evaluating the role of macrocycles in the susceptibility of hepatitis C virus NS3/4A protease inhibitors to drug resistance. ACS Chem Biol. 2013;8(7):1469–78.
139. HCV Phenotype Working Group, HCV Drug Development Advisory Group. Clinically relevant HCV drug resistance mutations. Ann Forum Collab HIV Res. 2012;14(3):10.
140. Berger KL, Lagacé L, Triki I, Cartier M, Marquis M, Lawetz C, Bethell R, Scherer J, Kukolj G. Viral resistance in hepatitis C virus genotype 1-infected patients receiving the NS3 protease inhibitor Faldaprevir (BI 201335) in a phase 1b multiple-rising-dose study. Antimicrob Agents Chemother. 2013;57(10):4928–36.
141. Lagace L, White PW, Bousquet C, Dansereau N, Do F, Llinas-Brunet M, Marquis M, Massariol MJ, Maurice R, Spickler C, et al. *In vitro* resistance profile of the hepatitis C virus NS3 protease inhibitor BI 201335. Antimicrob Agents Chemother. 2012; 56(1):569–72.
142. Susser S, Vermehren J, Forestier N, Welker MW, Grigorian N, Füller C, Perner D, Zeuzem S, Sarrazin C. Analysis of long-term persistence of resistance mutations within the hepatitis C virus NS3 protease after treatment with telaprevir or boceprevir. J Clin Virol. 2011;52(4):321–7.
143. McPhee F, Hernandez D, Yu F, Ueland J, Monikowski A, Carifa A, Falk P, Wang C, Fridell R, Eley T, et al. Resistance analysis of

hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. Hepatology. 2013;58(3):902–11.
144. Abbvie: Full prescribing information for VIEKIRA PAK. 2014. http://www.rxabbvie.com/pdf/viekirapak_pi.pdf
145. Kim AY, Timm J, Nolan BE, Reyor LL, Kane K, Berical AC, Zachary KC, Lauer GM, Kuntzen T, Allen TM. Temporal dynamics of a predominant protease inhibitor-resistance mutation in a treatment-naive, hepatitis C virus-infected individual. J Infect Dis. 2009;199(5):737–41.
146. Colson P, Gérolami R. Two years' persistence of naturally present substitution R155K within hepatitis C virus NS3 protease in the absence of protease inhibitor-based therapy. J Infect Dis. 2011;203(9):1341–2. Author reply 1342–1343.
147. Bryson PD, Cho NJ, Einav S, Lee C, Tai V, Bechtel J, Sivaraja M, Roberts C, Schmitz U, Glenn JS. A small molecule inhibits HCV replication and alters NS4B's subcellular distribution. Antiviral Res. 2010;87(1):1–8.
148. Zhang X, Zhang N, Chen G, Turpoff A, Ren H, Takasugi J, Morrill C, Zhu J, Li C, Lennox W, et al. Discovery of novel HCV inhibitors: synthesis and biological activity of 6-(indol-2-yl)pyridine-3-sulfonamides targeting hepatitis C virus NS4B. Bioorg Med Chem Lett. 2013;23(13):3947–53.
149. Tai VW, Garrido D, Price DJ, Maynard A, Pouliot JJ, Xiong Z, Seal 3rd JW, Creech KL, Kryn LH, Baughman TM, et al. Design and synthesis of spirocyclic compounds as HCV replication inhibitors by targeting viral NS4B protein. Bioorg Med Chem Lett. 2014;24(10):2288–94.
150. Shotwell JB, Baskaran S, Chong P, Creech KL, Crosby RM, Dickson H, Fang J, Garrido D, Mathis A, Maung J, et al. Imidazo[1,2-a]pyridines that directly interact with hepatitis C NS4B: initial preclinical characterization. ACS Med Chem Lett. 2012;3(7):565–9.
151. Gu Z, Graci JD, Lahser FC, Breslin JJ, Jung SP, Crona JH, McMonagle P, Xia E, Liu S, Karp G, et al. Identification of PTC725, an orally bioavailable small molecule that selectively targets the hepatitis C Virus NS4B protein. Antimicrob Agents Chemother. 2013;57(7):3250–61.
152. Dufner-Beattie J, O'Guin A, O'Guin S, Briley A, Wang B, Balsarotti J, Roth R, Starkey G, Slomczynska U, Noueiry A, et al. Identification of AP80978, a novel small-molecule inhibitor of hepatitis C virus replication that targets NS4B. Antimicrob Agents Chemother. 2014;58(6):3399–410.
153. Kakarla R, Liu J, Naduthambi D, Chang W, Mosley RT, Bao D, Steuer HM, Keilman M, Bansal S, Lam AM, et al. Discovery of a novel class of potent HCV NS4B inhibitors: SAR studies on piperazinone derivatives. J Med Chem. 2014;57(5):2136–60.
154. Esser-Nobis K, Romero-Brey I, Ganten TM, Gouttenoire J, Harak C, Klein R, Schemmer P, Binder M, Schnitzler P, Moradpour D, et al. Analysis of hepatitis C virus resistance to silibinin *in vitro* and *in vivo* points to a novel mechanism involving nonstructural protein 4B. Hepatology. 2013;57(3):953–63.
155. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol. 2004;85(Pt 9):2485–502.
156. Appel N, Zayas M, Miller S, Krijnse-Locker J, Schaller T, Friebe P, Kallis S, Engel U, Bartenschlager R. Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. PLoS Pathog. 2008;4(3):e1000035.
157. Tellinghuisen TL, Foss KL, Treadaway JC, Rice CM. Identification of residues required for RNA replication in domains II and III of the hepatitis C virus NS5A protein. J Virol. 2008;82(3):1073–83.
158. Manns M, Pol S, Jacobson IM, Marcellin P, Gordon SC, Peng CY, Chang TT, Everson GT, Heo J, Gerken G, et al. All-oral daclatasvir plus asunaprevir for hepatitis C virus genotype 1b: a multinational, phase 3, multicohort study. Lancet. 2014;384(9954):1597–605.
159. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, Reddy KR, Hassanein T, Jacobson I, Lawitz E, Lok AS, Hinestrosa F, Thuluvath PJ, et al. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. N Engl J Med. 2014;370(3):211–21.
160. Gilead Sciences. Full prescribing information for Harvoni. 2014. http://www.gilead.com/~/media/Files/pdfs/medicines/liver-disease/harvoni/harvoni_pi.pdf.
161. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, Serrano-Wu MH, Langley DR, Sun JH, O'Boyle 2nd DR, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature. 2010;465(7294):96–100.
162. Berger C, Romero-Brey I, Radujkovic D, Terreux R, Zayas M, Paul D, Harak C, Hoppe S, Gao M, Penin F, et al. Daclatasvir-like inhibitors of NS5A block early biogenesis of hepatitis C virus-induced membranous replication factories, independent of RNA replication. Gastroenterology. 2014;147(5):1094–105. e1025.
163. Nakamoto S, Kanda T, Wu S, Shirasawa H, Yokosuka O. Hepatitis C virus NS5A inhibitors and drug resistance mutations. World J Gastroenterol. 2014;20(11):2902–12.
164. Lawitz EJ, Gruener D, Hill JM, Marbury T, Moorehead L, Mathias A, Cheng G, Link JO, Wong KA, Mo H, et al. A phase 1, randomized, placebo-controlled, 3-day, dose-ranging study of GS-5885, an NS5A inhibitor, in patients with genotype 1 hepatitis C. J Hepatol. 2012;57(1):24–31.
165. Wong KA, Worth A, Martin R, Svarovskaia E, Brainard DM, Lawitz E, Miller MD, Mo H. Characterization of hepatitis C virus resistance from a multiple-dose clinical trial of the novel NS5A inhibitor GS-5885. Antimicrob Agents Chemother. 2013;57(12):6333–40.
166. Krishnan P, Beyer J, Mistry N, Koev G, Reisch T, DeGoey D, Kati W, Campbell A, Williams L, Xie W, et al. *In vitro* and *in vivo* antiviral activity and resistance profile of ombitasvir, an inhibitor of HCV NS5A. Antimicrob Agents Chemother. 2014;59:979–87.
167. DeGoey DA, Betebenner DA, Grampovnik DJ, Liu D, Pratt JK, Tufano MD, He W, Krishnan P, Pilot-Matias TJ, Marsh KC, et al. Discovery of pyrido[2,3-d]pyrimidine-based inhibitors of HCV NS5A. Bioorg Med Chem Lett. 2013;23(12):3627–30.
168. Vince B, Hill JM, Lawitz EJ, O'Riordan W, Webster LR, Gruener DM, Mofsen RS, Murillo A, Donovan E, Chen J, et al. A randomized, double-blind, multiple-dose study of the pan-genotypic NS5A inhibitor samatasvir in patients infected with hepatitis C virus genotype 1, 2, 3 or 4. J Hepatol. 2014;60(5):920–7.
169. Wang C, Jia L, O'Boyle DR, Sun JH, Rigat K, Valera L, Nower P, Huang X, Kienzle B, Roberts S, et al. Comparison of daclatasvir resistance barrier on NS5A from HCV genotypes 1-6: implications for cross-genotype activity. Antimicrob Agents Chemother. 2014;58:5155–63.
170. Wang C, Valera L, Jia L, Kirk MJ, Gao M, Fridell RA. *In vitro* activity of daclatasvir on hepatitis C virus genotype 3 NS5A. Antimicrob Agents Chemother. 2013;57(1):611–3.
171. Wang C, Jia L, Huang H, Qiu D, Valera L, Huang X, Sun JH, Nower PT, O'Boyle 2nd DR, Gao M, et al. *In vitro* activity of BMS-790052 on hepatitis C virus genotype 4 NS5A. Antimicrob Agents Chemother. 2012;56(3):1588–90.
172. Izumi N. Efficacy of daclatasvir in hepatitis C virus. Expert Rev Anti Infect Ther. 2014;12(9):1025–31.
173. Karino Y, Toyota J, Ikeda K, Suzuki F, Chayama K, Kawakami Y, Ishikawa H, Watanabe H, Hernandez D, Yu F, et al. Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir. J Hepatol. 2013;58(4):646–54.
174. Suzuki F, Sezaki H, Akuta N, Suzuki Y, Seko Y, Kawamura Y, Hosaka T, Kobayashi M, Saito S, Arase Y, et al. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J Clin Virol. 2012;54(4):352–4.
175. Itakura J, Kurosaki M, Takada H, Nakakuki N, Matsuda S, Gondou K, Asano Y, Hattori N, Itakura Y, Tamaki N, et al. Naturally occurring, resistance-associated hepatitis C virus NS5A

variants are linked to IL28B genotype and are sensitive to interferon-based therapy. Hepatol Res. 2015;45:E115–21.
176. Kuiken C, Yusim K, Boykin L, Richardson R. The Los Alamos hepatitis C sequence database. Bioinformatics. 2005;21(3):379–84.
177. McCormick AL, Wang L, Garcia-Diaz A, Macartney MJ, Webster DP, Haque T. Prevalence of baseline polymorphisms for potential resistance to NS5A inhibitors in drug-naive individuals infected with hepatitis C genotypes 1-4. Antivir Ther. 2014;20:81–5.
178. Murakami E, Niu C, Bao H, Micolochick Steuer HM, Whitaker T, Nachman T, Sofia MA, Wang P, Otto MJ, Furman PA. The mechanism of action of beta-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine involves a second metabolic pathway leading to beta-D-2′-deoxy-2′-fluoro-2′-C-methyluridine 5′-triphosphate, a potent inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. Antimicrob Agents Chemother. 2008;52(2):458–64.
179. Furman PA, Lam AM, Murakami E. Nucleoside analog inhibitors of hepatitis C viral replication: recent advances, challenges and trends. Future Med Chem. 2009;1(8):1429–52.
180. Tan CH, Jekle A, Kang H, Moy C, Deval J, Jin Z, Jiang M, Ardzinski A, Dyatkina N, Chanda SM, et al. Preclinical and *in vitro* resistance profile of potent nucleotide polymerase inhibitors of HCV: ALS-2200 and its single diastereomer, VX-135. Presented at the 8th international workshop on hepatitis C: resistance and new compounds. Boston, MA. 27–28 June 2013.
181. Migliaccio G, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhat B, Bartholomew L, Bosserman MR, Ceccacci A, Colwell LF, et al. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication *in vitro*. J Biol Chem. 2003;278(49):49164–70.
182. Feng JY, Cheng G, Perry J, Barauskas O, Xu Y, Fenaux M, Eng S, Tirunagari N, Peng B, Yu M, et al. Inhibition of hepatitis C virus replication by GS-6620, a potent C-nucleoside monophosphate prodrug. Antimicrob Agents Chemother. 2014;58(4):1930–42.
183. Lam AM, Murakami E, Espiritu C, Steuer HM, Niu C, Keilman M, Bao H, Zennou V, Bourne N, Julander JG, et al. PSI-7851, a pronucleotide of beta-D-2′-deoxy-2′-fluoro-2′-C-methyluridine monophosphate, is a potent and pan-genotype inhibitor of hepatitis C virus replication. Antimicrob Agents Chemother. 2010;54(8):3187–96.
184. Najera I. Resistance to HCV nucleoside analogue inhibitors of hepatitis C virus RNA-dependent RNA polymerase. Curr Opin Virol. 2013;3(5):508–13.
185. Margeridon-Thermet S, Le Pogam S, Li L, Liu TF, Shulman N, Shafer RW, Najera I. Similar prevalence of low-abundance drug-resistant variants in treatment-naive patients with genotype 1a and 1b hepatitis C virus infections as determined by ultradeep pyrosequencing. PLoS One. 2014;9(8):e105569.
186. Ji H, Kozak RA, Biondi MJ, Pilon R, Vallee D, Liang BB, La D, Kim J, Van Domselaar G, Leonard L, et al. Next generation sequencing of the hepatitis C virus NS5B gene reveals potential novel S282 drug resistance mutations. Virology. 2015;477:1–9.
187. Maimone S, Tripodi G, Musolino C, Cacciola I, Pollicino T, Raimondo G. Lack of the NS5B S282T mutation in HCV isolates from liver tissue of treatment-naive patients with HCV genotype-1b infection. Antivir Ther. 2015;20(2):245–7.
188. Ali S, Leveque V, Le Pogam S, Ma H, Philipp F, Inocencio N, Smith M, Alker A, Kang H, Najera I, et al. Selected replicon variants with low-level *in vitro* resistance to the hepatitis C virus NS5B polymerase inhibitor PSI-6130 lack cross-resistance with R1479. Antimicrob Agents Chemother. 2008;52(12):4356–69.
189. Pawlotsky JM. New hepatitis C therapies: the toolbox, strategies, and challenges. Gastroenterology. 2014;146(5):1176–92.
190. Olsen DB, Eldrup AB, Bartholomew L, Bhat B, Bosserman MR, Ceccacci A, Colwell LF, Fay JF, Flores OA, Getty KL, et al. A 7-deaza-adenosine analog is a potent and selective inhibitor of hepatitis C virus replication with excellent pharmacokinetic properties. Antimicrob Agents Chemother. 2004;48(10):3944–53.
191. Carroll SS, Ludmerer S, Handt L, Koeplinger K, Zhang NR, Graham D, Davies ME, MacCoss M, Hazuda D, Olsen DB. Robust antiviral efficacy upon administration of a nucleoside analog to hepatitis C virus-infected chimpanzees. Antimicrob Agents Chemother. 2009;53(3):926–34.
192. Berke JM, Vijgen L, Lachau-Durand S, Powdrill MH, Rawe S, Sjuvarsson E, Eriksson S, Gotte M, Fransen E, Dehertogh P, et al. Antiviral activity and mode of action of TMC647078, a novel nucleoside inhibitor of the hepatitis C virus NS5B polymerase. Antimicrob Agents Chemother. 2011;55(8):3812–20.
193. Ludmerer SW, Graham DJ, Boots E, Murray EM, Simcoe A, Markel EJ, Grobler JA, Flores OA, Olsen DB, Hazuda DJ, et al. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. Antimicrob Agents Chemother. 2005;49(5):2059–69.
194. Le Pogam S, Jiang WR, Leveque V, Rajyaguru S, Ma H, Kang H, Jiang S, Singer M, Ali S, Klumpp K, et al. *In vitro* selected Con1 subgenomic replicons resistant to 2′-C-methyl-cytidine or to R1479 show lack of cross resistance. Virology. 2006;351(2):349–59.
195. Dutartre H, Bussetta C, Boretto J, Canard B. General catalytic deficiency of hepatitis C virus RNA polymerase with an S282T mutation and mutually exclusive resistance towards 2′-modified nucleotide analogues. Antimicrob Agents Chemother. 2006;50(12):4161–9.
196. Cretton-Scott E, Perigaud C, Peyrottes S, Licklider L, Camire M, Larsson M, Colla ML, Hildebrand E, Lallos L, Bilello J, et al. *In vitro* antiviral activity and pharmacology of IDX184, a novel and potent inhibitor of HCV replication. Presented at the 43rd annual meeting of the European Association for the Study of Liver, Milan, Italy. 23–27 April 2008.
197. Chang W, Bao D, Chun BK, Naduthambi D, Nagarathnam D, Rachakonda S, Reddy PG, Ross BS, Zhang HR, Bansal S, et al. Discovery of PSI-353661, a novel purine nucleotide prodrug for the treatment of HCV infection. ACS Med Chem Lett. 2011;2(2):130–5.
198. Vernachio JH, Bleiman B, Bryant KD, Chamberlain S, Hunley D, Hutchins J, Ames B, Gorovits E, Ganguly B, Hall A, et al. INX-08189, a phosphoramidate prodrug of 6-O-methyl-2′-C-methyl guanosine, is a potent inhibitor of hepatitis C virus replication with excellent pharmacokinetic and pharmacodynamic properties. Antimicrob Agents Chemother. 2011;55(5):1843–51.
199. Bilello JP, Dousson CB, Pierra C, Surleraux D, LaColla M, Chapron C, Serra I, Golitsina N, Danehy F, Camire M, et al. The pharmacologic properties of IDX19368, a novel nucleotide prodrug, highlight its potential for use in a low-dose DAA regimen for HCV. Presented at the 19th international symposium on heptatits C virus and related viruses. Venice, Italy. 5–9 Oct 2012.
200. Du J, Bao D, Chun BK, Jiang Y, Reddy PG, Zhang HR, Ross BS, Bansal S, Bao H, Espiritu C, et al. beta-D-2′-alpha-F-2′-beta-C-Methyl-6-O-substituted 3′,5′-cyclic phosphate nucleotide prodrugs as inhibitors of hepatitis C virus replication: a structure-activity relationship study. Bioorg Med Chem Lett. 2012;22(18):5924–9.
201. Lalezari J, Asmuth D, Casiro A, Vargas H, Lawrence S, Dubuc-Patrick G, Chen J, McCarville J, Pietropaolo K, Zhou XJ, et al. Short-term monotherapy with IDX184, a liver-targeted nucleotide polymerase inhibitor, in patients with chronic hepatitis C virus infection. Antimicrob Agents Chemother. 2012;56(12):6372–8.
202. Lalezari J, Box T, O'Riordan W, Mehra P, Nguyen T, Poordad F, Dejesus E, Kwo P, Godofsky E, Lawrence S, et al. IDX184 in combination with pegylated interferon-alpha2a and ribavirin for 2

weeks in treatment-naive patients with chronic hepatitis C. Antivir Ther. 2013;18(6):755–64.
203. Gupta K, Seifer M, Serra I, Rashidzadeh H, Luo S, Colla ML, Pan-Zhou X-R, Chapron C, Hernandez-Santiago B, Parsy C, et al. Favorable preclinical profile of IDX21437, a novel uridine nucleotide prodrug, for use in a direct-acting antiviral (DAA) regimen for HCV. Presented at the 49th annual meeting of the European Association for the Study of the Liver (EASL). London, UK. 9–13 April 2014.
204. Yang W, Wiles J, Patel D, Podos S, Fabrycki J, Zhao Y, Jia L, Yang G, Rivera J, Elliot M, et al. Preclinical characteristics of ACH-3422: a potent uridine nucleotide prodrug for inhibition of hepatitis C virus NS5B RNA polymerase. Presented at the 64th annual meeting of the American Association for the Study of Liver Diseases (AASLD). Washington, D.C. 1–5 Nov 2013.
205. Tan H, Shaw K, Jekle A, Deval J, Jin Z, Fung A, Tam Y, Blatt L, Chanda S, Wang G, et al. Preclinical characterization of AL-516, a potent guanosine based nucleoside polymerase inhibitor for the treatment of chronic hepatitis C. Presented at the 65th annual meeting of the American Association for the Study of Liver Diseases (AASLD). Boston, MA. 7–11 November 2014.
206. Lam AM, Espiritu C, Murakami E, Zennou V, Bansal S, Micolochick Steuer HM, Niu C, Keilman M, Bao H, Bourne N, et al. Inhibition of hepatitis C virus replicon RNA synthesis by PSI-352938, a cyclic phosphate prodrug of beta-D-2′-deoxy-2′-alpha-fluoro-2′-beta-C-methylguanosine. Antimicrob Agents Chemother. 2011;55(6):2566–75.
207. Lam AM, Espiritu C, Bansal S, Micolochick Steuer HM, Zennou V, Otto MJ, Furman PA. Hepatitis C virus nucleotide inhibitors PSI-352938 and PSI-353661 exhibit a novel mechanism of resistance requiring multiple mutations within replicon RNA. J Virol. 2011;85(23):12334–42.
208. Le Pogam S, Seshaadri A, Ewing A, Kang H, Kosaka A, Yan JM, Berrey M, Symonds B, De La Rosa A, Cammack N, et al. RG7128 alone or in combination with pegylated interferon-alpha2a and ribavirin prevents hepatitis C virus (HCV) Replication and selection of resistant variants in HCV-infected patients. J Infect Dis. 2010;202(10):1510–9.
209. Pawlotsky JM, Najera I, Jacobson I. Resistance to mericitabine, a nucleoside analogue inhibitor of HCV RNA-dependent RNA polymerase. Antivir Ther. 2012;17(3):411–23.
210. Popa S, Berliba A, Ghicavii N, Patat A, Kauffman RS, Krop J, Garg V, Tong M, Chanda SM, Zhang Q, et al. VX-135, a once-daily nucleotide HCV polymerase inhibitor, was well tolerated and demonstrated potent antiviral activity when given with ribavirin in treatment-naïve patients with genotype 1 HCV. Presented at the 64th annual meeting of the American Association for the Study of Liver Diseases (AASLD). Washington, D.C. 1–5 Nov 2013.
211. Svarovskaia ES, Dvory-Sobol SH, Hebner C, Doehle B, Gontcharova V, Martin R, Gane EJ, Jacobson IM, Nelson DR, Lawitz E, et al. No resistance detected in four phase 3 clinical studies in HCV genotypes 1-6 of sufosbuvir + ribavirin with or without peginterferon. Presented at the 64th annual meeting of the American Association for the Study of Liver Diseases (AASLD). Washington DC. 1–4 Nov 2013.
212. Molina JM, Orkin C, Iser DM, Zamora FX, Nelson M, Stephan C, Massetto B, Gaggar A, Ni L, Svarovskaia E, et al. Sofosbuvir plus ribavirin for treatment of hepatitis C virus in patients co-infected with HIV (PHOTON-2): a multicentre, open-label, non-randomised, phase 3 study. Lancet. 2015;385(9973):1098–106.
213. Brown NA. Progress towards improving antiviral therapy for hepatitis C with hepatitis C virus polymerase inhibitors. Part I: nucleoside analogues. Expert Opin Investig Drugs. 2009;18(6):709–25.
214. Tong X, Li L, Haines K, Najera I. Identification of the NS5B S282T resistant variant and two novel amino acid substitutions that affect replication capacity in hepatitis C virus-infected patients treated with mericitabine and danoprevir. Antimicrob Agents Chemother. 2014;58(6):3105–14.
215. Tong X, Piso K, Haines K, Baronas V, Li L, Yan J-M, LePogam S, Kulkarni R, Thommes J, Klumpp K, et al. Lack of mercitibine resistance mutation NS5B S282T and detection and characterization of a novel mercitibine double resistance mutation NS5B L159F + L320F in patients treated with mercitibine plus peginterferon alfa-2a (40KD)/ribavirin provides further evidence for the high resistance barrier to mercitabine. Presented at the 65th annual meeting of the American Association for the Study of Liver Diseases (AASLD). Boston, Massachusetts, USA. 9–13 Nov 2012.
216. Tong X, Le Pogam S, Li L, Haines K, Piso K, Baronas V, Yan JM, So SS, Klumpp K, Najera I. *In vivo* emergence of a novel mutant L159F/L320F in the NS5B polymerase confers low-level resistance to the HCV polymerase inhibitors mericitabine and sofosbuvir. J Infect Dis. 2014;209(5):668–75.
217. Hedskog C, Gontcharova V, Han B, Martin R, Miller MD, Mo H, Svarovskaia ES. Evolution of the HCV viral population from the one patient with S282T detected at relapse after sufosbuvir monotherapy. Presented at the international workshop on HIV and hepatitis virus drug resistance and curative strategies. Toronto, Canada. 4–8 June 2013.
218. Lawitz E, Poordad FF, Pang PS, Hyland RH, Ding X, Mo H, Symonds WT, McHutchison JG, Membreno FE. Sofosbuvir and Ledipasvir fixed-dose combination with and without ribavirin in treatment-naïve and previously treated patients with genotype 1 hepatitis C: the LONESTAR study. Presented at the 64th annual meeting of the American Association for the Study of Liver Diseases (AASLD). Washington, D.C. 1–5 Nov 2013.
219. Schneider MD, Sarrazin C. Antiviral therapy of hepatitis C in 2014: do we need resistance testing? Antiviral Res. 2014;105:64–71.
220. Donaldson EF, Harrington PR, O'Rear JJ, Naeger LK. Clinical evidence and bioinformatics characterization of potential hepatitis C virus resistance pathways for sofosbuvir. Hepatology. 2015;61(1):56–65.
221. Gane E, Stedman C, Garg V, George S, Kieffer T, Krop J, Lan L, Rubin R, Lawal A. An interferon- and ribavirin-free 12-week tegimen of once-daily VX-135 and daclatasvir in treatment-naïve patients with genotype 1 HCV infection. Presented at the 49th annual meeting of the European Association for the Study of the Liver (EASL). London, UK. 9–13 April 2014.
222. Tigges A, Zhang E, Davis A, Spanks J, Dorrian J, Symons J, Bartels D, Fry J, Keiffer T. The S282T variant is not observed in treatment-naïve patients with genotype 1 HCV infection treated with the nucleotide inhibitor VX-135 in combination with ribavirin. Presented at the 49th annual meeting of the European Association for the Study of the Liver (EASL). London, UK. 9–13 April 2014.
223. Le Pogam S, Seshaadri A, Kosaka A, Chiu S, Kang H, Hu S, Rajyaguru S, Symons J, Cammack N, Najera I. Existence of hepatitis C virus NS5B variants naturally resistant to non-nucleoside, but not to nucleoside, polymerase inhibitors among untreated patients. J Antimicrob Chemother. 2008;61(6):1205–16.
224. Franco S, Casadella M, Noguera-Julian M, Clotet B, Tural C, Paredes R, Martinez MA. No detection of the NS5B S282T mutation in treatment-naive genotype 1 HCV/HIV-1 coinfected patients using deep sequencing. J Clin Virol. 2013;58(4):726–9.
225. Feld JJ. Interferon-free strategies with a nucleoside/nucleotide analogue. Semin Liver Dis. 2014;34(1):37–46.
226. Sarisky RT. Non-nucleoside inhibitors of the HCV polymerase. J Antimicrob Chemother. 2004;54(1):14–6.
227. Beaulieu PL, Bos M, Cordingley MG, Chabot C, Fazal G, Garneau M, Gillard JR, Jolicoeur E, LaPlante S, McKercher G, et al. Discovery of the first thumb pocket 1 NS5B polymerase inhibitor (BILB 1941) with demonstrated antiviral activity in patients

chronically infected with genotype 1 hepatitis C virus (HCV). J Med Chem. 2012;55(17):7650–66.
228. Tomei L, Altamura S, Bartholomew L, Biroccio A, Ceccacci A, Pacini L, Narjes F, Gennari N, Bisbocci M, Incitti I, et al. Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J Virol. 2003;77(24):13225–31.
229. Hirashima S, Suzuki T, Ishida T, Noji S, Yata S, Ando I, Komatsu M, Ikeda S, Hashimoto H. Benzimidazole derivatives bearing substituted biphenyls as hepatitis C virus NS5B RNA-dependent RNA polymerase inhibitors: structure-activity relationship studies and identification of a potent and highly selective inhibitor JTK-109. J Med Chem. 2006;49(15):4721–36.
230. Delang L, Froeyen M, Herdewijn P, Neyts J. Identification of a novel resistance mutation for benzimidazole inhibitors of the HCV RNA-dependent RNA polymerase. Antiviral Res. 2012;93(1):30–8.
231. Han D, Strommen K, Anton ED, Choe S, Chen M, Hazuda DJ, Petropoulos CJ, Barnard RJO, Reeves JD. Genotypic and phenotypic characterizations of natural and acquired reductions in susceptibility to the non-nucleoside inhibitor MK-3281. Presented at the 6th international workshop hepatitis C—resistance and new compound. Cambridge, MA. 23–24 June 2011.
232. Devogelaere B, Berke JM, Vijgen L, Dehertogh P, Fransen E, Cleiren E, van der Helm L, Nyanguile O, Tahri A, Amssoms K, et al. TMC647055, a potent nonnucleoside hepatitis C virus NS5B polymerase inhibitor with cross-genotypic coverage. Antimicrob Agents Chemother. 2012;56(9):4676–84.
233. Cummings MD, Lin TI, Hu L, Tahri A, McGowan D, Amssoms K, Last S, Devogelaere B, Rouan MC, Vijgen L, et al. Discovery and early development of TMC647055, a non-nucleoside inhibitor of the hepatitis C virus NS5B polymerase. J Med Chem. 2014;57(5):1880–92.
234. Rigat KL, Lu H, Wang YK, Argyrou A, Fanslau C, Beno B, Wang Y, Marcinkeviciene J, Ding M, Gentles RG, et al. Mechanism of inhibition for BMS-791325, a novel non-nucleoside inhibitor of hepatitis C virus NS5B polymerase. J Biol Chem. 2014;289(48):33456–68.
235. Liu M, Tuttle M, Gao M, Lemm JA. Potency and resistance analysis of hepatitis C virus NS5B polymerase inhibitor BMS-791325 on all major genotypes. Antimicrob Agents Chemother. 2014;58(12):7416–23.
236. Tatum H, Thuluvath PJ, Lawitz E, Martorell C, DeMicco M, Cohen S, Rustgi V, Ravendhran N, Ghalib R, Hanson J, et al. A randomized, placebo-controlled study of the NS5B inhibitor beclabuvir with peginterferon/ribavirin for HCV genotype 1. J Viral Hepat. 2014;22:658–64.
237. Shi ST, Herlihy KJ, Graham JP, Fuhrman SA, Doan C, Parge H, Hickey M, Gao J, Yu X, Chau F, et al. *In vitro* resistance study of AG-021541, a novel nonnucleoside inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. Antimicrob Agents Chemother. 2008;52(2):675–83.
238. Wang H, Guo C, Chen BZ, Ji M. Computational study on the drug resistance mechanism of HCV NS5B RNA-dependent RNA polymerase mutants V494I, V494A, M426A, and M423T to Filibuvir. Antiviral Res. 2015;113:79–92.
239. Howe AY, Cheng H, Thompson I, Chunduru SK, Herrmann S, O'Connell J, Agarwal A, Chopra R, Del Vecchio AM. Molecular mechanism of a thumb domain hepatitis C virus nonnucleoside RNA-dependent RNA polymerase inhibitor. Antimicrob Agents Chemother. 2006;50(12):4103–13.
240. May MM, Brohm D, Harrenga A, Marquardt T, Riedl B, Kreuter J, Zimmermann H, Ruebsamen-Schaeff H, Urban A. Discovery of substituted N-phenylbenzenesulphonamides as a novel class of non-nucleoside hepatitis C virus polymerase inhibitors. Antiviral Res. 2012;95(2):182–91.
241. Cooper C, Lawitz EJ, Ghali P, Rodriguez-Torres M, Anderson FH, Lee SS, Bedard J, Chauret N, Thibert R, Boivin I, et al. Evaluation of VCH-759 monotherapy in hepatitis C infection. J Hepatol. 2009;51(1):39–46.
242. Fenaux M, Eng S, Leavitt SA, Lee YJ, Mabery EM, Tian Y, Byun D, Canales E, Clarke MO, Doerffler E, et al. Preclinical characterization of GS-9669, a thumb site II inhibitor of the hepatitis C virus NS5B polymerase. Antimicrob Agents Chemother. 2013;57(2):804–10.
243. Gentile I, Buonomo AR, Zappulo E, Coppola N, Borgia G. GS-9669: a novel non-nucleoside inhibitor of viral polymerase for the treatment of hepatitis C virus infection. Expert Rev Anti Infect Ther. 2014;12(10):1179–86.
244. Lazerwith SE, Lew W, Zhang J, Morganelli P, Liu Q, Canales E, Clarke MO, Doerffler E, Byun D, Mertzman M, et al. Discovery of GS-9669, a thumb site II non-nucleoside inhibitor of NS5B for the treatment of genotype 1 chronic hepatitis C infection. J Med Chem. 2014;57(5):1893–901.
245. Biswal BK, Wang M, Cherney MM, Chan L, Yannopoulos CG, Bilimoria D, Bedard J, James MN. Non-nucleoside inhibitors binding to hepatitis C virus NS5B polymerase reveal a novel mechanism of inhibition. J Mol Biol. 2006;361(1):33–45.
246. Xue W, Jiao P, Liu H, Yao X. Molecular modeling and residue interaction network studies on the mechanism of binding and resistance of the HCV NS5B polymerase mutants to VX-222 and ANA598. Antiviral Res. 2014;104:40–51.
247. Kati W, Koev G, Irvin M, Beyer J, Liu Y, Krishnan P, Reisch T, Mondal R, Wagner R, Molla A, et al. *In vitro* activity and resistance profile of dasabuvir, a nonnucleoside hepatitis C virus polymerase inhibitor. Antimicrob Agents Chemother. 2015;59(3):1505–11.
248. Nguyen TT, Gates AT, Gutshall LL, Johnston VK, Gu B, Duffy KJ, Sarisky RT. Resistance profile of a hepatitis C virus RNA-dependent RNA polymerase benzothiadiazine inhibitor. Antimicrob Agents Chemother. 2003;47(11):3525–30.
249. Tomei L, Altamura S, Bartholomew L, Bisbocci M, Bailey C, Bosserman M, Cellucci A, Forte E, Incitti I, Orsatti L, et al. Characterization of the inhibition of hepatitis C virus RNA replication by nonnucleosides. J Virol. 2004;78(2):938–46.
250. Lu L, Mo H, Pilot-Matias TJ, Molla A. Evolution of resistant M414T mutants among hepatitis C virus replicon cells treated with polymerase inhibitor A-782759. Antimicrob Agents Chemother. 2007;51(6):1889–96.
251. Mo H, Lu L, Pilot-Matias T, Pithawalla R, Mondal R, Masse S, Dekhtyar T, Ng T, Koev G, Stoll V, et al. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor *in vitro*. Antimicrob Agents Chemother. 2005;49(10):4305–14.
252. Trivella JP, Gutierrez J, Martin P. Dasabuvir: a new direct antiviral agent for the treatment of hepatitis C. Expert Opin Pharmacother. 2015;16(4):617–24.
253. Lu L, Dekhtyar T, Masse S, Pithawalla R, Krishnan P, He W, Ng T, Koev G, Stewart K, Larson D, et al. Identification and characterization of mutations conferring resistance to an HCV RNA-dependent RNA polymerase inhibitor *in vitro*. Antiviral Res. 2007;76(1):93–7.
254. Thompson PA, Patel RA, Showalter RE, Li C, Appleman JR, Steffy KR. *In vitro* studies demonstrate that combinations of antiviral agents that include HCV polymerase inhibitor ANA598 have the potential to overcome viral resistance. Presented at the 59th annual meeting of the American Association for the Study of Liver Diseases (AASLD). San Francisco, CA. 31 October–1 November 2008.
255. Bilello JP, Colla ML, Serra I, Gillum JM, Soubasakos MA, Chapron C, Li B, Bonin A, Panzo RJ, Seifer M, et al. Enhanced antiviral activity of IDX375, a novel non-nucleoside inhibitor, in combination

with other HCV antiviral agents in the genotype 1b replicon *in vitro*. Presented at the 16th international symposium on hepatitis C virus and related viruses. Nice, France. 3–7 October 2009.
256. Ando I, Ogura N, Toyonaga Y, Hirahara K, Shibata T, Noguchi T. JTK-853, a novel non-nucleoside hepatitis C virus polymerase inhibitor, demonstrates a high genetic barrier to resistance *in vitro*. Intervirology. 2013;56(5):302–9.
257. Howe AY, Cheng H, Johann S, Mullen S, Chunduru SK, Young DC, Bard J, Chopra R, Krishnamurthy G, Mansour T, et al. Molecular mechanism of hepatitis C virus replicon variants with reduced susceptibility to a benzofuran inhibitor, HCV-796. Antimicrob Agents Chemother. 2008;52(9):3327–38.
258. Voitenleitner C, Crosby R, Walker J, Remlinger K, Vamathevan J, Wang A, You S, Johnson III J, Woldu E, Van Horn S, et al. *In vitro* characterization of GSK2485852, a novel hepatitis C virus polymerase inhibitor. Antimicrob Agents Chemother. 2013;57(11):5216–24.
259. Maynard A, Crosby RM, Ellis B, Hamatake R, Hong Z, Johns BA, Kahler KM, Koble C, Leivers A, Leivers MR, et al. Discovery of a potent boronic acid derived inhibitor of the HCV RNA-dependent RNA polymerase. J Med Chem. 2014;57(5):1902–13.
260. McCown MF, Rajyaguru S, Kular S, Cammack N, Najera I. GT-1a or GT-1b subtype-specific resistance profiles for hepatitis C virus inhibitors telaprevir and HCV-796. Antimicrob Agents Chemother. 2009;53(5):2129–32.
261. Shih IH, Vliegen I, Peng B, Yang H, Hebner C, Paeshuyse J, Purstinger G, Fenaux M, Tian Y, Mabery E, et al. Mechanistic characterization of GS-9190 (Tegobuvir), a novel nonnucleoside inhibitor of hepatitis C virus NS5B polymerase. Antimicrob Agents Chemother. 2011;55(9):4196–203.
262. Hebner CM, Han B, Brendza KM, Nash M, Sulfab M, Tian Y, Hung M, Fung W, Vivian RW, Trenkle J, et al. The HCV non-nucleoside inhibitor Tegobuvir utilizes a novel mechanism of action to inhibit NS5B polymerase function. PLoS One. 2012;7(6):e39163.
263. Wong KA, Xu S, Martin R, Miller MD, Mo H. Tegobuvir (GS-9190) potency against HCV chimeric replicons derived from consensus NS5B sequences from genotypes 2b, 3a, 4a, 5a, and 6a. Virology. 2012;429(1):57–62.
264. Stansfield I, Avolio S, Colarusso S, Gennari N, Narjes F, Pacini B, Ponzi S, Harper S. Active site inhibitors of HCV NS5B polymerase. The development and pharmacophore of 2-thienyl-5,6-dihydroxypyrimidine-4-carboxylic acid. Bioorg Med Chem Lett. 2004;14(20):5085–8.
265. Summa V, Petrocchi A, Matassa VG, Taliani M, Laufer R, De Francesco R, Altamura S, Pace P. HCV NS5b RNA-dependent RNA polymerase inhibitors: from alpha, gamma-diketoacids to 4,5-dihydroxypyrimidine- or 3-methyl-5-hydroxypyrimidinonecarboxylic acids. Design and synthesis. J Med Chem. 2004;47(22):5336–9.
266. Koch U, Attenni B, Malancona S, Colarusso S, Conte I, Di Filippo M, Harper S, Pacini B, Giomini C, Thomas S, et al. 2-(2-Thienyl)-5,6-dihydroxy-4-carboxypyrimidines as inhibitors of the hepatitis C virus NS5B polymerase: discovery, SAR, modeling, and mutagenesis. J Med Chem. 2006;49(5):1693–705.
267. Pacini B, Avolio S, Ercolani C, Koch U, Migliaccio G, Narjes F, Pacini L, Tomei L, Harper S. 2-(3-Thienyl)-5,6-dihydroxypyrimidine-4-carboxylic acids as inhibitors of HCV NS5B RdRp. Bioorg Med Chem Lett. 2009;19(21):6245–9.
268. Powdrill MH, Deval J, Narjes F, De Francesco R, Gotte M. Mechanism of hepatitis C virus RNA polymerase inhibition with dihydroxypyrimidines. Antimicrob Agents Chemother. 2010;54(3):977–83.

Part VI

Parasitic Drug Resistance: Mechanisms

Drug Resistance Mechanisms in *Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis,* and Opportunistic Anaerobic Protozoa

40

Consuelo Gómez García, Laurence A. Marchat, Lilia López-Cánovas, D. Guillermo Pérez Ishiwara, Mario A. Rodríguez, and Esther Orozco

1 Introduction

In protozoa, drug resistance is defined as the ability of a parasite strain to survive and multiply despite the administration and absorption of a drug supplemented in doses equal to or higher than those usually recommended [1]. Resistance of organisms to toxic agents is a survival mechanism fundamental for adaptation and evolution of life. As a counterpart, drug resistance is a medical trouble in cancer and infectious diseases, with no many alternatives available. Emergence and spread of drug resistance is one of the most important factors undermining protozoan parasite control programs in most of its endemic world. *Entamoeba histolytica*, *Giardia lamblia* (syn. *duodenalis* or *intestinalis*), and *Trichomonas vaginalis* (Fig. 40.1) are anaerobic and microaerophilic pathogens and their capability to develop drug resistance has been experimentally proved [2]. Over one billion individuals worldwide harbor these and other anaerobic protozoa such as *Blastocystis hominis*, *Cryptosporidium parvum*, *Isospora* spp., *Cyclospora* spp., and *Microsporidia* [3]. Most infected people live in poor countries. Regretful sanitary conditions, lack of public health policies, and poor health education are the main causes for protozoan infectious prevalence, even when they can be eradicated implementing drainage, parasite-free water supply, and health and sexual education for all people.

Intestinal parasites, *E. histolytica* and *G. lamblia* (Fig. 40.1a, b), enter humans by ingestion of cysts that come out with feces from infected individuals. The cysts, highly resistant to atmospheric conditions, are formed in the intestine and excreted with feces. They contaminate water and food, which serve as their vehicles to infect other hosts. Host factors induce transformation of cysts into trophozoites that cause the diseases. A high percentage of infected people do not present symptoms, but spread the parasites. *E. histolytica* invades mainly the gut and liver, but also the brain, lungs, skin, and genitals. On the other hand, *T. vaginalis* (Fig. 40.1c) is causative of the most common nonviral human sexually transmitted disease [4]; it mainly infects women, but men are also a target of the parasite. Between 25 and 50 % of infected people are asymptomatic; in women, infection provokes vaginitis with inflammatory discharge and predisposition to cervical neoplasia; it causes complications during pregnancy, low weight of newborns, preterm delivery, and respiratory diseases. In men, it produces urethritis, orchitis, oligoasthenoteratospermia, and hypogonadism [5]. Trichomonosis is linked to an increased risk of cytomegalovirus [6] and human immunodeficiency virus (HIV) transmission [7].

Anaerobic protozoa emerged very early in evolution and parasites have gained many characteristics through coevolution inside the host. They share some biological characteristics, but also present striking phenotypic and genotypic differences. *E. histolytica* has a cytoplasm full of vacuoles, and except for the nucleus, organelles are difficult to distinguish in the highly phagocytic trophozoites (Fig. 40.1a). *Giardia* has eight flagella, two nuclei, and a ventral disk formed by giardins and other cytoskeleton proteins that

C. Gómez García, Ph.D. • L.A. Marchat, Ph.D.
Programa Institucional de Biomedicina Molecular, Escuela Nacional de Medicina y Homeopatía (ENMyH), Instituto Politécnico Nacional, Guillermo Massieu Helguera # 239, Ciudad de México 07320, Mexico

L. López-Cánovas, Ph.D.
Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México, Plantel del Valle, San Lorenzo # 290, Ciudad de México 03100, Mexico

D.G. Pérez Ishiwara, Ph.D.
Centro de Investigación en Biotecnología Aplicada (CIBA), Instituto Politécnico Nacional, Ex-Hacienda San Juan Molino Carretera Estatal Tecuexcomac-Tepetitla Km 1.5, Tlaxcala 90700, Mexico

M.A. Rodríguez, Ph.D. • E. Orozco, Ph.D. (✉)
Departamento de Infectómica y Patogénesis Molecular, CINVESTAV, IPN, Ave. IPN # 2508, AP 14-740, Ciudad de México 07360, Mexico
e-mail: esther@mail.cinvestav.mx

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_40

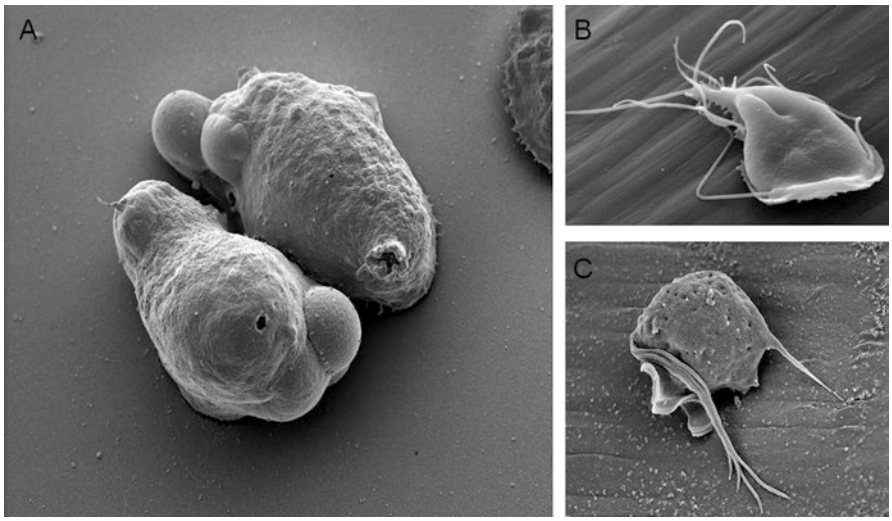


Fig. 40.1 Scanning electron microscopy of (**A**) *E. histolytica*, (**B**) *G. lamblia*, and (**C**) *T. vaginalis* trophozoites (micrographs were kindly given by Dr. Arturo González Robles, Departamento de Infectómica y Patogénesis Molecular, CINVESTAV IPN, Mexico)

allow the parasite adherence to epithelia (Fig. 40.1b). *T. vaginalis* has four anterior flagella and a recurrent flagellum incorporated into an undulating membrane, supported by a noncontractile costa (Fig. 40.1c). It can form pseudopodia to phagocyte epithelial cells. The three parasites have adherence molecules and cysteine proteases that function in colonization and damage production to tissues [8–10]. They do not *have bona fide* mitochondria and peroxisomes, organelles found in most eukaryotes, neither canonical mitochondrial processes. *E. histolytica* has a double-membrane limited organelle called EhkO, which contains DNA and pyruvate:ferredoxin oxidoreductase (PFOR) [11]. Additionally, mitochondrial-like enzymes have been found in other organelles called mitosome and crypton [12, 13]. Similarly, it has been reported that *G. lamblia* contains mitosomes that function in iron sulfur protein maturation [14]. *T. vaginalis* has hydrogenosomes, where both decarboxylation of pyruvate by PFOR and energy generation take place [15]. It is an anaerobic form of mitochondrion and produces H_2 during ATP synthesis [16]. Phylogenetic analysis suggests that *E. histolytica* and *G. lamblia* iron-hydrogenase genes were derived from a common eubacterial ancestor, distinct from the *T. vaginalis* iron-hydrogenase gene ancestor [17]. Similitude in their metabolism allows the use of common drugs against them such as the 5-nitroimidazoles (5-NI).

There are currently no effective vaccines available for prevention of parasitic diseases. By these reasons, the development of new antiprotozoal drugs is urgently required. Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), the preferred drug for the three mentioned parasites, enters into the cell by passive diffusion in an inactive form. It has a lower redox potential (−460 mV) than ferredoxin (Fd) (−320 mV) and gains electrons transferred by PFOR to Fd to be converted to toxic nitro or nitroso anions or intermediates, such as hydroxylamines (Fig. 40.2) generating a concentration gradient, favoring drug accumulation into the cell [18, 19]. Reduced metronidazole binds DNA and interferes with respiration and motility [18–20]. However, due to metronidazole toxicity and the emergence of metronidazole-resistant protozoa [21–24], discovery of new efficient drugs is needed.

Numerous pharmaco-biological factors contribute to the advent, spread, and intensification of drug-resistant parasites, such as immunological status of the host, drug characteristics, and environmental factors, among others [1]. Protozoa use various mechanisms to develop drug resistance, including DNA mutations, modulation of enzymes, and pump-like protein expression, such as the P-glycoproteins (PGPs) involved in the multidrug resistance phenotype (MDR), described in many organisms [25]. As poor countries cannot implement public health measures to prevent the dissemination of these parasites, they must understand the mechanisms that the parasites use to develop drug resistance, a fundamental tactic in the fight against these pathogens. Identifying resistance markers, finding a way to bypass them, as well as generating new drugs and vaccines to control infections provoked by anaerobic protozoa parasites are part of the approach to defeat them. Here, we review the current knowledge on drug susceptibility in anaerobic protozoa causative of human diseases.

2 *Entamoeba histolytica*

E. histolytica infects 500 million people, provoking 50 million cases of dysentery and liver abscesses, killing 100,000 persons each year [26]. Individuals harboring *E. histolytica* are asymptomatic or present diarrhea, bloody stools with mucus, colon abscesses, and dysentery. Liver abscesses, that may be lethal if not treated, occur in 3–9 % of infected patients [27]. Causes for the varied clinical symptoms lie in both parasite and host. Many authors have proposed that

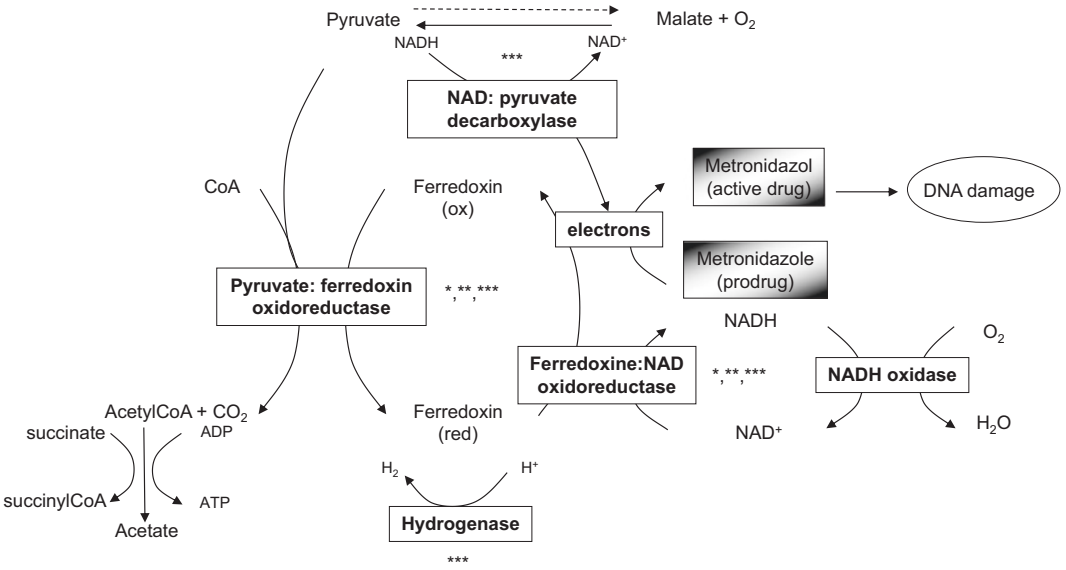


Fig. 40.2 Terminal part of the glycolytic pathway of anaerobic protozoa and its relation with metronidazole activation. (*Asterisk*) Represents the current knowledge on enzymes involved in drug activation. (*Single asterisk*) *E. histolytica*, (*double asterisk*) *G. lamblia*, (*triple asterisk*) *T. vaginalis* (figure created by authors from data in [3–19])

asymptomatic amoebiasis is due to *Entamoeba dispar* whereas the invasive disease is provoked by *E. histolytica* [28]. However, several studies have shown that *E. dispar* is able to destroy culture cells [29] and certain *E. histolytica* strains and clones have poor virulence, being almost unable to damage target cells [30]. If individuals harboring non-virulent *E. histolytica* or *E. dispar* should be medically treated, since they excrete potentially injurious cysts, is an unsolved question, because there are reports on people infected by asymptomatic carriers, which developed severe invasive amoebiasis [31]. Metronidazole is the most widely used antiamoebic drug and it is in the first-line drugs for invasive amoebiasis. However other nitroimidazoles as tinidazole and nitazoxanide, a nitrothiazoly-salicylamide derivative, have fewer side effects and are shorter treatment courses. Unfortunately, *E. histolytica* as many other parasites has established mechanisms to evade the drug pressure.

2.1 Antimicrobial Mechanism of Action in *E. histolytica*

The microaerotolerant trophozoites of *E. histolytica* grow in both the gut lumen and the rich-oxygenated epithelia. Agents currently used against amoebiasis are divided into tissue and luminal amoebicides (Table 40.1). Tissue amoebicides, such as metronidazole, tinidazole, and emetine kill trophozoites in tissues but have no effect against cysts. In contrast, luminal amoebicides, such as iodoquinol, diloxanide furoate, and paromomycin, are mainly active in the intestinal lumen, because they are poorly absorbed and are recommended to treat patient with asymptomatic infections following metronidazole [32]. Current chemotherapy of dysentery or extraintestinal abscesses consists of metronidazole or tinidazole, followed by a luminal amoebicide.

Trophozoites use fermentative metabolism for ATP production, which involves pyruvate decarboxylation by PFOR to acetyl CoA. *E. histolytica* possesses 2-oxoacid-reductase and biochemical assays identified the PFOR activity in cytoplasm [33], whereas antibodies against the recombinant PFOR localize it in cellular membranes and EhkO organelles [34]. Concomitantly to pyruvate decarboxylation, Fd is reduced. *E. histolytica* has two Fds: Fd1 and Fd2 and their amino acid sequences resemble clostridial type Fds. They have cysteine arrangement characteristic for the coordination of 2[4Fe–4S] clusters [35]. Inside the cell, an electron is transferred from Fd to the 5-nitro group of metronidazole to activate the drug and kill parasites.

Metronidazole provokes nausea and headache and is potentially carcinogenic in in vitro studies, but it has not been conclusively linked to the development of human malignancy. Emetine inhibits protein synthesis affecting ribosome movement along mRNA. It produces cardiac arrhythmia, gastrointestinal toxicity, and skin and neuromuscular reactions [36]. Diloxanide furoate is structurally related to chloramphenicol, whereas paromomycin, an aminoglycoside antibiotic, causes flatulence and diarrhea; both drugs inhibit protein synthesis. Iodoquinol is a halogenated hydroxyquinoline that chelates ferrous ions; its toxicity is associated with the iodine component producing neuropathy and blindness after prolonged administration [37].

Table 40.1 Drugs, target molecules, genes, and proteins involved in drug resistance in anaerobic protozoa

Parasite	Drug	Target molecule	Target location	Altered pathway	Genes and proteins involved in drug resistance
E. histolytica	Metronidazole	DNA	Nucleus	Electron transport	PFOR, SOD
	Emetine	Ribosome	Cytosol	Protein synthesis	*EhPgp1, EhPgp5, EhPgp6*
	Iodoquinol	DNA	DNA	Electron transport	ND
	Diloxanide furoate	ND	Cytosol	Protein synthesis	ND
T. vaginalis	Metronidazole	DNA	Nucleus	Electron transport	PFOR, Fd, NADP-dependent ME
G. lamblia	Metronidazole	DNA	Nucleus	Electron transport	PFOR, FdI
	Quinacrine	DNA, plasma membrane	Nucleus	ND	ND
	Tinidazole	DNA	Nucleus	Electron transport	Thiol-cycling enzymes
	Benzimidazole	β-Tubulin	Cytosol	Microtubule polymerization	NADH-oxidase
	Furazolidone	DNA	Nucleus	Protein synthesis	ND
	Paromomycin	ssRNA	Cytosol	Protein synthesis	ND

ND not determined

2.2 Mechanisms of Drug Resistance in *E. histolytica*

2.2.1 *E. histolytica* Metronidazole Resistance

Reluctance of amoebiasis to metronidazole treatment has been reported, mainly in patients with liver abscesses [38]. In vitro, metronidazole resistance has been induced by stepwise exposure to increasing drug amounts. Mutant lines growing in 40 μM metronidazole, a concentration almost fourfold the dose tolerated by sensitive amoebae [3, 23], overexpress the iron-containing superoxide dismutase (SOD) and peroxiredoxin; and they have a decreased expression of Fd1 and flavin reductase, but they do not exhibit PFOR downregulation or upregulation of PGPs involved in the multidrug resistance phenotype (MDR). Additionally, overexpression of SOD and peroxiredoxin by transfection assays produced cells with increased metronidazole resistance [23]. Of the three major anaerobic protozoa, *G. lamblia, T. vaginalis, and E. histolytica*, *E. histolytica* shows the lowest capacity to develop metronidazole resistance at high drug concentrations.

Another target for nitroimidazole-bearing drugs is the thioredoxin reductase. It reduces metronidazole and other nitro compound suggesting a central role for this enzyme in the treatment of infections caused by *E. histolytica* [39].

2.2.2 Trifluoromethionine Resistance

Trifluoromethionine is a fluorinated derivative of L-methionine and since 1966 it is known that the drug killed *E. histolytica* trophozoites 72 h after its addition to axenic cultures [40]. This compound is degraded by a methionine γ-lyase (MGL) into α-ketobutyrate, ammonia, and trifluoromethanethiol (CF3SH). The last product is unstable and generates carbonothionic difluoride (CSF2) that is toxic to cells [40]. However, trophozoites exposed to low levels of trifluoromethionine will become resistant to the drug and develop capacity to survive in the presence of the drug. They increase adhesion, decrease cytolysis, and repress *EhMGL* gene expression that is involved in the synthesis of methionine γ-lyase (MGL). Silencing of *EhMGL* gave as a result the developing of trifluoromethionine resistance [40]. Moreover comparison of transcriptional response of the wild-type and trifluoromethionine resistance trophozoites evidenced that *EhMGL* genes were downregulated whereas a trifluoromethionine gene was upregulated in resistant trophozoites [41]. Thus, resistant and wild-type trophozoites exposed to trifluoromethionine present a different transcriptional profile.

2.2.3 Multidrug Resistance in *E. histolytica*

MDR phenotype is a challenge in medicine and a barrier to beat cancer and protozoan infections. In *E. histolytica*, 10 μM emetine usually kills trophozoites, but emetine-resistant mutants grow in 220 μM emetine and show cross-resistance to colchicine, diloxanide furoate, and iodoquinol [42]. *E. histolytica* has six *EhPgp* (*mdr*) genes [43]. As in mammalian MDR cells, mutant trophozoites accumulate less amount of drug in their cytoplasm than wild-type cells, and their drug resistance is reverted by verapamil and transfection with *EhPgp1* confers the MDR phenotype [44]. *EhPgp1* gene transcript is overexpressed in mutants, independently of the drug concentration used; in contrast, *EhPgp5* and *EhPgp6* gene expression is induced by emetine and the amount of *EhPgp5* transcript is related to drug concentration [45]. Thus, tran-

scription regulation is involved in overexpression of *E. histolytica* MDR phenotype; however, few transcription factors have been cloned in this parasite making it difficult to establish the precise molecular mechanisms that cause this phenotype. It is known that a C/EBP-like nuclear protein forms a complex with the *EhPgp1* gene promoter and deletion of the DNA motif to which the protein is bound abolishes the promoter function [45, 46]. Additionally, functional activities of the *EhPgp1* gene promoter demonstrated that besides C/EBP, other sequences are crucial for promoter activity. Deletion or mutation of R9 repeats found at −203 to −211 and −218 to −226 bp produced 70 % reduction in CAT activity; in addition, these cis-acting elements activate *EhPgp1* gene expression and are recognized by the EhEBP1 protein [47].

In contrast, it has been identified in *EhPgp5* gene promoter a heat-shock element (HSE) that induces *EhPgp5 gene* expression in trophozoites exposed to emetine (personal communication Dr. Consuelo Gomez García). In addition, a putative heat-shock transcription factor (EhHSTS) family is present in *E. histolytica* trophozoites suggesting that some of the members of this family could bind to *EhPgp5* HSE and activate its expression when amoebae are growing under emetine pressure [48].

In addition, in trophozoites growing in 225 μM emetine, *EhPgp* gene amplification occurs [43]. Interestingly, half-life of *EhPgp5* mRNA is higher in trophozoites growing in 225 μM emetine than in parasites growing in 90 μM or without drug. The *EhPgp5* mRNA 3′ UTR length is heterogeneous, with different poly (A) tail length, which may influence mRNA half-life. Trophozoites grown without emetine could have factors that inhibit *EhPgp5* gene expression and maintain short poly (A) tail length; this may contribute to a shorter *EhPgp5* mRNA half-life. Some factors could not be expressed in the presence of emetine, and other emetine-responsive proteins could stimulate *EhPgp5* gene transcription and induce an enhanced polyadenylation of *EhPgp5* mRNAs [49].

Intriguingly, EhPGP proteins are located in the plasma membrane of trophozoites, but they have been found also out of the cells, suggesting that EhPGPs could be secreted by the amoebae. Thus, EhPGPs could be able to concentrate and drive the drug to the plasma membrane to expel it out of the cells [50]. It has also been described that EhPGP5 expressed in *Xenopus laevis* oocytes could function as a chloride channel or a chloride channel regulator [51]. In *E. histolytica* EhPGP5 overexpression alters chloride-dependent currents and causes trophozoite swelling [50, 51]. Moreover, Medel et al. [52] demonstrated that inhibition of EhPGP expression produces acidification of intracellular pH (ipH) and enhancement of programmed cell death (PCD) induced by G418. In contrast, PGP overexpression prevented intracellular acidification and circumvented the PCD [52].

EhPGP secretion is probably related to one or more of the multiple functions of this protein that are not investigated yet. MDR phenotype does not seem to be involved in metronidazole resistance in *E. histolytica*, but it is a barrier for the use of alternative agents against amoebiasis.

3 *Giardia lamblia*

G. lamblia is a protozoan parasite with a significant impact on public health worldwide whereby it was included in the "Neglected Disease Initiative" of the WHO in 2004 [53]. The prevalence worldwide of giardiasis has not changed in the last 5 years, with 280 million cases annually [2]. In developed countries, *G. lamblia* prevalence ranges from less than 1 to 8 % [54, 55], whereas in developing countries it ranges from 20 to 60 % [56, 57]. Furthermore, giardial infections contribute considerably to the 2.5 million annual deaths from diarrheal disease and about 500,000 new giardiasis cases are reported each year in Asia, Africa, and Latin America [2]. Epidemiology of the giardiasis varies depending on the region. In tropical developing regions with poor-quality drinking water the infection is almost universal in childhood and recurrent after treatment. In temperate developed areas the infections frequently occur as waterborne outbreaks that usually follow a seasonal pattern [54, 55]. Accordingly, rates of symptomatic disease have ranged from 100 % in travelers returning from endemic regions to completely asymptomatic excretion of cysts in children living in them [58, 59].

Giardia trophozoites live in the small intestine, graze on the mucosa through the giardins, and reproduce by binary fission covering the epithelia and avoiding nutrient absorption by the host. Due to the microenvironmental conditions, trophozoites develop into cysts that pass through the feces to other hosts. People are the most important reservoir hosts for human giardiasis, but the parasites also infect domestic and wildlife animals [60]. To date, eight major genetic groups have been identified in animals and two of them are found in both humans and animals. In humans, however, association between specific genotypes and clinical symptoms has not been conclusive [60].

Symptomatic giardiasis is characterized by mild-to-severe gastrointestinal signs. Some patients develop acute or chronic diarrhea, abdominal pain, greasy and foul-smelling stools, weakness, nausea, weight loss, decreased appetite, flatulence, vomiting, malabsorption, and growth retardation, whereas other individuals are asymptomatic carriers. This variability is attributed to host factors and host-parasite interactions.

Despite the prevalence of giardiasis there are no vaccines for humans or prophylactic drugs. Current antibiotics for giardiasis are the 5-NI which include metronidazole (the most common and the prototype of a prodrug) and other derivatives, as well as, alternative compounds derived from nitrofurans, benzimidazoles, acridine, aminoglycosides, and thiazolides (Tables 40.1 and 40.2) [3, 20, 61, 62]. Experimental trials have proven the effectiveness of some

Table 40.2 Main antigiardial drugs currently used

Class	Compound	Efficacy		Drug targets
		In vitro	In vivo	
Nitroimidazoles	Metronidazole	10^{-6} M	500–750 mg tid for 5–10 days	Enzymes of intermediary metabolism (PFOR, thioredoxin reductase and nitroreductases)
	Tinidazole		1–2 g mid, 1 day	
	Secnidazole		1–2 g mid, 1 day	
	Ornidazole		1–2 g mid, 1 day	
	Nimorazole		1–2 g mid, 1 day	
	Ronidazole		Not approved for human use	
Nitrofurans	Furazolidone	10^{-5}–10^{-6} M	400 mg/day for 7–10 days	Enzymes of intermediary metabolism (NADH oxidase)
Benzimidazoles	Albendazole	10^{-8} M	200–400 mg/day for a week	Cytoskeleton proteins (β-tubulin)
	Mebendazole	10^{-8} M	200 mg tid for 1–3 days	
Acridine	Quinacrine	10^{-7} M	300 mg/day for a week	Nucleic acids (AT-rich regions from DNA)
Aminoglycosides	Paromomycin	10^{-4}–10^{-5} M	500 mg tid for a week	Nucleic acids (16s rRNA)
Thiazolides	Nitazoxanide	10^{-6} M	500 mg mid for 3 days	Enzymes of intermediary metabolism (PFOR, thioredoxin reductase and nitroreductases)

mid once a day, *bid* twice a day, *tid* three times a day. Adapted from [20, 61, 62]

old known and new compounds [61, 63–68]. However, to date, there is no known highly effective alternative to metronidazole [66]. Randomized controlled trials performed until now have revealed a slight better efficacy of 5-NI in comparison to other antigiardial drugs and reasonable safety profiles [62]. Nevertheless, due to the increased rate of resistance or adverse effects of the current drugs, there is consensus about the need for discovering new drug targets and developing alternative antigiardial agents.

3.1 Antimicrobial Mechanism of Action in *G. lamblia*

According to a widely accepted hypothesis, in *G. lamblia*, like in *E. histolytica* and *T. vaginalis*, the PFOR converts pyruvate to acetyl-CoA with the transfer of a pair of electrons to Fd, which reduces metronidazole and other related 5-NI to a nitro radical and activates them into potent antigiardial agents (Figs. 40.2 and 40.3). Recent findings have shed light on other mechanisms involved in the reduction of metronidazole in which participate proteins of the thioredoxin reductase pathway of *G. lamblia* and nitroreductases [69, 70].

A line of evidence for PFOR as a main target for 5-NI in *Giardia* was obtained from metronidazole-resistant isolates, some of which exhibit PFOR lower expression levels. These findings were reproduced in vitro generating metronidazole-resistant phenotypes by blocking the PFOR gene expression in *Giardia* [71]. *G. lamblia* PFOR is a homodimer of 135 kDa subunits, whose activity is 75–80 % membrane associated. It resists low temperatures (−70 °C) and transfers electrons to purified FdI but not to either NAD^+ or $NADP^+$. *Giardia* has three Fds with iron-sulfur clusters: FdI, FdII, and FdIII [72]. FdI, the major one, differs from other protozoan Fds in size, amino acid sequence, and iron-sulfur cluster. Its molecular mass was calculated in 5.7 and 5.9 kDa by mass spectrometry and amino acid sequencing, respectively. Consistent with the amino acid profile of other Fds, methionine, arginine, histidine, and tyrosine residues are absent from *Giardia* FdI, but it has 16.4 % of acidic residues whereas other Fds have about 30 %. The N-terminus contains a potential iron-sulfur-binding motif $\left(-C1-X-X-A-X-X-C3\cdots C4-\right)$ with a nonconservative substitution of alanine for the second cysteine. *Giardia* FdI contains a $[3Fe–4S]^{(1+,\,0)}$ cluster, while Fd from *E. histolytica* two $[4Fe–4S]^{(2+,\,1+)}$ and *T. vaginalis* Fd has a single $[2Fe–2S]^{(2+,\,1+)}$ cluster [72]. Only FdI interacts with PFOR and is involved in metronidazole activation in vitro [72]. The 5-NIs reduced are bound to DNA, altering the helical structure, breaking the strands, provoking DNA cross-linking, and interfering with mitosis. The binding makes DNA unable to segregate or it modifies genes involved in mitosis, arresting cell cycle in G2 + M phase and inducing programmed cell death [73]. Further, ultrastructural studies have brought evidences of damage to the dorsal surface, the ventral disk, and the lateral flange of the parasite upon treatment with metronidazole [68].

However, the 5-NI resistance in *Giardia* clones and strains may occur without downregulation of PFOR [70, 74].

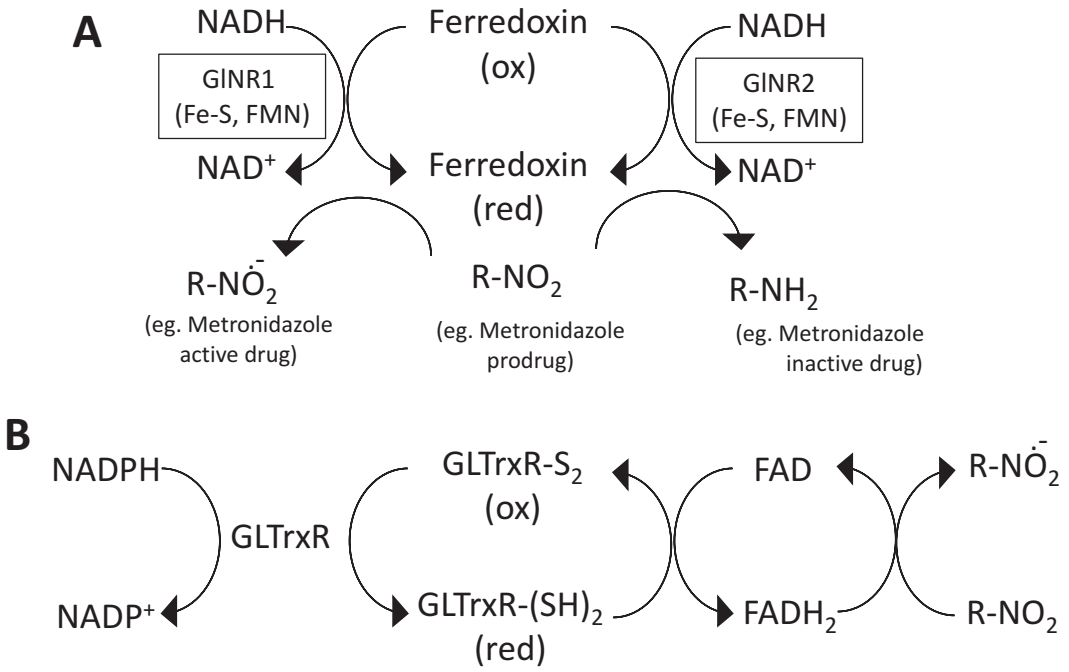


Fig. 40.3 Other mechanisms of reduction of 5-NI compounds in *G. lamblia*. (**a**) The reduction of the nitro group ($R–NO_2$) of 5-NI drugs by nitroreductases can activate or inactivate the drug. G1NR1 and G1NR2: *G. lamblia* nitroreductase 1 and 2, respectively, with Fe–S clusters and a nitro-flavin mononucleotide (FMN) domain. (**b**) Thioredoxin reductase of *G. lamblia* (GLTrxR) also reduces the nitro group of 5-NI compounds mediated by flavin dinucleotides (FAD). *ox* oxidated, *red* reduced (adapted from [69])

Reduction of the nitro group by the Fds is also catalyzed by nitroreductases, which activates nitro compounds inducing DNA damage (Fig. 40.3). Two genes encoding nitroreductases (*g1nR1* and *g1nR2*) have been identified and characterized in *G. lamblia* [70, 75]. The polypeptide sequences of GlNR1 and GlNR2 are very similar [70]. Both proteins possess a Fd domain with four Fe–S clusters at their N-terminus and a nitro-flavin mononucleotide (FMN) reductase domain at their C-terminus. However, GlNR1 and GlNR2 have different modes of action. Fd domain of both nitroreductases transfers electrons from a donor (NADH) to the FMN in the active center, but GlNR1 reduces partially the nitro compounds yielding toxic intermediates, while GlNR2 reduces them entirely yielding a nontoxic end product, e.g., the corresponding amine [70]. mRNA levels of *G. lamblia* GlNR1 are much lower than the GlNR2 in 5-NI-resistant strains. Trophozoites overexpressing GlNR1 have a higher susceptibility to nitazoxanide and metronidazole, which also support that GlNR1 activates 5-NI via reduction yielding a cytotoxic product [70]. GlNR1, but not GlNR2, provokes nitric oxide and DNA repair responses, even in the absence of 5-NI [76]. Thus, susceptibility to 5-NI may depend not only on activation, but also on inactivation of the drugs by specific nitroreductases [76] (Fig. 40.3).

Flavin-dependent thioredoxin reductase of *G. lamblia* (GLTrxR), like TrxR of other anaerobic protozoa, also reduces 5-NI compounds to nitro radical anions and generates superoxide under aerobic conditions [69]. Evidences that come from proteomic analyses with 5-NI-treated trophozoites of *G. lamblia* show that metronidazole and tinidazole are bound to GLTrxR and other proteins. GLTrxR activity is strongly diminished after 5-NI treatment, which results in impaired removal of hydrogen peroxide by peroxidases [69]. Furthermore, 5-NIs deplete intracellular thiol pools in *G. lamblia*, but metronidazole, in contrast to what happens in *E. histolytica* and *T. vaginalis*, has the slightest effect [69, 76–78]. Also, in contrast to the other anaerobic protozoan, none of the other proteins forming covalent adducts with 5-NI are candidates for being involved in the TrxR-mediated redox network of *G. lamblia*, instead of some of them are closely associated with PFOR [75]. Others, like the translation elongation factor EF-1c, an important factor in protein synthesis, are widely degraded upon treatment with 5-NI. These findings suggest that the TrxR pathway is involved in the metabolism of 5-NI in *G. lamblia* in a different way than in *E. histolytica* and *T. vaginalis* [69, 76].

The 5-NI drugs are activated in the *Giardia* cytosol. Until now there are no evidences of the involvement of the *G. lamblia* mitosome in the mechanisms of 5-NI activation. The mitosome proteome is reduced and limited to a single metabolic pathway for FeS cluster assembly [79].

Furazolidone is activated inside the cell by NADH oxidase to generate toxic products that interfere with DNA synthesis. NADH oxidase, a 46 kDa monomeric flavoprotein, contains FAD in a 1:1 molecular ratio with the polypeptide and it is responsible for the high level of NADPH-NADH turnover in *Giardia* [77]. NADPH and NADH donate electrons to NADH oxidase, which also accepts electrons from oxygen to produce H_2O as an end product, but not from reduced Fd [77]. Furazolidone arrests the trophozoites in S phase and eventually in G2 + M phase preventing DNA synthesis and cell cycle completion, possibly due to DNA damage [73].

Quinacrine intercalates in DNA, inhibiting nucleic acid synthesis. However, there are studies showing no quinacrine accumulation in trophozoite nuclei exposed to the drug; instead of this, the plasma membrane appeared fragile after overnight drug exposure, suggesting that it may be another target for the drug [78]. In addition, quinacrine inhibits NADH oxidase and cholinesterase activities and produces a decreased excystation in in vitro and patient-derived cysts.

Benzimidazoles bind to β-tubulin in the same site that colchicine does, altering the cytoskeleton. After mebendazole or albendazole exposure, trophozoites detach from the substrate, exhibiting striking modifications of their overall morphology, including ventral disk disassembly [80].

Paromomycin, as in higher eukaryotes, interferes with *Giardia* 16S-like small-subunit (SS) RNA, causing mRNA codon misreading and protein synthesis inhibition.

In vivo, nitazoxanide is deacetylated to tizoxanide, which has equal effectiveness, and exhibits a mode of action similar to that of metronidazole via PFOR [81]. Besides that, nitazoxanide binds to the nitroreductase GlNR1 inhibiting its activity. In the presence of nitazoxanide and its derivatives, specific activity of GlNR1 decreases in a concentration-dependent manner, but its transcriptional expression is not affected [81]. Other findings have correlated the resistance to nitazoxanide and metronidazole with altered expression of genes coding heat-shock proteins [74].

3.2 Drug Resistance Mechanisms in *G. lamblia*

Single and multidrug resistance to some of the current antigiardial used drugs, including metronidazole, has been reported in human patients and can be induced in vitro by stepwise exposure to increasing drug concentrations [74, 77, 82, 83]. Multiple mechanisms have been implicated in 5-NI drug resistance, including a diminished ability to reduce and activate 5-NI prodrugs [69, 71] and to detoxify nitro radicals [39]. Although the treatment failure in patients is occurring more frequently with all the compounds used, it is not always due to the development of resistance by the parasite. Other causes are invoked like (1) low compliance with drug therapy, (2) immunosuppression, (3) reinfestation, and (4) post-*Giardia* lactose intolerance, which is the most common of the disaccharide deficiencies associated with giardiasis [84]. Clinical metronidazole resistance prevalence levels are as high as 20 % with recurrence rates up to 90 % and the average success rates of albendazole are 62–95 % [3]. In addition, metronidazole is inactive against *Giardia* cysts [76].

3.2.1 Metronidazole Resistance in *G. lamblia*

Despite of the documented clinical resistance to metronidazole [3], the resistant *Giardia* clones (Mzr) have rarely been isolated from patients [85]. This can be explained, at least partly, by the loss of parasite attachment and infectivity observed in Mzr *Giardia* cell lines in vivo and in vitro. This phenomenon has been related to impaired glucose metabolism, since the noninfectious Mzr lines consume less glucose, and glucose promotes ATP-independent parasite attachment in the parental lines [85]. In addition, glucose-metabolizing pathways are important for activation of metronidazole and then a fitness trade-off may be able to exist between diminished metronidazole activation and reduced infectivity as it was suggested [85]. However, Mzr does not always interfere with in vivo infectivity of *Giardia*, as well as some, but not all, Mzr *Giardia* cell lines have decreased PFOR levels and activity of Fd [71, 72]. Downregulation of PFOR most probably affects the glycolytic metabolism in Mzr *Giardia*, but it has not been determined conclusively [85]. Then, the mechanisms of metronidazole resistance appear to be as diverse as the mechanisms of nitro drug activation are.

Giardia cell lines resistant to a derivative of 5-NI, that is at least 14-fold more active than the metronidazole, are also highly resistant to metronidazole (ID90 values, concentration of drug at which 10 % of control parasite ATP levels are detected, for metronidazole >200 μM, 20-fold more than susceptible isolates) [86]. However, such highly resistant lines have normal levels of PFOR, but a decreasing activity of NADPH oxidase and a suppressed reduction of flavins, suggesting that flavin metabolism is also linked to 5-NIs resistance in *G. lamblia* [69]. It has also been observed in *T. vaginalis* that MTZ inactivation of proteins related to the TrxR pathway is overcome in resistant cells by reregulating PFOR [70]. Then, downregulation of PFOR in *Giardia* could be also a consequence, rather than a prerequisite, of resistance formation. In addition, in *G. lamblia* the nitroreductases not only activate but also inactivate metronidazole [39, 76].

Metronidazole resistance is also associated with chromosomal rearrangements and gene duplications [87]. However, *G. lamblia* genome shows a high diversity that can rise up to 30–50 % in genes and in intergenic regulatory regions [88], which has hindered the association of specific genotypes with drug sensitivity [3]. Additionally, decreased level of metronidazole in *Giardia* cytoplasm is consistent with changes in uptake, transport, and efflux of fluorescent analogues as observed in MDR [87].

3.2.2 Resistance Mechanism to Other Compounds in *G. lamblia*

Interestingly, furazolidone resistance correlates with reduced drug entry and increased levels of thiol-cycling enzymes which defend *Giardia* against toxic radicals, suggesting that efficient thiol cycling may be involved in furazolidone reduction [89]. Additionally, quinacrine is actively excluded from resistant trophozoites [78] and albendazole resistance is associated with cytoskeleton changes, particularly in the ventral disk. However, resistant trophozoites do not have the mutation in phenylalanine 200 in β-tubulin, found in albendazole-resistant helminthes and fungi [83].

3.3 Cross-Resistance in *G. lamblia*

Cross-resistance is a concern with all commonly used antigiardial drugs and it has been documented between all currently used 5-NI drugs and metronidazole [2]. Albendazole resistance also developed more readily in furazolidone-resistant or metronidazole-resistant and therefore these strains are multidrug resistant [3]. Brasseur et al. [22] reported two patients that did not respond to successive treatments with metronidazole, albendazole, and quinacrine, showing the existence of clinical cross-resistance also among different classes of antigiardial compounds. The BRIS/83/HEPU/106-2ID$_{10}$ line is resistant to albendazole and tinidazole [83]; and furazolidone-resistant *Giardia* strains adapt more readily to quinacrine [78] and albendazole [83]. There are various mechanisms involved in cross-resistance in giardiasis, since drugs target different parasite pathways and molecules. *G. lamblia* genome sequence completion has helped to identify multidrug resistance (*mdr*) genes like those coding for the mdr-associated protein 1 (Gl50803_28379) and mdr-like protein (Gl40224) [90, 91]. Recently, six ATP-binding cassette (ABC) transporter genes have been identified in *G. lamblia* and their overexpression was demonstrated in trophozoites treated with albendazole, nitazoxanide, and a derivative of albendazole. At least one of them (ABC1) has an architecture of P-glycoprotein (PGP) [92]. Further studies are needed to characterize such *mdr* genes or identify new ones.

4 *Trichomonas vaginalis*

T. vaginalis infects 180 million people each year worldwide and there are about 50% of asymptomatic carriers [4]. Trophozoites are the unique stage in its life cycle and no cysts have been identified, so transmission only occurs via trophozoites by sexual contact. However, cyst-like cells have been found in *T. vaginalis* under environmental stress [93]. This parasite contains more than 400 distinct proteinase genes in its genome and 220 correspond to the cysteine type (CP) which are involved in cytoadherence [94]. Eradication of trichomonosis is considered as an effective means for controlling HIV transmission, because 24% of HIV infections are attributable to *T. vaginalis* infection [95]. Additionally, the development of this parasitosis has been positively associated with subsequent incidence of prostate cancer [96]. Trichomonosis is controlled by metronidazole (Table 40.1), although other 5-NIs are also dispensed and used as prophylactic agents in gynecological surgery and topical intravaginal treatments, using a single 1.5–2 g metronidazole in oral dose to 500 mg twice daily over 7 days [97].

4.1 Antimicrobial Mechanism of Action in *T. vaginalis*

In *T. vaginalis*, glycolysis occurs in the cytosol, producing pyruvate that is then decarboxylated by PFOR in hydrogenosome to form acetyl-CoA that is transferred to succinate, resulting in acetate and ATP production. Simultaneously, PFOR reduces Fd and electrons are finally given by Fe-hydrogenase to hydrogen ions forming hydrogen as an end product (Fig. 40.2). Hydrogenosomes contain one PFOR embedded in the hydrogenosomal membrane, one [2Fe–2S]–Fd that is similar to mitochondrial Fds [98] and three [Fe]-hydrogenases [99]. Alternatively, the hydrogenosomal NAD(P)-dependent malic enzyme (ME) can form pyruvate from malate, with the reduction of Fd by NAD-Fd oxidoreductase (NADH:FOR) [15]. In the presence of metronidazole, Fd can transfer electrons to metronidazole to convert it to the toxic form of the drug, which binds transiently to DNA, disrupting or breaking the strands and leading to cell death. In addition, there is a reduced hydrogen production and an increased intracellular hydrogen peroxide [100]. The flavin enzyme thioredoxin reductase can also contribute to metronidazole activation in *Trichomonas* [101].

4.2 Drug Resistance Mechanisms in *T. vaginalis*

4.2.1 Metronidazole Resistance in *T. vaginalis*

Although metronidazole resistance is rare in trichomonosis, resistance rates can exceed 15% in some populations [102]. However, the mechanism of metronidazole resistance in *T. vaginalis* is almost unknown. Two types of metronidazole resistance have been initially described. The aerobic resistance that occurs in parasites isolated from patients with treatment failures is characterized by a reduction of oxygen-scavenging processes which impairs metronidazole activation; in addition, activated metronidazole can be oxidized back to the inactive drug through the so-called futile cycle [103]. Resistant clinical

isolates have a reduced or absence of flavin reductase (FR) activity, which is related to the elevated intracellular oxygen levels [76], but they do not exhibit reduced expression of the PFOR or Fd genes [104]. Additionally, targeted Fd gene replacement does not lead to metronidazole resistance [105]. On the other hand, anaerobic metronidazole resistance that has been demonstrated in cultured strains [106] relies on elimination or inactivation of PFOR and Fd responsible for reductive activation of metronidazole, as well as shrinking of the hydrogenosome [107]. Parasites grown in anaerobic conditions at relatively low drug concentrations (3 μg/mL) develop first aerobic resistance up to 200 μg/mL of drug. Longer exposure to increasing drug concentrations allows the generation of anaerobic metronidazole-resistant mutants being able to grow at 1120–1425 μg/mL of drug [108]. However, these strains do not multiply under anaerobiosis. In these experiments, some anaerobic drug-resistant strains presented high decrease of PFOR, Fd, and hydrogenase activities, while ME and NADH:FOR progressively decreased when the anaerobic level of resistance increased. Lactate and other main end products of carbohydrate metabolism increased in drug-resistant cells, whereas hydrogenosomal metabolites such as acetate and hydrogen dramatically lowered. All these findings revealed that aerobic and anaerobic resistance events, considered to be unrelated, are developed in a common continuous process in *T. vaginalis.* They also confirmed that total anaerobic resistance results from the lack of both PFOR and ME that are involved in metronidazole activation. It seems that generation of metronidazole resistance is given through a multistep process, suggesting that several mutations in various hydrogenosomal proteins involved in drug activation might occur [97, 108]. Recent works also revealed the loss of other enzymes involved in metronidazole reduction, namely thioredoxin reductase and free flavins [101, 109], as well as the flavin reductase 1 (FR1) [76], which has a role in intracellular oxygen removal [15] and toxic hydrogen peroxide formation (Table 40.1) [110]. Moreover, single-nucleotide polymorphisms in two nitroreductase genes ($ntr4_{Tv}$ and $ntr6_{Tv}$) were recently associated with metronidazole resistance in *T. vaginalis* [111].

4.2.2 Multiple Drug Resistance in *T. vaginalis*

The *Tvpgp1* gene encodes a 589-amino acid protein with an amino terminal hydrophobic region, a carboxy-terminal ATP-binding site and six transmembrane segments, which corresponds to half size of mammalian and *E. histolytica* PGPs. *T. vaginalis* genome has two *Tvpgp1* copies, but only one was detected in four of seven drug-resistant strains studied. Moreover, several clinical metronidazole-resistant isolates overexpress *Tvpgp1* mRNA to levels ranging from 2- to 20-fold more than the wild type. However, no correlation was found between the *Tvpgp1* mRNA amount and *Tvpgp1* gene copy number with drug resistance levels [112]. Moreover the gene is not amplified in any of the drug-resistant strains [112]. This does not necessarily mean that *Tvpgp1* gene is not involved in drug resistance. In *E. histolytica* drug-resistant mutants, the *EhPgp1* mRNA overexpression confers drug resistance but the transcript amount and the gene copy number do not correlate with drug resistance levels [43]. Additionally, resistance to metronidazole is also reverted by verapamil [97]. Thus, more studies are necessary to define the role of *Tvpgp1* gene in *T. vaginalis* drug resistance.

4.3 Cross-Resistance in *T. vaginalis*

Currently, metronidazole and other 5-NI like tinidazole, ornidazole, and secnidazole are the only recommended drugs for standard treatment of *T. vaginalis* infection [113]. Although most patients are cured with standard treatment with single or week-long courses of metronidazole, organisms resistant to these therapies have been reported [114]. Even more, metronidazole-resistant *T. vaginalis* isolates have been increasingly reported as 2.4–9.5 % of cases around the world [115]. Cross-resistance to different 5-NI has also been reported, but it is unknown why some metronidazole-resistant isolates display cross-resistance to tinidazole whereas others not [76]. Evaluation of 104 clinically metronidazole-resistant isolates showed that almost all samples were cross-resistant to tinidazole in aerobic conditions. Interestingly, isolates with higher metronidazole resistance have decreased sensitivity to tinidazole, suggesting that increased metronidazole resistance may correlate with increased tinidazole resistance. However, metronidazole-refractory cases have finally been cured by very high doses of tinidazole [116]. Several cases of metronidazole resistance were reported to be also resistant to ornidazole [3]. Recently, the activities of two enzymes, the thioredoxin reductase and flavin reductase, were evaluated in four metronidazole susceptible and five metronidazole- and tinidazole-resistant isolates. Interestingly, thioredoxin reductase activity was similar in all nine isolates, while the flavin reductase activity decreased in all isolates with lowered sensitivity to metronidazole and it was absent in strains which display the highest level of metronidazole resistance. Moreover, the downregulation of the alcohol dehydrogenase 1 (ADH1) was detected only in metronidazole-resistant isolates. Unfortunately, differentiation between metronidazole-resistant isolates that are cross-resistant to tinidazole, and such which are not, was not possible [76].

This may be because the common 5-NI drugs have different simple side chains at the one-position of the imidazole; while metronidazole possesses a hydroxyethyl group, the tinidazole has an ethylsulfonylethyl group. These modifications mostly affect the pharmacokinetic properties of the drugs but have only limited influence on drug potency or abil-

ity to overcome resistance [85]. As in *E. histolytica* and *G. lamblia*, new alternative treatment regiments also need to be developed to have more treatments against trichomonosis.

5 Other Anaerobic Opportunistic Protozoan Parasites

B. hominis, *C. parvum*, *Isospora* spp., and *Cyclospora* spp. invade preferentially the gastrointestinal mucosa. *Microsporidia*, amitochondriate intracellular parasites closely related to fungi, produce intestinal, pulmonary, ocular, muscular, and renal diseases. Five *Microsporidia* genera (*Enterocytozoon* spp., *Encephalitozoon* spp., *Septata* spp., *Pleistophora* spp., and *Nosema* spp.) and one unclassified genus (referred to as *Microsporidium*) are associated with human diseases. These microorganisms are considered as emerging opportunistic parasites, causing diarrhea, lethal wasting, and other symptoms in immunocompetent and immunocompromised hosts, mainly in HIV patients and malnourished infants [3].

5.1 Antimicrobial Treatments Against Opportunistic Protozoan Parasites

Metronidazole is the drug of choice against *B. hominis* [117], but trimethoprim-sulfamethoxazole (TMP-SMX), iodoquinol, emetine, pentamidine, quinacrine, furazolidone, and 5-NI derivatives are also used [118]. Metronidazole appears to be the most effective drug, and TMP-SMX and nitazoxanide may be considered as second-choice drugs in metronidazole treatment failure [119], although TMP-SMX is considered to be superior to metronidazole in the treatment without the side effects [119].

The current treatment for *C. parvum* infection options is limited to one approved drug, nitazoxanide, which hastens the resolution of symptoms in immunocompetent patients [120], but it is less efficacious in malnourished children and shows no benefit in immunocompromised patients [121]. Paromomycin has also been used, but a study on hospitalized children showed that nitazoxanide is more effective than paromomycin in cryptosporidiosis [122]. Importantly, the target of nitazoxanide is undefined in *Cryptosporidium*, so no clinically validated targets exist for the treatment of cryptosporidiosis.

The drug of choice for *Isospora belli* treatment is trimethoprim-sulfamethoxazole. In patients allergic to sulfonamides, pyrimethamine is given as treatment [123]. Albendazole and its sulfoxide and sulfone metabolites are the drugs of choice against most *Microsporidia* [123], but they are ineffective against *Enterocytozoon bieneusi*, which is controlled by fumagillin, but it has toxic side effects [124], so more effective and safer drugs are needed. Albendazole, fumagillin, 5-fluorouracil, sparfloxacin, oxibendazole, and propamidineisethionate inhibit *Encephalitozoon cuniculi* growth in vitro and synthetic polyamine analogues that bind to nucleic acids are effective antimicrosporidial agents in vitro and in vivo [125].

5.2 Drug Resistance in *B. hominis*, *C. parvum*, and *Microsporidia*

There are case reports of metronidazole treatment failures in *Blastocystis* infections, and *B. hominis* isolates from different geographical origin have distinct levels of metronidazole resistance [126]. A study on subtype-dependent variation in drug susceptibilities of *Blastocystis* revealed that subtype 7 is resistant to metronidazole but sensitive to emetine, whereas subtype 4 is sensitive to metronidazole but resistant to emetine, indicating that unknown mechanisms of activation and resistance may be involved [127]. In addition, an association between *Blastocystis* infection and irritable bowel syndrome (IBS) has been suggested. Interestingly, in most of the IBS patients *Blastocystis* is resistant to metronidazole treatment [128]. Remarkably, in this parasite, it has been observed that emetine resistance could occur along with metronidazole resistance, suggesting that multidrug-resistant phenotypes might be present in the parasite [119]. Accordingly, some genes coding for multidrug resistance pump proteins (ABC transporters) were identified in the *Blastocystis* sp. ST7 genome [129]. Similarly, *C. parvum* contains a family of ABC transporters that resemble the PGPs described in other organisms and the membrane protein CpABC is located in the host-parasite boundary [130], suggesting a possible role in drug resistance. However, its ability to efflux drugs has not been fully investigated. In addition, *C. parvum* dihydrofolate reductase (DHFR) contains amino acid residue changes at positions analogous to those at which point mutations produce antifolate resistance in other parasites, suggesting that *C. parvum* DHFR may be intrinsically resistant to antifolate DHFR inhibitors [131]. This can explain why it is refractory to treatment with common antibacterial and antiprotozoal antifolates.

Chronic relapsing *I. belli* infections have been reported in AIDS patients despite treatment with trimethoprim-sulfamethoxazole and immune reconstitution. In these patients, a double daily dose of trimethoprim-sulfamethoxazole should be given up to 2 years [132]. In addition, nitazoxanide resistance has been reported in hosts infected by *Isospora* [133], but it is not clear how resistance occurs.

Encephalitozoon infections resistant to conventional treatments with albendazole and fluconazole have been cured by the antifungal itraconazole and fumagillin [134]. Interestingly, it has been shown that the *Encephalitozoon intestinalis* genome contains two sequences (*EiABC1* and *EiABC2*) encoding different ATP-binding cassette genes, including a *Pgp* that could be implicated in multidrug resistance [135], but its participation in drug resistance has

not been studied. On the other hand, *Nosema bombycis* is resistant to itraconazole and metronidazole in vitro, while it is sensitive to fumagillin [136].

5.3 Alternative Drugs Against *E. histolytica*, *G. lamblia*, *T. vaginalis*, and Opportunistic Protozoa

The lack of a useful alternative class of molecules against amoebiasis, trichomoniasis, and giardiasis as well as to opportunistic protozoa provides impetus to the efforts to identify and exploit alternative antiprotozoan parasite therapies. Thus, research on novel drugs and vaccines against protozoa might be strongly supported if we want to eradicate these infection diseases. Recently, auranofin, a drug used therapeutically for rheumatoid arthritis, was given orally to hamsters infected with *E. histolytica* trophozoites, and resulted a potential drug anti-*E. histolytica* because it is ten times more potent than metronidazole and less toxic [137]. However, it has not been widely tested in humans to be used as an alternative yet. Transcriptional profiling and biochemical assays suggested that auranofin targets *E. histolytica* thioredoxin reductase, preventing the reduction of thioredoxin and enhancing sensitivity of trophozoites to reactive oxygen-mediated killing [137].

Additionally preliminary studies suggest that cationic antimicrobial peptides (AMPs) represent a promising route towards developing new, efficient antiparasitic therapies [138, 139]. Preet et al. [140] demonstrated that cryptdin-2 exerts amoebicidal activity by inducing striking morphological changes in *E. histolytica* which is consistent with its membrane-dependent mechanism of action [140]. In addition to membrane permeabilization, its amoebicidal mechanism involves inhibition of DNA, RNA, and protein synthesis. In the same way, various cryptdin isoforms have been reported to exhibit parasiticidal activity against *G. lamblia* and it has been suggested that cryptdin-2 possesses the most potent giardicidal activity due to the relative efficacy of binding to the trophozoite surface [141]. Moreover, several experimental trials have proven the effectiveness of disulfiram, orlistat, miltefosine, antihelminths, and antiprotozoan drug combinations, compounds of natural origin and metronidazole analogues against *G. lamblia* [61, 63–68].

Now, the reliance on a single drug class for treating *T. vaginalis* infections may be problematic if resistance to nitroimidazole becomes widespread in *T. vaginalis* strains. Additionally, effective alternatives to the 5-nitroimidazole drugs are needed for patients with drug allergy. Compounds obtained from betulinic acid as the piperazine exhibited a significant anti-*T. vaginalis* activity against ATCC 30236 and fresh clinical isolates. Regarding mechanism of action, this triterpene was probably able to cause rupture of cellular membranes [142].

In the same way, several studies that show anti-*T. vaginalis* activity of natural products have been described [143–147], most of them highlighting triterpenes', saponins', and alkaloids' potentials. However, the in vitro antitrichomonal activity of the natural polyphenol resveratrol (RESV) demonstrates that the antiparasitic mechanism of this polyphenol occurs through induction of hydrogenosomal metabolism alteration. This effect on trichomonal energy metabolism leads to a profound dysfunction of the hydrogenosome, which has deleterious effects on the parasite [16]. Other antiprotozoan drugs that kill trichomonads in vitro and might have efficacy against *T. vaginalis* infections include nitazoxanide (approved for use against giardiasis and cryptosporidiosis) and miltefosine (approved for treatment of human visceral leishmaniasis). However, nitazoxanide is poorly absorbed from the intestinal tract and therefore may only be an option for intravaginal treatment of trichomoniasis [148].

On the other hand, there have been several studies examining the use of alternative agents for the treatment of *Blastocystis* infection, but their inhibitory activity was not as great as with similar concentrations of metronidazole [119]. However, *Blastocystis* isolates from IBS patients have demonstrated increased susceptibility to garlic at 0.01 mg/mL [149].

Probiotics are live organisms which when administered in adequate amounts confer a health benefit to the host. In this sense, some probiotics have been analyzed in the treatment of infections by opportunistic protozoa. One study showed that the *Saccharomyces boulardii* treatment on *Blastocystis*-infected children was as effective as metronidazole therapy [150]. In addition, *Lactobacillus acidophilus* CH1 bacteriocin showed potent effect against intestinal microsporidiosis in immunosuppressed mice [151].

Microtubule formation in *Cryptosporidium* is another potential drug target. Dinitroanilines, including trifluralin, are herbicides that block microtubule formation and inhibit cryptosporidial growth in vitro and in vivo [152]. In addition, a cysteine protease inhibitor (K11777) inhibits *C. parvum* growth in vitro and showed a potent anticryptosporidial activity in an animal model [153].

The requirement for a robust and sustained development of antiparasitic drugs is imperative if we are to control parasite infections in the developed world and turn the tide on the multitude of infectious agents that continue to thwart society in the developing world.

6 Concluding Remarks

Metronidazole, the drug of choice for amoebiasis, giardiasis, trichomonosis, and other opportunistic diseases produced by anaerobic protozoan parasites, can soon become outdated because of its excessive use in chemotherapy and prophylaxis. The emergence of metronidazole resistance is a serious

challenge to eradicate these infections and the fact that protozoa are able to develop resistance to most antiparasitic drugs indicates that further investigation is necessary on the drug resistance mechanisms and the way to overcome this problem with novel antiparasite products. Our knowledge on the cellular pathways used by protozoa to bypass drug effects and survive inside the host is still limited. Although several genes, proteins, and cellular pathways involved in drug action and drug resistance have been discovered (Tables 40.1 and 40.2), it is urgent to continue with the study of mechanisms responsible for the emergence of resistant parasites to overcome this problem and design new chemotherapeutic strategies. We also need to know the prevalence of resistance in specific geographical areas to look for better alternatives and avoid the use of toxic and obsolete drugs for patients infected with resistant protozoa. The current advances and novel genomics, proteomics, and metabolomics high-throughput tools to detect genes and proteins involved in parasite virulence and drug resistance mechanisms may help finding new and efficient therapeutic alternatives, such as vaccines and better drugs. The understanding of resistance mechanisms and mechanism of action of drugs may also point the way to more rational use of drugs and drug combinations to minimize development of resistance and to achieve more effective chemotherapy.

Acknowledgments The authors thank Dr. Arturo González Robles, Departamento de Infectómica y Patogénesis Molecular, CINVESTAV IPN, Mexico, for the parasite micrographs. The work on *E. histolytica* was supported by CONACYT, Mexico.

References

1. Creek DJ, Barret MP. Determination of antiprotozoal drug mechanisms by metabolomics approaches. Parasitology. 2014;141:83–92.
2. Debnath A, Ndao M, Reed SL. Reprofiled drug targets ancient protozoans. Gut Microbes. 2013;4:66–71.
3. Upcroft P, Upcroft JA. Drug targets and mechanisms of resistance in the anaerobic protozoa. Clin Microbiol Rev. 2001;14:150–64.
4. W.H.O. An overview of selected curable sexually transmitted diseases. Global program on AIDS. 1995.
5. Lloyd GL, Case JR, De Frias D, Brannigan RE. *Trichomonas vaginalis* orchitis with associated severe oligoasthenoteratospermia and hypogonadism. J Urol. 2003;170:924.
6. Fowler KB, Pass RF. Sexually transmitted diseases in mothers of neonates with congenital cytomegalovirus infection. J Infect Dis. 1991;164:259–64.
7. Laga M, Manoka A, Kivuvu M, et al. Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. AIDS. 1993;7:95–102.
8. Garcia-Rivera G, Rodriguez MA, Ocadiz R. *Entamoeba histolytica*: a novel cysteine protease and an adhesin form the 112 kDa surface protein. Mol Microbiol. 1999;33:556–68.
9. Garcia AF, Chang TH, Benchimol M, et al. Iron and contact with host cells induce expression of adhesins on surface of *Trichomonas vaginalis*. Mol Microbiol. 2003;47:1207–24.
10. Sousa MC, Goncalves CA, Bairos VA, Poiares-Da-Silva J. Adherence of *Giardia lamblia* trophozoites to Int-407 human intestinal cells. Clin Diagn Lab Immunol. 2001;8:258–65.
11. Luna-Arias JP, Sanchez T, Herrera-Aguirre ME, et al. Purification of *Entamoeba histolytica* DNA containing organelles (EkhOs): a further characterization. J Eukaryot Microbiol. 2003;50:706–8.
12. Tovar J, Fischer A, Clark CG. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. Mol Microbiol. 1999;32:1013–21.
13. Mai Z, Ghosh S, Frisardi M, et al. Hsp60 is targeted to a cryptic mitochondrion-derived organelle ("crypton") in the microaerophilic protozoan parasite *Entamoeba histolytica*. Mol Cell Biol. 1999;19:2198–205.
14. Tovar J, Leon-Avila G, Sanchez LB, et al. Mitochondrial remnant organelles of *Giardia* function in iron-sulphur protein maturation. Nature. 2003;426:172–6.
15. Kulda J. Trichomonads, hydrogenosomes and drug resistance. Int J Parasitol. 1999;29:199–212.
16. Mallo N, Lamas J, Leiro JM. Hydrogenosome metabolism is the key target for antiparasitic activity of resveratrol against *Trichomonas vaginalis*. Antimicrob Agents Chemother. 2013; 57(6):2476–84.
17. Nixon JE, Field J, McArthur AG, et al. Iron-dependent hydrogenases of *Entamoeba histolytica* and *Giardia lamblia*: activity of the recombinant entamoebic enzyme and evidence for lateral gene transfer. Biol Bull. 2003;204:1–9.
18. Muller M. Mode of action of metronidazole on anaerobic bacteria and protozoa. Surgery. 1983;93:165–71.
19. Land KM, Clemens DL, Johnson PJ. Loss of multiple hydrogenosomal proteins associated with organelle metabolism and high-level drug resistance in trichomonads. Exp Parasitol. 2001;97:102–10.
20. Müller J, Andrew H, Müller N. Treatment of giardiasis and drug resistance. In Vitro. 2011;10:10–21.
21. Samarawickrema NA, Brown DM, Upcroft JA, et al. Involvement of superoxide dismutase and pyruvate:ferredoxin oxidoreductase in mechanisms of metronidazole resistance in *Entamoeba histolytica*. J Antimicrob Chemother. 1997;40:833–40.
22. Brasseur P, Favennec L. Two cases of giardiasis unsuccessfully treated by albendazole. Parasite. 1995;2:422.
23. Wassmann C, Hellberg A, Tannich E, et al. Metronidazole resistance in the protozoan parasite *Entamoeba histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. J Biol Chem. 1999;274:26051–6.
24. Yarlett N, Yarlett NC, Lloyd D. Metronidazole-resistant clinical isolates of *Trichomonas vaginalis* have lowered oxygen affinities. Mol Biochem Parasitol. 1986;19:111–6.
25. Sauvage V, Aubert D, Escotte-Binet S. The role of ATP-binding cassette (ABC) proteins in protozoan parasites. Mol Biochem Parasitol. 2009;167:81–94.
26. WHO. Amoebiasis. Wkly Epidemiol Rec. 1997;72:97–100.
27. Peters RS, Gitlin N, Libke RD. Amebic liver abscess. Annu Rev Med. 1981;32:161–74.
28. Jackson TF. *Entamoeba histolytica* and *Entamoeba dispar* are distinct species; clinical, epidemiological and serological evidence. Int J Parasitol. 1998;28:181–6.
29. Espinosa-Cantellano M, Gonzalez-Robles A, Chavez B, et al. *Entamoeba dispar:* ultrastructure, surface properties and cytopathic effect. J Eukaryot Microbiol. 1998;45:265–72.
30. Orozco E, Guarneros G, Martinez-Palomo A, et al. *Entamoeba histolytica.* Phagocytosis as a virulence factor. J Exp Med. 1983;158:1511–21.

31. Gatti S, Cevini C, Bruno A, et al. Transmission of *Entamoeba histolytica* within a family complex. Trans R Soc Trop Med Hyg. 1995;89:403–5.
32. Haque R, Huston CD, Hughes M, et al. Amebiasis. N Engl J Med. 2003;348:1565–73.
33. Reeves RE. Metabolism of *Entamoeba histolytica* Schaudinn, 1903. Adv Parasitol. 1984;23:105–42.
34. Rodriguez MA, Garcia-Perez RM, Mendoza L, et al. The pyruvate:ferredoxin oxidoreductase enzyme is located in the plasma membrane and in a cytoplasmic structure in *Entamoeba*. Microb Pathog. 1998;25:1–10.
35. Huber M, Garfinkel L, Gitler C, et al. Nucleotide sequence analysis of an *Entamoeba histolytica* ferredoxin gene. Mol Biochem Parasitol. 1988;31:27–33.
36. Agrawal A, Singh LM, Sagar P. Sensitivity of protein and RNA synthesis to emetine in axenic *Entamoeba histolytica*. Zentralbl Bakteriol Mikrobiol Hyg A. 1987;266:575–9.
37. Khaw M, Panosian CB. Human antiprotozoal therapy: past, present, and future. Clin Microbiol Rev. 1995;8:427–39.
38. Hanna RM, Dahniya MH, Badr SS, et al. Percutaneous catheter drainage in drug-resistant amoebic liver abscess. Trop Med Int Health. 2000;5:578–81.
39. Pal D, Banerjee S, Cui J, et al. *Giardia*, *Entamoeba* and *Trichomonas* enzymes activate metronidazole (nitroreductases) and inactivate metronidazole (nitroimidazole reductases). Antimicrob Agents Chemother. 2009;53:458–64.
40. Penuliar GM, Furukawa A, Sato D, et al. Mechanism of trifluoromethionine resistance in *Entamoeba histolytica*. J Antimicrob Chemother. 2011;66:2045–52.
41. Penuliar GM, Furukawa A, Nakada-Tsukui K, et al. Transcriptional and functional analysis of trifluoromethionine resistance in *Entamoeba histolytica*. J Antimicrob Chemother. 2012;67:375–86.
42. Samuelson JC, Burke A, Courval JM. Susceptibility of an emetine-resistant mutant of *Entamoeba histolytica* to multiple drugs and to channel blockers. Antimicrob Agents Chemother. 1992;36:2392–7.
43. Orozco E, Lopez C, Gomez C, et al. Multidrug resistance in the protozoan parasite *Entamoeba histolytica*. Parasitol Int. 2002;51:353–9.
44. Ghosh SK, Lohia A, Kumar A, et al. Overexpression of P-glycoprotein gene 1 by transfected *Entamoeba histolytica* confers emetine-resistance. Mol Biochem Parasitol. 1996;82:257–60.
45. Gomez C, Perez DG, Lopez-Bayghen E, et al. Transcriptional analysis of the *EhPgp1* promoter of *Entamoeba histolytica* multidrug-resistant mutant. J Biol Chem. 1998;273:7277–84.
46. Marchat LA, Gomez C, Perez DG, et al. Two CCAAT/enhancer binding protein sites are cis-activator elements of the *Entamoeba histolytica EhPgp1* (*mdr*-like) gene expression. Cell Microbiol. 2002;4:725–37.
47. Ramírez ME, Pérez DG, Náder E. Transcriptional activation by an URE4-like sequence in the *EhPgp1* gene core promoter. J Bacteriol Parasitol. 2012;3(6):148–54.
48. Gómez C, Macías ML, Pérez-Ishiwara DG, et al. A novel heat shock transcription factor family in *Entamoeba histolytica*. Am J Infect Dis. 2007;3(2):115–22.
49. Lopez-Camarillo C, Luna-Arias JP, Marchat LA, et al. *EhPgp5* mRNA stability is a regulatory event in the *Entamoeba histolytica* multidrug resistance phenotype. J Biol Chem. 2003;278:11273–80.
50. Bañuelos C, Orozco E, Gomez C, et al. Cellular location and function of the P-glycoproteins (EhPgps) in *Entamoeba histolytica* multidrug-resistant trophozoites. Microb Drug Resist. 2002;8:291–300.
51. Delgadillo DM, Pérez DG, Gómez C, et al. The *Entamoeba histolytica* EhPgp5 (MDR-like) protein induces swelling of the trophozoites and chloride dependent currents in *Xenopus laevis* oocytes. Microb Drug Resist. 2002;8(1):15–26.
52. Medel O, Gómez C, Sánchez V, et al. *Entamoeba histolytica* P-glycoprotein (EhPgp) inhibition, induce trophozoite acidification and enhance programmed cell death. Exp Parasitol. 2013;135(3):532–40.
53. Savioli L, Smith H, Thompson A. *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. Trends Parasitol. 2006;22:203–8.
54. European Centre for Disease Prevention and Control. Annual Epidemiological Report (2013). Reporting on 2011 surveillance data and 2012 epidemic intelligence data. Stockholm: ECDC; 2013
55. Yoder JS, Gargano JW, Wallace RM, et al. Giardiasis surveillance—United States, 2009–2010. MMWR Surveill Summ. 2012;61:13–23.
56. Leclerc H, Schwartzbrod L, Dei-Cas E. Microbial agents associated with waterborne diseases. Crit Rev Microbiol. 2002;28:371–409.
57. Santos CK, Grama DF, Limongi JE, et al. Epidemiological, parasitological and molecular aspects of *Giardia duodenalis* infection in children attending public daycare centers in southeastern Brazil. Trans R Soc Trop Med Hyg. 2012;106:473–9.
58. Gascón J. Epidemiology, etiology and pathophysiology of traveler's diarrhea. Digestion. 2006;73:102–8.
59. Swaminathan A, Torresi J, Schlagenhauf P, et al. A global study of pathogens and host risk factors associated with infectious gastrointestinal disease in returned international travellers. J Infect. 2009;59:19–27.
60. Ryan U, Cacciò SM. Zoonotic potential of Giardia. Int J Parasitol. 2013;43:943–56.
61. Busatti HG, Santos JF, Gomes MA. The old and new therapeutic approaches to the treatment of giardiasis: where are we? Biologics. 2009;3:273.
62. Pasupuleti V, Escobedo AA, Deshpande A, et al. Efficacy of 5-nitroimidazoles for the treatment of giardiasis: a systematic review of randomized controlled trials. PLoS Negl Trop Dis. 2014;8:e2733. doi:10.1371/journal.pntd.0002733.
63. Tejman-Yarden N, Miyamoto Y, Leitsch D, et al. A reprofiled drug, auranofin, is effective against metronidazole-resistant *Giardia lamblia*. Antimicrob Agents Chemother. 2013;57:2029–35.
64. Galkin A, Kulakova L, Lim K, et al. Structural basis for inactivation of *Giardia lamblia* carbamate kinase by disulfiram. J Biol Chem. 2014;289:10502–9.
65. Hahn J, Seeber F, Kolodziej H, et al. High sensitivity of *Giardia duodenalis* to tetrahydrolipstatin (orlistat) *in vitro*. PLoS One. 2013;8:e71597. doi:10.1371/journal.pone.0071597.
66. Eissa MM, Amer EI. *Giardia lamblia*: a new target for miltefosine. Int J Parasitol. 2012;42:443–52.
67. Hausen MA, Menna-Barreto RF, Lira DC, et al. Synergic effect of metronidazole and pyrantel pamoate on *Giardia lamblia*. Parasitol Int. 2011;60:54–8.
68. Busatti HG, Alves RJ, Santana-Anjos KG, et al. Effects of metronidazole analogues on *Giardia lamblia*: experimental infection and cell organization. Diagn Microbiol Infect Dis. 2013;75:160–4.
69. Leitsch D, Burgess AG, Dunn LA, et al. Pyruvate: ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in *Giardia lamblia*. J Antimicrob Chemoth. 2011;66:1756–65.
70. Müller J, Schildknecht P, Müller N. Metabolism of nitro drugs metronidazole and nitazoxanide in *Giardia lamblia*: characterization of a novel nitroreductase (GlNR2). J Antimicrob Chemother. 2013;68:1781–9.
71. Dan M, Wang AL, Wang CC. Inhibition of pyruvate-ferredoxin oxidoreductase gene expression in *Giardia lamblia* by a virus-mediated hammerhead ribozyme. Mol Microbiol. 2000;36:447–56.
72. Townson SM, Hanson GR, Upcroft JA, et al. A purified ferredoxin from *Giardia duodenalis*. Eur J Biochem. 1994;220:439–46.
73. Bagchi S, Oniku AE, Topping K, et al. Programmed cell death in *Giardia*. Parasitology. 2012;139:894–903.

74. Müller J, Ley S, Felger I, et al. Identification of differentially expressed genes in a *Giardia lamblia* WB C6 clone resistant to nitazoxanide and metronidazole. J Antimicrob Chemother. 2008;62:72–82.
75. Nillius D, Müller J, Müller N. Nitroreductase (GlNR1) increases susceptibility of *Giardia lamblia a*nd *Escherichia coli* to nitro drugs. J Antimicrob Chemother. 2011;66:1029–35.
76. Leitsch D, Schlosser S, Burgess A, et al. Nitroimidazole drugs vary in their mode of action in the human parasite *Giardia lamblia*. Int J Parasitol Drugs Drug Resist. 2012;2:166–70.
77. Brown DM, Upcroft JA, Upcroft P. A H_2O-producing NADH oxidase from the protozoan parasite *Giardia duodenalis*. Eur J Biochem. 1996;241:155–61.
78. Upcroft JA, Campbell RW, Upcroft P. Quinacrine-resistant *Giardia duodenalis*. Parasitology. 1996;112:309–13.
79. Jedelský PL, Doležal P, Rada P, et al. The minimal proteome in the reduced mitochondrion of the parasitic protist *Giardia intestinalis*. PLoS One. 2011;6:e17285. doi:10.1371/journal.pone.0017285.
80. Morga UM, Reynoldson JA, Thompson RC. Activities of several benzimidazoles and tubulin inhibitors against *Giardia* spp. *in vitro*. Antimicrob Agents Chemother. 1993;37:328–31.
81. Muller J, Wastling J, Sanderson S, et al. A novel *Giardia lamblia* nitroreductase, GlNr1, interacts with nitazoxanide and other thiazolides. Antimicrob Agents Chemother. 2001;51:1979–86.
82. Boreham PF, Phillips RE, Shepherd RW. Altered uptake of metronidazole *in vitro* by stocks of *Giardia intestinalis* with different drug sensitivities. Trans R Soc Trop Med Hyg. 1988;82:104–6.
83. Upcroft J, Mitchell R, Chen N, et al. Albendazole resistance in *Giardia* is correlated with cytoskeletal changes but not with a mutation at amino acid 200 in beta-tubulin. Microb Drug Resist. 1996;2:303–8.
84. Meltzer E, Lachish T, Schwartz E. Treatment of giardiasis after nonresponse to nitroimidazole. Emerg Infect Dis. 2014;20:1742.
85. Tejman-Yarden N, Millman M, Lauwaet T, et al. Impaired parasite attachment as fitness cost of metronidazole resistance in *Giardia lamblia*. Antimicrob Agents Chemother. 2011;55:4643–51.
86. Dunn LA, Burgess AG, Krauer KG, et al. A new-generation 5-nitroimidazole can induce highly metronidazole-resistant *Giardia lamblia in vitro*. Int J Antimicrob Agents. 2010;36:37–42.
87. Upcroft JA, Healey A, Murray DG, et al. A gene associated with cell division and drug resistance in *Giardia duodenalis*. Parasitology. 1992;104:397–405.
88. Upcroft P, Upcroft JA. Organization and structure of the *Giardia* genome. Protist. 1999;150:17–23.
89. Smith NC, Bryant C, Boreham PF. Possible roles for pyruvate:ferredoxin oxidoreductase and thiol-dependent peroxidase and reductase activities in resistance to nitroheterocyclic drugs in *Giardia intestinalis*. Int J Parasitol. 1988;18:991–7.
90. GL50803_28379 Multidrug resistance-associated protein 1 [*Giardia lamblia* ATCC 50803-28379]—Gene–NCBI. 2014. http://www.uniprot.org/uniprot/A8B3T0.
91. Wampfler PB, Tosevski V, Nanni P, et al. Proteomics of secretory and endocytic organelles in *Giardia lamblia*. PLoS One. 2014;9:e94089. doi:10.1371/journal.pone.0094089.
92. Rodríguez-Ochoa NT, Jiménez-Gutiérrez A, Hernández JM, et al. (2014). Análisis de la expresión de genes MDR en trofozoítos de *Giardia duodenalis* tratados con nitazoxanida, albendazol y JVG9. Poster presented at XIX national congress of biochemical engineering, VIII international congress of biochemical engineering and XII biomedicine and molecular biotechnology scientific meetings, Colegio Mexicano de Ingeniería Bioquímica, Mazatlan, Sinaloa, Mexico, 9–11 April 2014.
93. Ribeiro KC, Pereira-Neves A, Benchimol M. The mitotic spindle and associated membranes in the closed mitosis of trichomonads. Biol Cell. 2002;94:157–72.
94. Rendón-Gandarilla FJ, Ramón-Luing L, Ortega-López J, et al. The TvLEGU-1, a legumain-like cysteine proteinase, plays a key role in *Trichomonas vaginalis* cytoadherence. Biomed Res Int. 2013;561979. doi:10.1155/2013/561979.
95. Sorvillo F, Smith L, Kerndt P, et al. *Trichomonas vaginalis*, HIV, and African-Americans. Emerg Infect Dis. 2001;7:927–32.
96. Stark JR, Judson G, Alderete JF, et al. Prospective study of Trichomonas vaginalis infection and prostate cancer incidence and mortality: Physicians' Health Study. J Natl Cancer Inst. 2009;101:1406–11.
97. Dunne RL, Dunn LA, Upcroft P, et al. Drug resistance in the sexually transmitted protozoan *Trichomonas vaginalis*. Cell Res. 2003;13:239–49.
98. Johnson PJ, d'Oliveira CE, Gorrell TE, et al. Molecular analysis of the hydrogenosomal ferredoxin of the anaerobic protist *Trichomonas vaginalis*. Proc Natl Acad Sci U S A. 1990;87:6097–101.
99. Horner DS, Foster PG, Embley TM. Iron hydrogenases and the evolution of anaerobic eukaryotes. Mol Biol Evol. 2000;17:1695–709.
100. Edwards DI. Nitroimidazole drugs—action and resistance mechanisms. I. Mechanisms of action. J Antimicrob Chemother. 1993;31:9–20.
101. Leitsc D, Kolarich D, Binder M, et al. *Trichomonas vaginalis*: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. Mol Microbiol. 2009;72:518–36.
102. Upcroft JA, Dunn L, Wal T, et al. Metronidazole resistance in *Trichomonas vaginalis* from highland women in Papua New Guinea. Sex Health. 2009;6:334–8.
103. Rasoloson D, Tomkova E, Cammack R, et al. Metronidazole-resistant strains of *Trichomonas vaginalis* display increased susceptibility to oxygen. Parasitology. 2001;123:45–56.
104. Mead JR, Fernandez M, Romagnoli PA, et al. Use of *Trichomonas vaginalis* clinical isolates to evaluate correlation of gene expression and metronidazole resistance. J Parasitol. 2006;92:196–9.
105. Land KM, Delgadillo-Correa MG, Tachezy J, et al. Targeted gene replacement of a ferredoxin gene in *Trichomonas vaginalis* does not lead to metronidazole resistance. Mol Microbiol. 2004;51:115–22.
106. Brown DM, Upcroft JA, Dodd HN, et al. Alternative 2-keto acid oxidoreductase activities in *Trichomonas vaginalis*. Mol Biochem Parasitol. 1999;98:203–14.
107. Wright JM, Webb RI, O'Donoghue P, et al. Hydrogenosomes of laboratory-induced metronidazole-resistant *Trichomonas vaginalis* lines are downsized while those from clinically metronidazole-resistant isolates are not. J Eukaryot Microbiol. 2010;57:171–6.
108. Rasoloson D, Vanacova S, Tomkova E, et al. Mechanisms of *in vitro* development of resistance to metronidazole in *Trichomonas vaginalis*. Microbiology. 2002;148:2467–77.
109. Leitsch D, Kolarich D, Duchene M. The flavin inhibitor diphenyleneiodonium renders *Trichomonas vaginalis* resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways. Mol Biochem Parasitol. 2010;171:17–24.
110. Chapman A, Linstead DJ, Lloyd D. Hydrogen peroxide is a product of oxygen consumption by *Trichomonas vaginalis*. J Biosci. 1999;24:339–44.
111. Paulish-Miller TE, Augostini P, Schuyler JA, et al. *Trichomonas vaginalis* metronidazole resistance is associated with single nucleotide polymorphisms in the nitroreductase genes ntr4Tv and ntr6Tv. Antimicrob Agents Chemother. 2014;28:2938–43.
112. Johnson PJ, Schuck BL, Delgadillo MG. Analysis of a single-domain P-glycoprotein-like gene in the early-diverging protist *Trichomonas vaginalis*. Mol Biochem Parasitol. 1994;66:127–37.
113. Centers for Disease Control and Prevention. Sexually transmitted disease treatment guidelines, 2010. MMWR Recomm Rep. 2010;59:1–110.

114. Nyirjesy P, Gilbert J, Mulcahy LJ. Resistant trichomoniasis: successful treatment with combination therapy. Sex Transm Dis. 2011;38(10):962–3.
115. Kirkcaldy RD, Augostini P, Asbel LE, et al. *Trichomonas vaginalis* antimicrobial drug resistance in 6 US Cities, STD surveillance network, 2009–2010. Emerg Infect Dis. 2012;18(6):939–43.
116. Crowell AL, Sanders-Lewis KA, Secor WE. *In vitro* metronidazole and tinidazole activities against metronidazole-resistant strains of *Trichomonas vaginalis*. Antimicrob Agents Chemother. 2003;47:1407–9.
117. Nigro L, Larocca L, Massarelli L, et al. A placebo-controlled treatment trial of *Blastocystis hominis* infection with metronidazole. J Travel Med. 2003;10:128–30.
118. Coyle CM, Varughese J, Weiss LM, et al. *Blastocystis*: to treat or not to treat. Clin Infect Dis. 2012;54:105–10.
119. Sekar U, Shanthi M. *Blastocystis*: consensus of treatment and controversies. Trop Parasitol. 2013;3:35–9.
120. Rossignol JF, Kabil SM, el-Gohary Y, et al. Effect of nitazoxanide in diarrhea and enteritis caused by Cryptosporidium species. Clin Gastroenterol Hepatol. 2006;4:320–4.
121. Cabada MM, White Jr AC. Treatment of cryptosporidiosis: do we know what we think we know? Curr Opin Infect Dis. 2010;23:494–9.
122. Hussein SM, Abdella OH, Abu-Hashim AH, et al. Comparative study between the effect of nitazoxanide and paromomycin in treatment of cryptosporidiosis in hospitalized children. J Egypt Soc Parasitol. 2013;43:463–70.
123. Chawla R, Ichhpujani RL. Enteric spore-forming opportunistic parasites in HIV/AIDS. Trop Parasitol. 2011;1:15–9.
124. Didier ES, Weiss LM. Microsporidiosis: not just in AIDS patients. Curr Opin Infect Dis. 2011;24:490–5.
125. Franssen FF, Lumeijm JT, van Knapen F. Susceptibility of *Encephalitozoon cuniculi* to several drugs *in vitro*. Antimicrob Agents Chemother. 1995;39:1265–8.
126. Haresh K, Suresh K, Khairul Anus A, et al. Isolate resistance of *Blastocystis hominis* to metronidazole. Trop Med Int Health. 1999;4:274–7.
127. Mirza H, Teo JD, Upcroft J, et al. A rapid, high-throughput viability assay for *Blastocystis* spp. reveals metronidazole resistance and extensive subtype-dependent variations in drug susceptibilities. Antimicrob Agents Chemother. 2011;55:637–48.
128. Yakoob J, Jafri W, Jafri N, et al. *In vitro* susceptibility of *Blastocystis hominis* isolated from patients with irritable bowel syndrome. Br J Biomed Sci. 2004;61:75–7.
129. Denoeud F, Roussel M, Noel B, et al. Genome sequence of the stramenopile *Blastocystis*, a human anaerobic parasite. Genome Biol. 2011;12:R29.
130. Perkins ME, Riojas YA, Wu TW, et al. CpABC, a *Cryptosporidium parvum* ATP-binding cassette protein at the host-parasite boundary in intracellular stages. Proc Natl Acad Sci U S A. 1999;96:5734–9.
131. Vasquez JR, Gooze L, Kim K, et al. Potential antifolate resistance determinants and genotypic variation in the bifunctional dihydrofolate reductase-thymidylate synthase gene from human and bovine isolates of *Cryptosporidium parvum*. Mol Biochem Parasitol. 1996;79:153–65.
132. Boyles TH, Black J, Meintjes G, et al. Failure to eradicate *Isospora belli* diarrhoea despite immune reconstitution in adults with HIV—a case series. PLoS One. 2012;7:e42844.
133. Bialek R, Overkamp D, Retting I, et al. Case report: nitazoxanide treatment failure in chronic isosporiasis. Am J Trop Med Hyg. 2001;65:94–5.
134. Rossi P, Urbani C, Donelli G, et al. Resolution of microsporidial sinusitis and keratoconjunctivitis by itraconazole treatment. Am J Ophthalmol. 1999;127:210–2.
135. Bonafonte MT, Srewart J, Mead JR. Identification of two putative ATP-cassette genes in *Encephalitozoon intestinalis*. Int J Parasitol. 2001;31:1681–5.
136. Canning EU, Hollister WS. *In vitro* and *in vivo* investigations of human microsporidia. J Protozool. 1991;38:631–15.
137. Debnath A, Parsonage D, Andrade RM, et al. A high-throughput drug screen for *E. histolytica* identifies a new lead and target. Nat Med. 2012;18:956–60.
138. Downey AS, Graczyk TK, Sullivan DJ. In vitro activity of pyrvinium pamoate against *Entamoeba histolytica* and *Giardia intestinalis* using radiolabelled thymidine incorporation and an SYBR Green I-based fluorescence assay. J Antimicrob Chemother. 2009;64:751–4.
139. Mor A. Multifunctional host defense peptides: antiparasitic activities. FEBS J. 2009;276:6474–82.
140. Preet S, Bharati S, Shukla G, et al. Evaluation of amoebicidal potential of paneth cell cryptdin-2 against Entamoeba histolytica. PLoS Negl Trop Dis. 2011;5(12):1386–95.
141. Aley SB, Zimmerman M, Hetsko M, et al. Killing of *Giardia lamblia* by cryptdins and cationic neutrophil peptides. Infect Immun. 1994;62:5397–403.
142. Innocente AM, de Brum P, Piccoli A, et al. Anti-Trichomonas vaginalis activity from triterpenoid derivatives. Parasitol Res. 2014;113:2933–40.
143. Adebajo AC, Ayoola OF, Iwalewa EO, et al. Anti-trichomonal biochemical and toxicological activities of methanolic extract and some carbazole alkaloids from the leaves of Murraya koenigii growing in Nigeria. Phytomedicine. 2006;4:246–54.
144. Giordani RB, Vieira PDB, Weizenmann M, et al. Lycorine induces cell death in the amitochondriate parasite, *Trichomonas vaginalis*, via an alternative non-apoptotic death pathway. Phytochemistry. 2011;72:545–650.
145. Rocha T, De Brum VP, Gnoatto SC, et al. Anti-*Trichomonas vaginalis* of saponins from Quillaja, Passiflora, and Ilex species. Parasitol Res. 2012;110:2551–6.
146. Rocha DAS, Rosa IA, Souza W, et al. Evaluation of the effect of miltefosine on *Trichomonas vaginalis*. Parasitol Res. 2014;113: 1041–7.
147. Ibrahim AN. Comparison of *in vitro* activity of metronidazole and garlic-based product (Tomex®) on *Trichomonas vaginalis*. Parasitol Res. 2014;112:2063–7.
148. Secor WE. *Trichomonas vaginalis*: treatment questions and challenges. Expert Rev Anti Infect Ther. 2012;10(2):107–9.
149. Yakoob J, Abbas Z, Beg MA, et al. *In vitro* sensitivity of *Blastocystis hominis* to garlic, ginger, white cumin, and black pepper used in diet. Parasitol Res. 2011;109:379–85.
150. Dinleyici EC, Eren M, Dogan N, et al. Clinical efficacy of *Saccharomyces boulardii* or metronidazole in symptomatic children with *Blastocystis hominis* infection. Parasitol Res. 2011;108:541–5.
151. Mossallam SF, Amer E, Diab RG. Potentiated anti-microsporidial activity of *Lactobacillus acidophilus* CH1 bacteriocin using gold nanoparticles. Exp Parasitol. 2014;144:14–21.
152. Armson A, Sargent K, MacDonald LM, et al. A comparison of the effects of two dinitroanilines against *Cryptosporidium parvum in vitro* and *in vivo* in neonatal mice and rats. FEMS Immunol Med Microbiol. 1999;26:109–13.
153. Ndao M, Nath-Chowdhury M, Sajid M, et al. A cysteine protease inhibitor rescues mice from a lethal *Cryptosporidium parvum* infection. Antimicrob Agents Chemother. 2013;57:6063–73.

Mechanisms of Antimalarial Drug Resistance

41

Giancarlo A. Biagini and Stephen A. Ward

1 Introduction

It has been estimated that in 2013 there were approximately 198 million cases of malaria (with an uncertainty range of 124–283 million) and an estimated 584,000 deaths (with an uncertainty range of 367,000–755,000), with the majority of deaths amongst African children under 5 years of age [1]. As a result of global efforts, including in transmission control (e.g. removal of breeding sites using insecticides and prevention of human contact through screens and bed nets), improved antimalarial chemotherapy and early effective case management, malaria mortality rates have fallen by 47 % globally and by 54 % in Africa since 2000 [1].

Effective chemotherapy remains central to malaria control/elimination and as such first-line antimalarials exert significant selection pressure on malaria parasites to evolve resistance. The past 60 years have seen waves of newly deployed antimalarials countered by the evolution of drug resistance by malaria parasites, often leading to significant global rises in malaria-associated morbidity and mortality [2] (Table 41.1).

The introduction of chloroquine (CQ) shortly after World War II had a tremendous impact on global health; however today resistance to the drug has been observed in every region where *P. falciparum* occurs [3]. Resistance developed from a number of independent foci including South America, Southeast Asia and Papua New Guinea [4, 5]. Gradually over the next 20 years, resistance spread throughout South America and Southeast Asia arriving in East Africa in the late 1970s. Chloroquine resistance has since spread across all of sub-Saharan Africa. As drug resistance is genetically determined, it spreads by active malaria transmission, as gametocytes from resistant isolates will produce resistant offspring. Many African countries switched their first-line drug from CQ to the antifolate combination of sulphadoxine-pyrimethamine (SP); however resistance to SP also grew and spread very quickly, especially in Southeast Asia, South America [3, 6] and then in sub-Saharan Africa [7]. Amodiaquine (AQ), an active analogue of CQ, replaced CQ and SP in many areas in Africa but this drug was also subject to resistance-mediated failures [8–13]. A switch to mefloquine (MQ) proved successful at first; however resistance was reported as early as 5 years after its introduction as a prophylactic treatment in parts of Thailand [3] (Table 41.1) with cure rates in some regions of Thailand dropping to below 41 % [14]. Resistance to mitochondrial bc_1 inhibitor atovaquone was even more rapid, emerging in the same year as its launch [3].

In 2001 the WHO recommended treating uncomplicated malaria with combinations of two unrelated drugs, with a recommendation that one of which should be an artemisinin derivative. The recommended frontline treatments, so-called artemisinin combination therapies (ACTs)—combine fast-acting artemisinin or semi-synthetic artemisinins with antimalarials possessing longer therapeutic half-lives such as lumefantrine, amodiaquine, mefloquine, piperaquine, pyronaridine and sulphadoxine-pyrimethamine [12].

In 2006, the first signs of parasite "resistance" to artemisinin were observed in Southeast Asia, in the border region between Thailand and Cambodia [15, 16], although rumours of a loss of parasite sensitivity to these drugs in the region actually emerged some 6 years earlier. Parasites from this region were observed to have a delayed parasite clearance phenotype following either artesunate monotherapy or an ACT. The delayed parasite clearance phenotype is not resistance as defined by the WHO (see Box 41.1) and does not necessarily lead to treatment failure [17]. Treatment failure in the Greater Mekong Subregion following treatment with an ACT has only been observed where resistance to the partner drug exists regardless of the presence of artemisinin resistance [17]; however the slow clearance phenotype gives rise to concern that the last effective class of currently registered antimalarials, and the only one that offers rapid biomass reduction, is likely to have a limited shelf life.

G.A. Biagini, Ph.D. (✉) • S.A. Ward, Ph.D.
Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK
e-mail: Giancarlo.Biagini@lstmed.ac.uk; saward@liverpool.ac.uk

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_41

Table 41.1 Development of malaria parasite resistance to antimalarial drugs[a]

Drug	Introduced	First reported resistance	Difference (years)
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulphadoxine-pyrimethamine	1967	1967	0
Mefloquine	1977	1982	5
Atovaquone	1996	1996	0

[a]Data taken from [3]

Box 41.1 WHO definitions

Drug resistance: defined by the WHO in 1967 as "the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject" [213], later amended to include: "The form of the drug active against the parasite must be able to gain access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action" [214].

Treatment failure: "The inability to clear malarial parasitemia or resolve clinical symptoms despite administration of an antimalarial medicine" [12].

Early treatment failure: Defined as (i) danger signs or severe malaria on day 1, 2 or 3 in the presence of parasitaemia, (ii) parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature, (iii) parasitaemia on day 3 with axillary temperature ≥37.5 °C; or parasitaemia on day 3 ≥25 % of count on day 0 [12].

Late clinical failure: danger signs or severe malaria in the presence of parasitaemia on any day between days 4 and 28 (day 42) in patients who did not previously meet any of the criteria for early treatment failure; or presence of parasitaemia on any day between days 4 and 28 (day 42) with axillary temperature ≥37.5 °C in patients who did not previously meet any of the criteria for early treatment failure [12].

Late parasitological failure: presence of parasitaemia on any day between days 7 and 28 (day 42) with axillary temperature <37.5 °C in patients who did not previously meet any of the criteria for early treatment failure or late clinical failure [12].

Adequate clinical and parasitological response: absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria for early treatment failure, late clinical failure or late parasitological failure [12].

Understanding malaria parasite resistance mechanisms is critical to efforts aimed at delivering the next generation of effective antimalarials (e.g. mmv.org) and in tracking resistance (e.g. wwarn.org) to aid control programmes and inform policymakers. With mortality still at ca. 0.5 million per year, mainly in African children under the age of 5 years old, and the emergence of multidrug-resistant parasites resistant to all known antimalarial drug classes, the need for the development of new drugs which circumvent current parasite resistance mechanisms remains an urgent priority. However, a comprehensive knowledge of drug resistance mechanisms is required to support the development of such strategies. Here we review the latest genetic, biochemical and physiological data that underpin current theories of resistance mechanisms to the major classes of antimalarial drugs.

2 Resistance Mechanisms to Quinoline and Quinoline-Based Antimalarials

The quinoline-based antimalarials such as chloroquine (CQ), amodiaquine (AQ), quinine (Q) and mefloquine (MQ) were the defensive bastions against malaria for many years (Fig. 41.1). The success of these drugs is based on their excellent clinical efficacy, limited host toxicity, ease of use and cost-effective synthesis. Although it took over 20 years to appear, resistance to quinoline antimalarials is now ubiquitous in malaria-endemic countries [12]. The exact mode of action of quinolines has not fully been elucidated but it is accepted that a crucial step in this process is the binding of the drug to ferriprotoporphyrin IX (FP, or haem) a by-product of haemoglobin degradation, which occurs in the parasite digestive food vacuole (DV). The uncertainty of the mode of action of quinolines, together with a poor understanding of parasite physiology, leaves the mechanism of CQ resistance in *P. falciparum* for the most part enigmatic.

2.1 Access to Haematin Is the Biochemical Basis of CQ-Resistance

The exquisite specificity of CQ for malaria parasites stems from the parasite-specific accumulation of the drug to levels far greater than are seen with mammalian cells. CQ is a weak base and depending on the surrounding pH can take the form

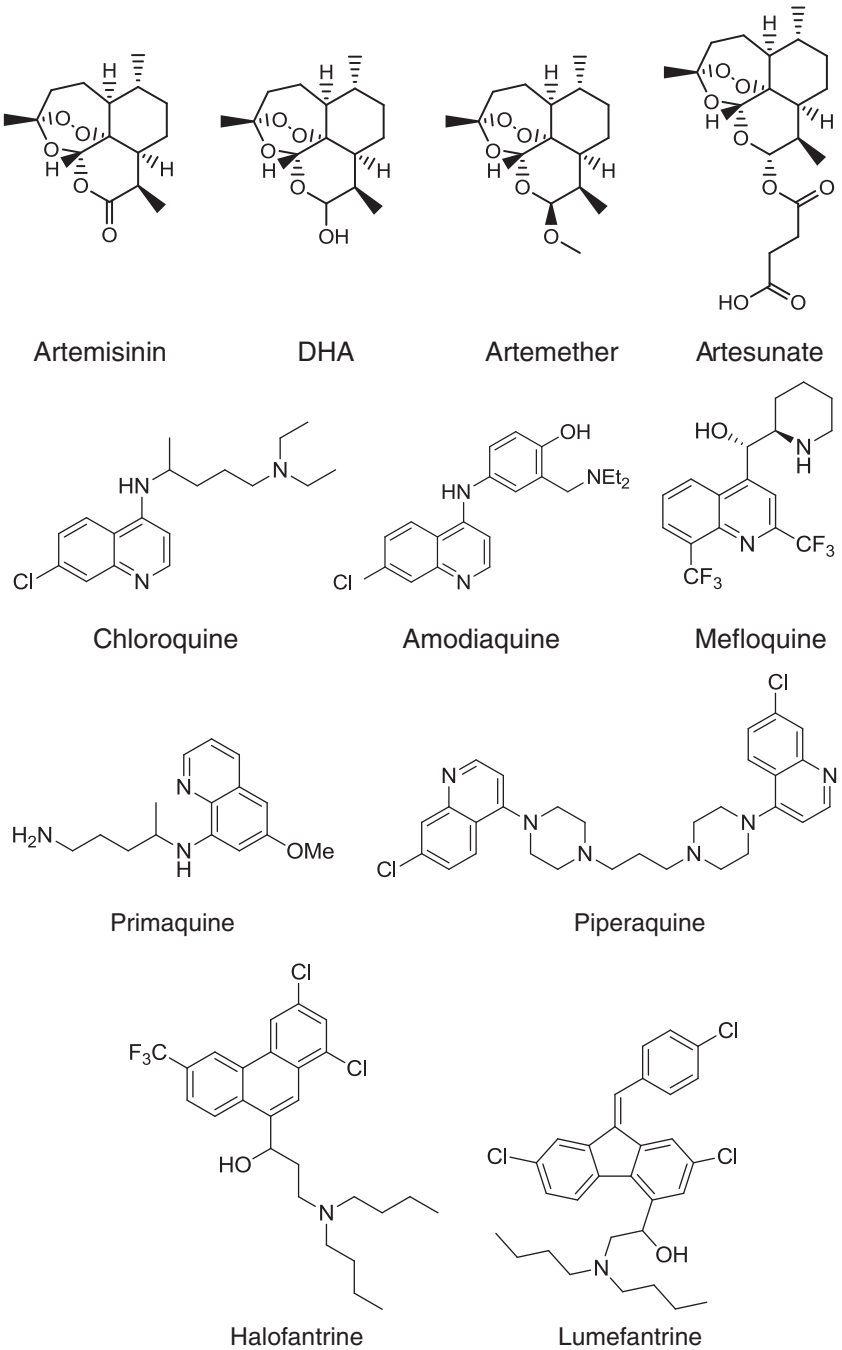


Fig. 41.1 Chemical structure of common first-line antimalarials

of the un- (CQ), singly (CQ^+) or doubly protonated (CQ^{++}) species, owing to the protonatable diethylamine nitrogen side chain (pK_a 10.2) and the quinoline-ring heteroatom nitrogen (pK_a 8.1, [18]). The un-protonated (uncharged) species of CQ is membrane permeable and is able to distribute equally across all cellular compartments whilst the protonated species is relatively impermeable to membranes [19]. In the parasite DV (estimated pH ~5.2–5.8 [20, 21]) a high concentration of CQ^{++} is trapped in its doubly protonated and membrane-impermeable form [19]. The "proton trapping" of CQ potentially results in this drug accumulating several thousandfold with concentrations possibly reaching mM levels in the food vacuole of the parasite [22]. However, many mammalian cells contain large acidic vacuoles and yet accumulate much less CQ than malaria parasites. These data indicate that proton trapping cannot be the only mechanism driving CQ uptake into the parasite. To elucidate the full mechanism of CQ uptake, the role of the DV in digesting

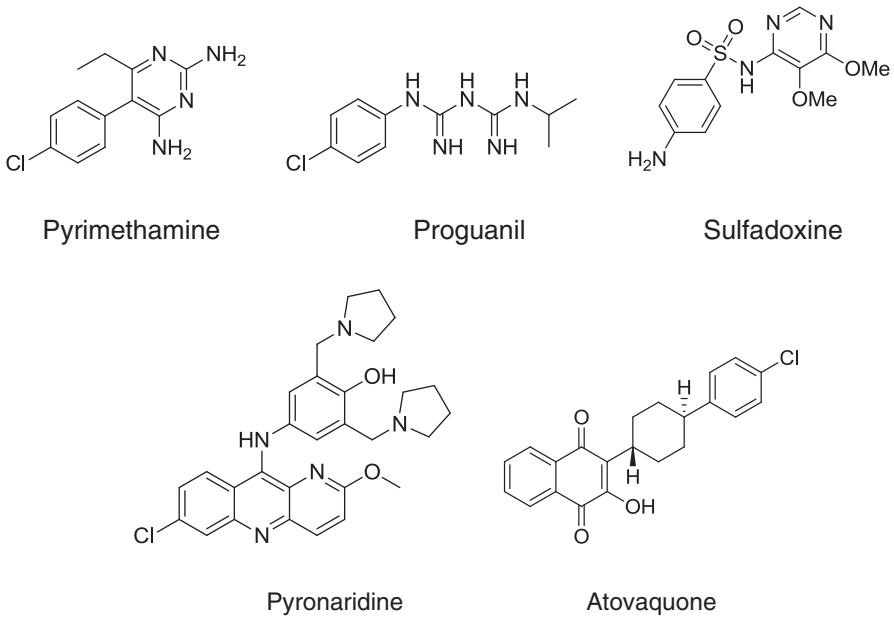


Fig. 41.1 (continued)

host cell haemoglobin also needs to be considered. The process of haemoglobin digestion releases large quantities of haem or ferriprotoporphyrin IX (FP) which is toxic in its free form. In the malaria parasite, FP is oxidised and dimerised to beta haematin before biocrystallisation into haemozoin or malarial pigment, which is non-toxic to the parasite [23, 24]. One hypothesis is that protonated CQ binds to FP inhibiting the haemozoin biocrystallisation process and causing a build-up of free FP and/or CQ-FP complex, leading ultimately to parasite death [25, 26]. CQ binds to FP with high affinity, both in the test tube and in the intracellular parasite [26–28]. The consensus view is that the parasite-specific hyper-accumulation of CQ is probably due to a combination of proton trapping in the acid DV and binding to FP in the same organelle. For all species of *Plasmodium*, CQ-resistant parasites are observed to accumulate much lower levels of CQ than their CQ-sensitive counterparts [27, 29–34]. This observation, together with the demonstration that CQ-resistant and CQ-sensitive parasites contain similar amounts of the FP target, limits the potential mechanisms of CQR to those that reduce the access of CQ to its haematin target [27].

Many hypotheses have been proposed to account for the observed reduction of CQ uptake in CQ-resistant parasites. It was thought for a long time that CQ-resistant parasites have a smaller ΔpH (DV_{IN} vs. DV_{OUT}), e.g. a more alkaline DV lumen compared to CQ-sensitive parasites, reducing the level of trapping of the charged CQ species (CQ^{+} & CQ^{++}). Because each molecule of CQ can potentially associate with two protons, relatively small changes in DV pH can have a dramatic effect on the concentration of CQ in this organelle. For example, increasing the pH of the DV from 5.2 to 5.7 will decrease the amount of protonated CQ tenfold, enough to explain the reduced susceptibility of CQ-resistant parasites [33]. Although at first an appealing theory, it has subsequently received no evidence to support it. Measuring the pH of the DV is technically very demanding [35–37] and until more recently this hypothesis had not been tested. Ironically, the first reports of a comparison of DV pH of CQ-sensitive and CQ-resistant parasite lines suggested that CQ-resistant parasites may have a *more acidic* DV than CQ-sensitive parasites [21, 38, 39]. Several workers [37, 40] have expressed reservations with regard to the experimental design adopted in the initial studies purporting to report DV pH values [38, 39]; however subsequent studies using more robust pH measurement techniques again reported a more acidic DV pH in CQ-resistant lines [21]. CQ-sensitive lines were reported to have a DV pH of around 5.7 and this was found to fall to around 5.2 in CQR lines. If so, this would be expected to increase the amount of CQ accumulated in the DV of CQ-resistant parasites by about tenfold. In an attempt to reconcile these physiological data with the sixfold reduced CQ uptake that is actually measured, it was proposed that increased aggregation of μ-oxo-dimeric FP at lower pH causes a reduction in CQ accumulation due to the lower affinity of binding of CQ to aggregated versus soluble species of FP [41]. This hypothesis would predict that CQ-resistant parasites have an altered steady-state CQ-binding capacity compared to CQ-sensitive lines. However the reverse is true; a study comparing CQ-resistant

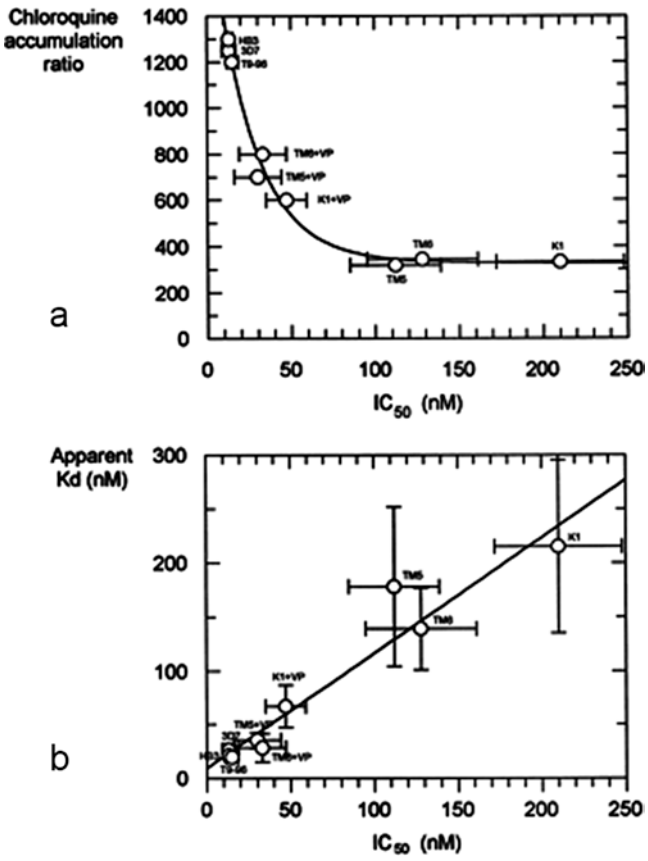


Fig. 41.2 Analysis of the antimalarial activity and saturable binding characteristics of chloroquine (CQ). The antimalarial activity of CQ is linearly related to the *apparent* receptor K_d (**b**) rather than the total cellular CQ accumulation (**a**)

and -sensitive lines revealed an equal number of CQ-binding sites [27]. CQ uptake into *P. falciparum* consists of both a saturable and non-saturable component [27, 30]. The saturable component of CQ uptake is evident at drug concentrations which are pharmacologically relevant, suggesting that this is the component that is relevant to the antimalarial activity of the drug. Detailed analysis of equilibrium CQ uptake in several lines of CQ-sensitive and -resistant parasites was performed and modelled [27]. It was demonstrated that no significant differences were found in either the non-saturable component of CQ uptake or in the capacity (B_{max}) of the saturable component. Notably however, the sensitivity of parasites to CQ (as measured by the IC_{50}, the concentration of CQ required to inhibit parasite proliferation by 50 %) was found to be directly proportional ($r^2 = 0.93$) to the apparent affinity (K_d) of the saturable uptake component (Fig. 41.2a, b). In lay terms, this study established that the concentration of the drug target haematin does not change between CQ-sensitive and -resistant parasites and that instead the *apparent* affinity for the target changes. This was subsequently confirmed in a study that directly measured the haemozoin content of a panel of isolates with a large variation in sensitivity to CQ. Only small differences in the rate of haemozoin generation were observed, and these small differences were unrelated to the CQ sensitivity of the isolates [42]. These data are very difficult to reconcile with a reduced DV pH that would accelerate the rate of haemozoin (malarial pigment) generation in CQ-resistant isolates [41]. Rather than changes in DV pH reducing the amount of target molecules, it is far more likely that CQR results from a transport process that reduces the local concentration of the drug available to bind the FP target [27, 43, 44]. This hypothesis has been supported by independent analysis of CQ-binding parameters [43, 45] and subsequent molecular studies which have pinpointed the genetic determinant for CQ resistance.

2.2 PfCRT Is the Genetic Determinant of CQ Resistance

In the late 1980s an intricate study was initiated to localise the molecular loci which harbours gene(s) responsible for CQ resistance. This approach involved a genetic cross of a cloned CQ-resistant (Dd2) and a cloned sensitive (HB3) parasite isolate using a primate model [46]. Phenotypic typing of the resulting progeny of the cross and mapping of loci using RFLP and microsatellite markers [47–49] localised a key determinant to a region on chromosome 7. An open reading frame which was termed *cg2* for "candidate gene 2" was identified as a possible candidate for CQ resistance [50]; however subsequent transfection studies showed that the *cg2* gene did not confer CQ resistance in transformed parasites [51]. Further analysis of the 36 kb region on chromosome 7 eventually yielded a highly fragmented (13 exons) open reading frame, named *pfcrt* for CQ resistance transporter, which showed highly significant linkage to over 40 CQ-resistant parasite lines examined [52]. Genetic mutations in *pfcrt* were reported to be associated with reduced in vitro susceptibilities to chloroquine in laboratory lines and field isolates [52–55]. Subsequent allelic exchange experiments have now shown without doubt that polymorphisms in *pfcrt* confer CQ resistance [56].

PfCRT contains 424-amino acid residues with a predicted ten transmembrane domains (Fig. 41.3) that localises the protein to the DV membrane [52], and is believed to be dependent on phosphorylation of residue 416 [57]. There are some ten non-synonymous variants of *pfcrt* from different geographical origins. Broadly speaking, the CQ-resistant parasite isolates from Southeast Asia and Africa have *pfcrt* genes with seven to nine mutated codons, and their mutated codons are represented by the amino acid residue pattern of CIETH(L)SEST(I)I, from positions 72 to 371 [4, 52, 58]. The CQ-resistant parasites from South America and Papua New Guinea possess *pfcrt* genes with four to five mutated codons forming patterns of S(C)MN(E)TQSDLR [4, 54, 58].

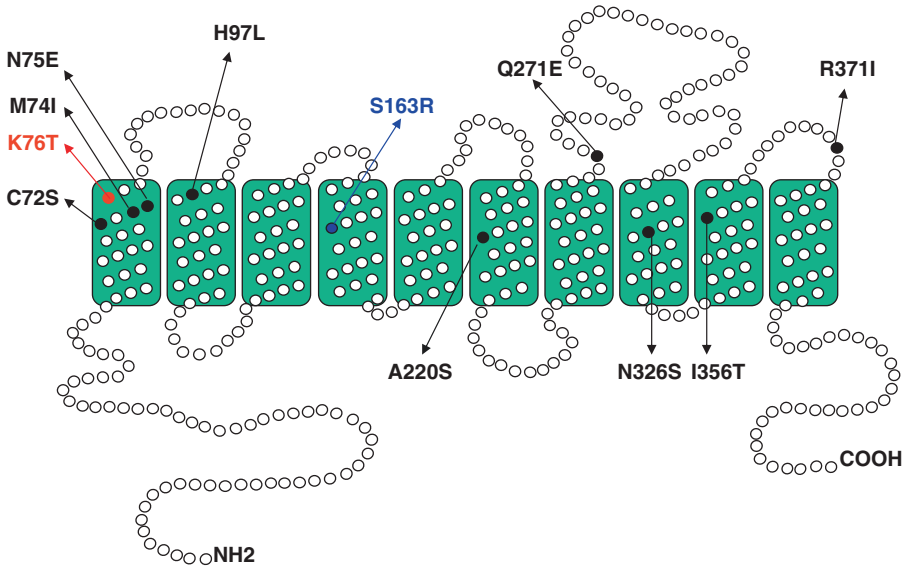


Fig. 41.3 Schematic representation of the protein structure of PfCRT. The scheme highlights the ten transmembrane domains with known polymorphisms conferring chloroquine resistance represented by the *black dots*. The vital K76T mutation found in all known CQ-resistant isolates is shown in the *red dot* and the novel S163R mutation which can confer CQ sensitivity and the loss of the verapamil effect is shown in *blue* (adapted and reproduced with permission from Elsevier Science)

Altogether there are some 30 known variant residues of *pfcrt* that have been identified, with the minimum number of non-synonymous mutations reported in *pfcrt* of CQ-resistant parasites being four, namely C72S, K76T, N326D and I356L [4]. Mutation K76T is found in all CQ-resistant parasites and A220S is observed in most CQ-resistant isolates, signifying their essential role in CQ resistance [58]. Mutations in the *P. vivax* homologue of *pfcrt* are not associated with CQ resistance [59], suggesting a genetic basis for CQ resistance in *P. vivax* that is different from that in *P. falciparum*.

The epidemiological evidence to support the theory that *pfcrt* is the critical determinant of CQ resistance is strong. Meta-analyses of clinical studies demonstrate that the presence of PfCRT K76T on the day 0 of treatment increases the risk of late treatment failure with CQ by between two- and sevenfold, depending on whether follow-up is 14 or 28 day-post-treatment [60]. However, although mutant *pfcrt* is detected in the majority of treatment failures, the presence of mutant *pfcrt* alone cannot predict treatment outcome—that is to say an adequate clinical and parasitological response (ACPR, see Box 41.1) can be observed even in the presence of mutant *pfcrt*. It is hypothesised that this phenomenon is due to a combination of (1) host immunity, which contributes to parasite clearance, and (2) one or more parasite mechanism(s), including *pfmdr1* (see below) which can moderate the degree of CQ resistance (e.g. 2.7-fold in CQ IC_{50} range reported in one study [61]) in parasites harbouring mutant *pfcrt* [58].

Evidence suggests that *pfcrt* is critical for parasite survival with *pfcrt* knockout experiments so far proving lethal. In addition the fitness of *pfcrt* mutants appears to be reduced. Detailed studies from Malawi have revealed a progressive loss of the mutant allele over a decade since the replacement of CQ with SP as first-line treatment and the effective elimination of CQ usage within that population [62]. Similar trends have been reported in China and in Kenya [63, 64].

2.3 Proposed Functional Roles for PfCRT in CQ Resistance

Although localised to the DV membrane [52], the physiological role of the PfCRT transporter in *P. falciparum* physiology is currently unknown and for this reason the role of PfCRT in CQ resistance mechanisms remains elusive. This deficiency however has not deterred assiduous workers in proposing a variety of putative resistance mechanisms. Three main theories have evolved: the first proposes that PfCRT influences CQ distribution indirectly, by altering ion gradients across the DV membrane such as chloride [39, 65, 66]. The second hypothesis proposes that CQ is effluxed out of the DV by an ATP-dependent primary active transport process [43, 67, 68]. The third hypothesis, known as the "charged drug leak model", proposes that PfCRT facilitates the movement of protonated CQ (CQ^{++}) down its concentration gradient out of the DV [44, 69, 70].

In support of the first hypothesis, studies which have heterologously expressed PfCRT into yeast (*Pichia pastoris* [65]) and *Xenopus oocytes* [71] indicate that PfCRT is able to modulate host transport systems. In the yeast, PfCRT is reported to function in the passive movement of Cl^- [65], whilst in the *Xenopus* system, PfCRT-expressing oocytes exhibit a depolarised resting membrane potential (Ψ_m) and a higher intracellular pH (pH_i), compared to control oocytes [71]. However, the fact that PfCRT "modulates" other transport process is somewhat vague. There is considerable dis-

tinction to be drawn between the scenario whereby PfCRT actively regulates other transporters and whereby it merely acts consequentially on other transport processes by the perturbation of ion (e.g. Ca^{2+}, Cl^{-}, K^{+}, Na^{+}, H^{+}) homeodynamics. A further problem faced by these studies is that due to the high A/T content of *P. falciparum* genes, the coding elements of the *pfcrt* gene had to be reconstructed to allow for protein translation. It is not known therefore how these changes affect the function of the heterologously expressed protein.

Evidence for an energy-dependent CQ transporter as described in the second hypothesis was first proposed by Krogstad and colleagues [67, 68]. It was demonstrated that steady-state accumulation of CQ by CQ-resistant parasites is reduced by adding glucose to the medium. By contrast, adding glucose to suspensions of CQ-sensitive parasites markedly stimulated the accumulation of CQ [68]. The simplest interpretation of these data is that CQ-sensitive parasites have an energy-dependent CQ uptake mechanism (energy is required both to maintain the DV proton gradient and to traffic and digest haemoglobin, releasing FPIX) and that CQ-resistant parasites have an additional energy-dependent CQ efflux mechanism. In addition, a recent study has demonstrated that CQ uptake can be trans-stimulated and that in CQ-resistant parasites this effect is energy dependent [43]. Based on these observations, these authors suggested that an ATP-dependent primary active efflux transporter is responsible for CQ resistance [43]. There are however other explanations for these data and currently this theory is yet to be widely accepted.

The "charged drug leak" hypothesis [44, 69, 70, 72] was initially supported from two independent studies indicating that PfCRT is a member of the drug/metabolite transporter superfamily [73, 74] that may therefore be able to transport CQ directly. Transporters of this class are not directly energised by ATP and transport is often modulated by the transmembrane Ψ_m. The charged drug leak hypothesis provides a potential explanation as to how polymorphisms in *pfcrt* may directly mediate CQ resistance. The critical mutations associated with the development of CQ resistance are located on the food vacuole side and in the membrane (Fig. 41.3). These mutations are associated with a loss of basic and hydrophobic residues. Since CQ is diprotonated at the pH of the food vacuole, the loss of a basic residue at the opening of the channel in mutated PfCRT may allow the positively charged CQ to diffuse through an aqueous pore into the parasite cytoplasm. The release of CQ will be aided by both the protonated CQ (CQ^{++}) concentration and proton gradients across the food vacuole membrane (Fig. 41.4). In addition, it provides a potential explanation for the observed "reversal"

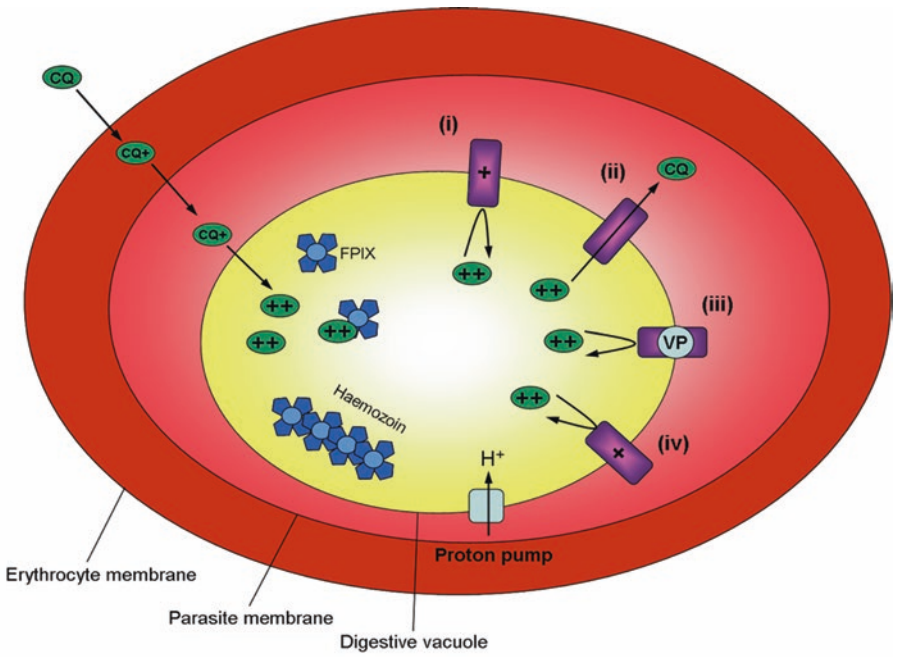


Fig. 41.4 The "charged drug leak" model for CQ resistance. Allelic exchange studies have shown a definite role for PfCRT in CQ resistance. (*i*) In the wild-type state (CQ-sensitive, K76) the positive charge on the K (lysine) residue may prevent the movement of the di-protonated CQ (CQ^{++}) through PfCRT. (*ii*) Replacement of this residue in the CQ-resistant parasites by the K76T mutation (replacement with the neutral residue threonine) might allow the flux of CQ^{++} through PfCRT, thus lowering the concentration of CQ in the digestive vacuole (DV) away from the haem target. (*iii*) Verapamil (VP) may work by reintroducing the positive charge to the barrel of the PfCRT protein, thus preventing the flux of CQ out of PfCRT, resulting in an increased sensitivity to CQ. (*iv*) The selection for the novel S163R mutation potentially mimics the effects of both VP and the normal K (lysine) residue at codon 76 by introducing a positive charge to the barrel of PfCRT, thereby preventing the flux of CQ through PfCRT (reproduced with permission from [44])

of CQ resistance by a wide variety of structurally unrelated compounds whose only common features are hydrophobicity and positive charge [75]. It is predicted that such compounds at high concentrations could sit in the hydrophobic core of the transporter, replace the positive charge and block the leak of charged CQ (e.g. verapamil, Fig. 41.4). In support of this, a study that introduced a novel mutation in PfCRT (S163R), which acts to replace a positive charge inside the barrel of the PfCRT transporter, resulted in returning the parasites to a CQ-sensitive status and abolishing verapamil reversibility while retaining all of the mutations, including K76T and A220S, associated with resistance [44, 70, 72].

Support of a general role for PfCRT in transporting CQ was provided by a further heterologous expression study performed with *Xenopus* oocytes. In this study, *pfcrt* was modified by both codon optimisation and deletion of the putative trafficking motif [76]. Subsequent transport assays showed that CQ transport could only be measured with mutant PfCRT containing the critical K76T mutation [76]. Furthermore, CQ transport in mutated PfCRT could be inhibited by verapamil, quinine, amodiaquine and charged peptides [76]. Plant homologues of PfCRT have been shown to be required for glutathione (GSH) homeostasis [77], and in a recent study using isogenic parasite lines, evidence was provided to support the hypothesis that PfCRT has a dual role in CQR, facilitating both efflux of CQ from the DV and influx of GSH into the DV [78]. It is proposed that this dual function of mutated PfCRT allows elevated levels of GSH in the DV in order to reduce the level of free haem available for CQ binding [78]. This potential additional haem detoxification function would appear to resonate with the broader view that differences in the ability of parasites to detoxify haem could account for the different levels of CQ resistance observed in parasites with different genetic backgrounds [79].

2.4 Pfmdr1 and Resistance Mechanisms to Mefloquine, Amodiaquine, Piperaquine, Lumefantrine Halofantrine and Quinine

It was hypothesised that analogous with mammalian tumour cells exhibiting multidrug resistance (mdr) phenotypes by virtue of the up-regulation of ATP-dependent P-glycoproteins, it was possible that drug-resistant *P. falciparum* lines may also harbour similar multidrug-efflux transporters. Subsequently, two genes showing homology with human *mdr*-type genes were identified and named *pfmdr1* and *pfmdr2* [80, 81]. Further analysis of *pfmdr2* indicated that there was no up-regulation or polymorphisms which correlated with *P. falciparum* drug resistance [81, 82] and in addition it was shown that structurally this gene product differed significantly from mammalian *mdr*-encoded proteins [83]. Polymorphisms in *pfmr1* however were shown to correlate with CQ-resistant parasites [84], although further surveys did not always show such a good correlation [85–87]. Nevertheless, the localisation of the *pfmdr1* gene product, Pgh1 (for P-glycoprotein homologue), in the membrane of the parasite DV [88] suggested an involvement in quinoline and quinoline-related drug resistance.

The polymorphisms found in the *pfmdr1* gene which correlate with drug resistance include N86Y, Y184F, S1034C, N1042D and D1246Y. The mutation N86Y shows an association with CQ resistance; however it is absent from a large number of South American CQ-resistant strains (e.g. [84, 89]). The discrepancies surrounding the involvement of *pfmdr1* in resistance to CQ and related quinolines were eventually resolved in a study by Cowman and colleagues using allelic exchange techniques [90]. Variant *pfmdr1* genes from a drug-resistant line (7G8) carrying the mutations 1034C, 1042D and 1246Y were transfected into a CQ-sensitive *P. falciparum* strain (D10) carrying the wild-type sensitive residues (1034S, 1042N and 1246D). The variant *pfmdr1* genes from the drug-resistant line did not confer resistance to CQ but did confer resistance to quinine [90]. However, removal of the *pfmr1* mutations from the CQ-resistant strain did increase sensitivity to CQ and confer resistance to mefloquine and halofantrine. These data conclusively demonstrated that *pfmdr1* was a genetic determinant for mefloquine, quinine and halofantrine but not for CQ. In order to explain the "CQ modulation" effect of Pgh1, it was proposed that Pgh1 can act in concert with another system (now known to be PfCRT) which confers CQ resistance.

In addition to polymorphisms arising from point mutations, gene amplification of *pfmdr1* has also long been suggested as a possible cause for antimalarial drug resistance [91], and a causal link between halofantrine, mefloquine and quinine resistance was inferred [85, 92]. Subsequently it was shown that gene amplification of *pfmdr1* was correlated to mefloquine resistance in vivo [93]. It was concluded that increased copy number of *pfmdr1* was the most important determinant of mefloquine resistance. Interestingly, single-nucleotide polymorphisms in *pfmdr1* were only associated with increased mefloquine susceptibility in vitro, and not in vivo. An increase in *pfmdr1* copy number has also been associated with increase in treatment failure with artesunate-mefloquine (AS-MQ) and artemether-lumefantrine (AL) [94]. In Southeast Asia, the presence of the N86Y is considered as a negative marker for gene amplification [12].

Evidence that CQ and amodiaquine result in cross-resistance, both in vitro and in vivo, has been demonstrated and linked to mutations in both *pfcrt* mutations and *pfmdr1* [13], with *pfcrt* mutations in codons 72–76 observed in South America linked to greater resistance to amodiaquine compared to Southeast Asian or African isolates [13]. In terms of

Pfmdr1, mutations N86Y and N1042D are reported as being most linked to amodiaquine resistance [13].

In a recent meta-analysis, the relationship between *pfcrt* and *pfmdr1* was assessed from clinical trial data based on therapeutic responses to artesunate-amodiaquine (AS-AQ) and AL [95]. Individual patient data from 31 clinical trials, which included more than 7000 patients, were analysed to assess relationships between parasite polymorphisms in *pfcrt* and *pfmdr1* and clinically relevant outcomes after treatment with AL or AS-AQ. Mutation N86Y in *pfmdr1* and increased *pfmdr1* copy number were significant risk factors for treatment failure in patients treated with AL. However, as shown in previous in vitro allelic exchange studies [90], mutations in *pfcrt* and *pfmdr1* exert opposing selective effects and this was observed clinically for AL compared to ASAQ [95].

In 1978, piperaquine was introduced as a first-line monotherapy in China due to the high prevalence of CQ resistance [96]. In the 1980s emergence of piperaquine resistance reduced its use and it was later re-introduced as a combination therapy known as CV4 (China-Vietnam 4), consisting of dihydroartemisinin (DHA), trimethoprim, piperaquine and primaquine [96]. Piperaquine has subsequently been reformulated again as DHA-piperaquine (DHA-PPQ) developed by MMV as Eurartesim®.

Piperaquine resistance was first reported in China, where patient isolates were recovered with increased parasite in vitro IC_{50} phenotypes [97, 98]. The mechanism by which resistance is mediated, however, remains unknown. Using comparative whole-genome hybridization analyses, copy number variation in a region (825,600–888,300) on chromosome 5 has been associated with piperaquine resistance [99], although hitherto individual genes have not been identified. In addition, in a clinical trial held in Burkina Faso, significant selection for PfCRT K76T was observed following treatment with AL and DHA-PPQ, as well as selection of *pfmdr1*-N86Y after AL but not DHA-PPQ treatment, suggesting reverse selection of the *pfcrt* gene by PPQ [100]. However in a recent in vitro study of 280 *P. falciparum* isolates piperaquine susceptibility was not associated with *pfcrt* [101].

Quinine remains effective against *P. falciparum* but decreasing efficacy has been reported in the many malaria-endemic areas [102–104]. It is assumed that quinine resistance shares some of the mechanisms associated with CQ and mefloquine resistance. As described above, it was shown that polymorphisms in *pfmdr1* increase resistance to quinine [90], and in addition mutations in PfCRT and in particular K76T also confer a quinine-resistant phenotype [105]. Interestingly, it was observed that the K76I mutation greatly increased sensitivity to quinine but reduced sensitivity to its enantiomer quinidine, indicative of a unique stereo-specific response not observed in other CQ-resistant lines [105]. In an experiment whereby genetically crossed *P. falciparum* lines were analysed using quantitative trait loci (QTL) associated with quinine resistance, three main loci were identified on chromosomes 5, 7 and 13 [61]. The mapped segments on chromosomes 5 and 7 are consistent with the involvement of *pfmdr1* and *pfcrt*, respectively; however the chromosome 13 segment implies the involvement of another novel genetic determinant. Several candidate genes have been analysed and some correlation has been demonstrated between quinine resistance and polymorphisms in *pfnhe-1*, a putative Na^+/H^+ exchanger. However in a global analysis of *pfnhe-1* polymorphisms to determine its usefulness as a marker for quinine resistance, it was concluded that there were marked geographic disparities and that candidate polymorphisms in the *pfnhe-1* gene, as molecular markers for quinine resistance, appear limited [106].

3 Resistance Mechanisms to the Antifolates

Folate is an essential vitamin which cannot be synthesised by humans. The *de novo* folate synthetic pathway is however present in the malaria parasite and for this reason it has been a most attractive drug target for decades (Fig. 41.5). In particular, two enzymes in the *P. falciparum* folate biosynthetic pathway have been targeted for antimalarial chemotherapy, the first is dihydropteroate synthase (DHPS) and the second is dihydrofolate reductase (DHFR). DHPS is not found in mammalian cells and in *P. falciparum* it is the C-terminal domain of a bifunctional protein combined with 7,8-dihydro-6-hydroxy-methylpterin pyrophosphokinase (or pfPPPK-DHPS [107, 108]). The DHFR in *P. falciparum* is quite unlike that of mammalian cells and more akin to that found in other protozoa and plants in that it is only one domain of a bifunctional enzyme that also contains thymidylate synthase (or TS [109]). DHPS is susceptible to sulphonamides such as sulphadoxine (SD) and dapsone (DDS) whilst DHFR is susceptible to antimalarials such as pyrimethamine (PYR) and biguanides such as proguanil (PG) and chlorproguanil (CPG) through their cyclic metabolites.

Combinations of DHPS and DHFR inhibitors act synergistically [110], a fact that has been successfully exploited clinically by combining PYR and SD in the drug Fansidar™ (or SP) and more recently by combining CPG with DDS, known as LapDap™ [111–113]. Inhibition of these enzymes leads to a depletion of parasite intracellular folates which further interferes with methionine and pyrimidine biosynthesis eventually leading to parasite death (Fig. 41.5).

As a result of growing resistance to chloroquine (CQ) treatment, SP became a first-line drug of choice for the treatment of uncomplicated malaria in many countries with an existing burden of CQ resistance. However, resistance to SP grew and spread very quickly (Table 41.1), especially in Southeast Asia, South America [3, 6] and subsequently Africa [7].

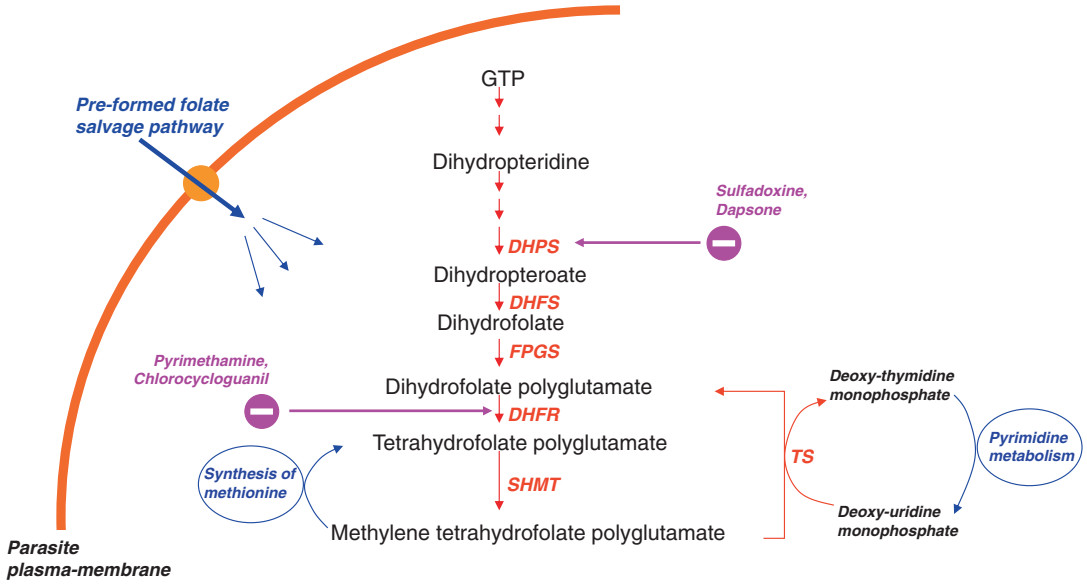


Fig. 41.5 The de novo folate synthetic pathway of *P. falciparum*. Enzymes: DHPS (dihydropteroate synthase), DHFS (dihydrofolate synthase), FPGS (folyl polyglutamate synthase), DHFR (dihydrofolate reductase), SHMT (serine hydroxymethyltransferase), TS (thymidylate synthase). DHPS is susceptible to sulphonamides such as sulphadoxine (SD) and dapsone (DDS) whilst DHFR is susceptible to pyrimethamine (PYR) and the biguanides proguanil (PG) and chloroproguanil (CPG)

3.1 Resistance Caused by Mutations in DHPS and DHFR

A number of mechanisms have been evoked to explain antifolate resistance in *P. falciparum*, with the principal mechanisms involving point mutations in DHPS and DHFR. Point mutation in the DHPS domain of the *pppk-dhps* gene confers resistance to sulphadoxine and dapsone [7, 107, 114–119] whilst point mutations in the DHFR domain of the *dhfr-ts* gene confer resistance to pyrimethamine and the cyclic active metabolites of the biguanides [120–123].

Variations in five amino acids within *P. falciparum dhps* have been shown in the laboratory and in the field to be associated with increased IC_{50} values for sulphadoxine and a number of other related sulphonamides and sulphones including dapsone. Amongst these, the A437G mutation has been shown by transfection experiments to be the initial mutation, causing a fivefold increase in IC_{50}. Higher IC_{50} levels were associated with the additional mutations S436F/A, K540E, A581G and A613S [116]. Mutations affecting positions 436, 437 and 540 can each occur singly; however the A581G variation is always associated with A437G and similarly the A613S/T alteration is always coupled with changes in either residue 436 or 437 [116], apparently reflecting steric constraints of the enzyme. All of the five polymorphic residues are believed to form a part of a solvent-accessible channel connecting the catalytic centre [116, 124]; however detailed structural knowledge remains elusive until the DHPS can be fully crystallised.

Studies on *P. chabaudi* performed over 30 years ago first suggested that alterations in the DHFR enzyme led to reduced pyrimethamine binding and consequently resistance [125]. The *dhfr* gene point mutations that confer resistance to pyrimethamine were subsequently described [120, 121, 126] with conclusive proof linking the point mutations and pyrimethamine resistance provided by transfection studies [122]. Compared with the wild-type *dhfr*, S108N increases resistance to pyrimethamine by about 100-fold [120, 121]. Succeeding mutations N51I, C59R and I164L progressively increase resistance to pyrimethamine up to a further order of magnitude to about 1000-fold compared to the wild type [7]. It has been observed in the field that the mutations N51I, C59R and I164L do not occur alone in the absence of the S108N mutation. Structural studies on the crystallised *P. falciparum* DHFR-TS [127] have shown that the mutations occur in the active site of the DHFR domain. These *dhfr* mutations also confer resistance to the active cyclic metabolites of proguanil (PG) and chlorproguanil (CPG), namely cycloguanil and chlorcycloguanil, respectively.

As described the high prevalence (>50 %) of the DHPS K540E mutation, which is found almost exclusively as the quintuple mutant haplotype (DHFR; N51I, C59R, S108N and DHPS; A437G, K540E), results in SD-PYR treatment failures in non-pregnant adults, and children, and when used as intermittent preventive treatment in infants (IPTi) [128, 129]. However, it is reported that SD-PYR may retain value and efficacy for use in intermittent preventive treatment in pregnancy (IPTp) [130, 131]. The reason for the ability of

SD-PYR to clear resistant parasite infection during IPTp remains unclear but it is reasoned to be due to a significant contribution by partial host immunity, as seen in many African settings [131, 132].

3.2 Further Putative Antifolate Resistance Mechanisms

Studies in *P. falciparum* have shown that the addition of folic acid or folate derivatives decreases the activity of antifolate drugs both in vitro and in vivo [133, 134]. Similarly, lowering the folate concentration enhances the in vitro activity of antifolate drugs [135]. Taken together these observations indicate the presence of a folate salvage phenotype in *P. falciparum* (Fig. 41.5). Additionally it was shown that a range of antifolates could be potentiated by probenecid (believed to be an inhibitor of the salvage pathway) both in vitro and in vivo at therapeutically relevant concentrations [136, 137] highlighting the potential of the folate salvage pathway as an auxiliary drug target. Two parasite transporters, PfFT1 and PfFT2, have recently been identified and characterised as being responsible in folate salvage [138].

Several studies have also looked at whether *P. falciparum* is able to increase expression of target proteins (DHPS and DHFR) in order to "dilute" the effect of the antifolates. Increased expression can occur by either up-regulation at the transcriptional level and/or the translational level. No evidence of a drug-induced increase of expression at the transcriptional level has been observed [139]; however translational up-regulation of the DHFR-TS is reported to be induced by antifolates and inhibitors of TS [140]. It is not clear at this stage, however, how much these observed changes in translation contribute to antifolate resistance and whether they have any clinical relevance.

4 Resistance Mechanisms to Naphthoquinones

Atovaquone is a naphthoquinone developed to selectively compete for ubiquinone (CoQ) in the mitochondrial electron transport chain of *P. falciparum* (Fig. 41.1, [141]). Atovaquone which is 1000-fold more active against parasite compared with mammalian mitochondria [142] specifically acts by binding to the CoQ oxidation site in the cytochrome bc_1 complex [143, 144] (Fig. 41.6).

During the intra-erythrocytic stage of infection, a key role of the parasite mitochondrion is to provide orotate for pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase (DHODH). Consistent with this, inhibition of the bc_1 complex by atovaquone affects the concentrations of metabolites in the pyrimidine biosynthetic pathway [145, 146]. In support of the biochemical evidence, transgenic

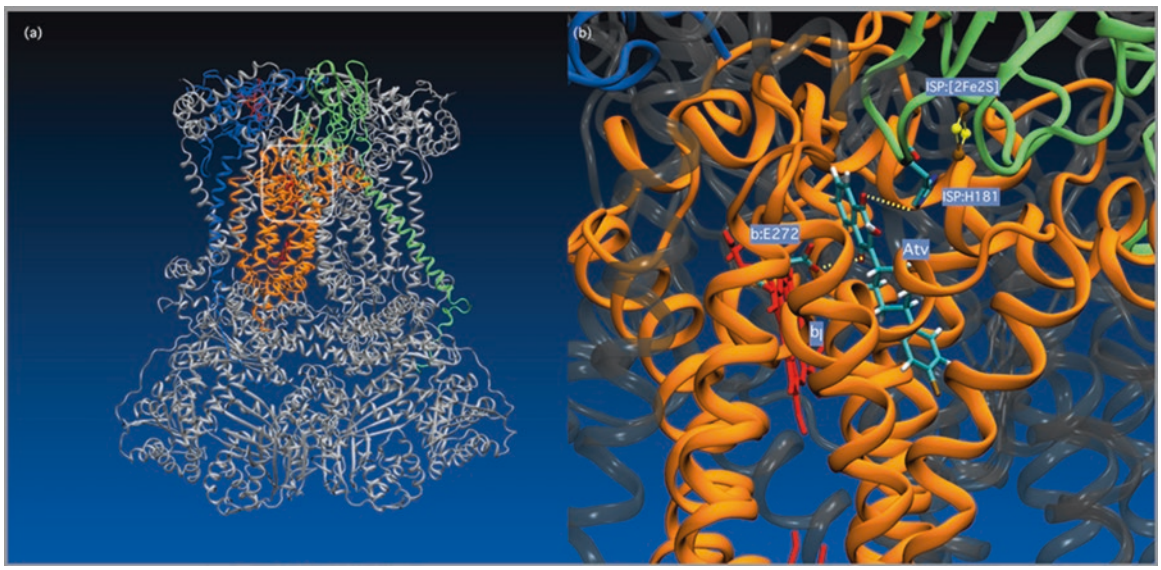


Fig. 41.6 (**a**) Representation of the yeast cytochrome bc_1 complex (3CX5.PDB), with atovaquone modelled at the Q_o site (*boxed area*) [215]. The bc_1 complex is a structural and functional homodimer with a molecular mass of approximately 480 kDa, consisting of ten discrete subunits per monomer in yeast and *P. falciparum*.28 The electron-transferring catalytic unit of one monomer is highlighted; cytochrome *b* is represented in *orange*, cytochrome c_1 in *blue* and the Rieske iron-sulphur protein (ISP) in *green*. Haem groups (cyt *b* and cyt c_1) are shown in *red*. The remaining subunits of the complex are rendered in *grey*. (**b**) Molecular model of atovaquone (ATO) bound to the Q_o site of the bc_1 complex. Subunits are coloured as in (**a**). Atovaquone was modelled into the Q_o site of cytochrome *b* as described by Fisher et al. [165]. Hydrogen-bonding interactions between the naphthoquinone headgroup of atovaquone and side chains of Glu-272 (cyt b) and His-181 (ISP) are indicated by *yellow lines*. The positions of haem bl (cyt b) and the ISP [2Fe2S] cluster are also shown

P. falciparum parasites expressing ubiquinone-independent yeast DHODH were shown to display an atovaquone-resistant phenotype [147]. In addition, a recent study suggests that a further cellular consequence of mitochondrial inhibition by atovaquone is the inhibition of purine biosynthesis [148]. Blood-stage parasite death as a result of atovaquone is relatively slow compared to other antimalarials such as artemisinin and chloroquine [149–151]. This feature appears to be consistent with other mitochondrial acting antimalarials and is possibly due to the drug acting only on late trophozoites and not on the earlier "ring" stages [149]. Atovaquone is however active against liver stages, resulting in its utility as a prophylaxis drug, although it is not believed to be active against "dormant" *Plasmodium vivax* hypnozoites [152, 153].

When used as a single agent, resistance to atovaquone was quickly observed both in vitro [154, 155] and in mice models [156]. Initial clinical trials demonstrated a 30 % treatment failure within 28 days of treatment [157, 158]. Significant synergy in antimalarial activity was achieved when atovaquone was combined with the biguanide proguanil (Malarone™). This synergistic effect is unrelated to the inhibition of folate metabolism or the cyclic metabolite of proguanil, cycloguanil. The combination was shown to be successful in significantly reducing the number of treatment failures [159, 160]. Nevertheless, atovaquone-resistant parasites are equally resistant to atovaquone/proguanil combinations [161]. In species of plasmodium, resistance to atovaquone is associated with missense mutations around the Q_o (CoQ oxidation site) region of the cytochrome bc_1 gene, especially near the highly conserved PEWY sequence [143, 156, 162, 163]. Atovaquone-resistant *P. falciparum* lines, generated in the laboratory, were polymorphic at codons 133, 272 and 280 [162]. Whilst in vivo, the first cases of Malarone-treatment failure were associated with mutations at codon 268, namely Y268N [163] and Y268S [164]. Position 268 in cytochrome *b* is highly conserved across all phyla and is located within the "ef" helix component of the Q_o site which is putatively involved in ubiquinol binding. The resultant atovaquone-resistant growth IC_{50} phenotype of these mutants is some 1000-fold higher than susceptible strains; however this is accompanied by a ~40 % reduction in the V_{max} of the bc_1 complex, suggestive of a significant fitness cost to the parasite [165].

The described mutations at codon 268 are considered useful tools for the surveillance Malarone resistance [166, 167]; however there is evidence in the field of Malarone resistance in the absence of the 268 mutation [168]. Furthermore, it has been reported that an in vitro atovaquone-resistant parasite line has been generated in the laboratory possessing wild-type cyt *b* [169]. The mechanism underpinning the parasite's atovaquone-resistant phenotype in this strain remains to be elucidated.

As described, de novo resistance to atovaquone occurs very rapidly in vitro and in vivo [157, 158]. The reason for this phenomenon in not known but may include PK/PD considerations [141] related to the physicochemical properties of atovaquone combined with a slow rate of sterilisation, as well as genetic considerations, related to the increased mutation rate of mitochondrially encoded genes such as cytochrome *b* compared to nuclear encoded genes [141, 170].

5 Resistance Mechanisms to Artemisinin

Artemisinins possess potent antimalarial activity and the World Health Organisation (WHO) recommends their use in artemisinin-based combination therapy (ACT) for first-line therapy of *Plasmodium falciparum* malaria worldwide [12]. Artemisinin and its derivatives (artesunate (AS), artemether (A), arteether (AE) and dihydroartemisinin (DHA)) represent a very unique class of antimalarial compounds (Fig. 41.1) developed from an ancient Chinese herbal remedy from the sweet wormwood *Artemisia annua* or "qinghao". Artemisinins are endoperoxides (containing a peroxide bridge, C–O–O–C, Fig. 41.1) and this feature is believed to be the key to their mode of action [171], although the mechanism(s) of activation and subsequent biological target(s) of endoperoxides continue to be debated [172]. Endoperoxide bioactivation cleavage generates short-lived cytotoxic oxy-radicals in the presence of haem iron or free iron Fe^{2+} [173, 174] that have a lethal effect on the parasite.

Three models of bioactivation are proposed; in the first model known as the "reductive scission" model, evidence has been submitted to support the generation of oxygen-centred radicals that are rearranged to more stable carbon-centred radicals [175, 176] by ferrous iron binding to either O1 or O2. This generates oxy-radical intermediates which subsequently rearrange to primary or secondary carbon-centred radicals via either β-scission or a [1,5]-H shift. In support of this hypothesis, evidence for the formation of these carbon-centred radical intermediates has been provided using electron paramagnetic resonance (EPR) spin-trapping techniques [177–179]. It has been proposed that these C-centred radicals are capable of haem and/or protein alkylation.

In the second model, it is hypothesised that iron acts as a Lewis acid to facilitate ionic activation of antimalarial trioxanes generating downstream reactive oxygen species [180, 181]. The ring opening involves heterolytic cleavage of the endoperoxide bridge followed by interaction with water generating an open unsaturated hydroperoxide, capable of direct oxidation of protein residues. Fenton degradation of the oxygen-centred radical intermediate can provide hydroxyl

radicals (HO•) highly reactive against amino acids, lipids or nucleic acids.

A third model, known as the "cofactor model", proposes that bioactivation of endoperoxides occurs via redox-active flavoenzymes, resulting in the perturbation of redox homeostasis coupled with the generation of ROS [182]. Bioactivation by electron transport chain (ETC) components has been previously suggested [183, 184], but the cofactor model of artemisinin activation is not restricted to mitochondrial flavoenzymes but rather implicates cytosolic flavoenzymes and also rejects the direct requirement for either Fe^{2+} and/or non-haem iron activation.

For the first two models, the origin of the iron available for bioactivation is also a point of debate and experimental evidence has been presented for both haem and non-haem iron bioactivation [185, 186] (see [172] for in-depth review).

Once activated, endoperoxide antimalarials have been reported to disrupt a number of parasite functions and enzymes, including the haem detoxification pathway [187], the translationally controlled tumour protein (*Pf*TCTP) [188], the sarco/endoplasmic reticulum membrane calcium *Pf*ATPase6 [189] and the parasite mitochondrion [183, 184, 190–195].

Irrespective of the mode of bioactivation and the biological target(s) for the cytotoxic oxy-radicals, a recent study demonstrated that a primary physiological event leading to rapid parasite death is the depolarisation of both parasite plasma and mitochondrial membrane potential ($\Delta\Psi_p$ and $\Delta\Psi_m$) via an iron-mediated generation of reactive oxygen species (ROS) [196]; however it was not discernable from this study whether iron plays a role in endoperoxide bioactivation and/or iron-mediated oxidative stress.

As described in Section 1, since 2001, ACTs have been the WHO-recommended first-line drugs for the treatment of uncomplicated malaria [12]. Treatment failures have been recorded to ACTs in Southeast Asia, and although treatment failures only occur where resistance to the partner drug exists [17], the delayed parasite clearance phenotype [15, 16], attributed to artemisinin "resistance" (but not resistance as defined by the WHO, see Box 41.1), has given rise to concern about the therapeutic lifespan of the first-generation artemisinins.

In 2006, the first signs of parasite "resistance" to artemisinin were observed in Southeast Asia, in the border region between Thailand and Cambodia [15, 16]. Parasites from this region were observed to have a delayed parasite clearance phenotype following either artesunate monotherapy or an ACT. The delayed parasite clearance phenotype is not resistance as defined by the WHO (see Box 41.1) and does not necessarily lead to treatment failure [17]. Treatment failure in the Greater Mekong Subregion following treatment with an ACT has only been observed where resistance to the partner drug exists regardless of the presence of artemisinin resistance [17].

The reduced parasite clearance rate can also be expressed as an increased parasite clearance half-life [197, 198] or more practically by the microscopic detection of parasites on day 3 of ACT use [12]. However, the traditional 48-h inhibition of proliferation assay (IC_{50}) cannot discern any difference in artemisinin/DHA sensitivity in parasites displaying the clinical slow clearance phenotype compared to wild-type parasites [199]. This lack of in vitro phenotype proved to be a bottleneck to understanding the molecular mechanisms underpinning the clinical findings. A breakthrough came with the development of a modified growth inhibition assay, known as the ring-stage survival assay ($RSA_{0–3\ h}$), which measures the survival rate of early ring-staged parasites to exposure to pharmacologically relevant DHA (700 nM for 6 h), which was able to correlate increased $RSA_{0–3\ h}$ values to parasites displaying increased clinical parasite clearance half-lives [200, 201]. The $RSA_{0–3\ h}$ was used to monitor parasites in vitro following a 5-year-long regimen of artemisinin pressure. Whole-genome sequencing was used to analyse the temporal acquisition of mutations associated with increased $RSA_{0–3\ h}$ survival rates, culminating in the identification of mutations in PF3D7_1343700 kelch propeller domain (K13-propeller) as a molecular marker(s) for artemisinin "resistance" [202]. The K13-propeller gene is located on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates [203–205]. A genomic analysis of Cambodian isolates further identified four prevalent K13-propeller mutations, Y493H, R539T, I543T and C580Y, that were associated with increased parasite clearance half-lives (>5 h) and increased $RSA_{0–3\ h}$ survival [202, 206]. Definitive evidence for a role of the K13-propeller mutations in artemisinin "resistance" was provided by a subsequent zinc-finger nuclease-based transfection study whereby K13 mutations were introduced into wild-type strains resulting in increased $RSA_{0–3\ h}$ survival rates and conversely Cambodian isolates with mutant K13-propeller, when reverted to wild type, resulted in decreased $RSA_{0–3\ h}$ survival rates [207]. However in the same study, introduction of one of the key mutations, C580Y, into parasites with different genetic backgrounds resulted in marked differences in the degree of resistance to DHA as measured by $RSA_{0–3\ h}$ [207]. It was observed that survival rates conferred greater levels of resistance in three Cambodian isolates as compared with Dd2 and FCB parasites [207], strongly indicating that additional parasite features are involved in mediating DHA resistance.

As to the role of the K13-propeller, this is yet to be determined, but in a recent study, biochemical and cellular evidence is presented that indicates that artemisinins are inhibitors of the malaria parasite phosphatidylinositol-3-kinase (PfPI3K, [208]). In addition, increased PfPI3K was associated with the K13-propeller C580Y mutation. Evidence is presented to support the hypothesis that the K13-

propeller C580Y mutation reduces polyubiquitination of PfPI3K, thereby limiting proteolysis resulting in increased levels of the kinase, as well as its lipid product phosphatidylinositol-3-phosphate (PI3P). The authors hypothesise that PI3P must then play a pivotal role in artemisinin resistance by influencing one or more cellular mechanisms such as host cell remodelling, organelle functions (apicoplast and digestive food vacuole) and/or redox, transcriptional and DNA repair pathways. This is the first study to link the K13-propeller to a cellular function and it will be of great interest to see if future studies are able to corroborate and expand these findings.

As described, the artemisinin resistance mechanism appears to be restricted to the ring stage of asexual development with artemisinin-induced dormancy in ring stages observed in both sensitive and resistant parasites [209, 210]. The trophozoite stages have been shown to be more susceptible to artemisinin exposure [211], and the ability of ring stages to arrest development following drug exposure, and then continue development once the drug has been eliminated, appears to be central to the parasites' in vivo resistance mechanism(s). In support of this, a recent population transcriptomics study identified up-regulation of genes involved in extended phase of ring-stage development in artemisinin-resistant isolates as well as genes involved in unfolded protein response (UPR) [212].

In summary, the current hypotheses regarding the mechanisms of parasite artemisinin resistance appear to reflect the PK/PD features of the drug. Artemisinins have a short therapeutic half-life and induce death via mechanisms leading to lipid peroxidation [196]. Malaria parasites counter this by shortening the period of trophozoite development and by up-regulation and scaling up (by hitherto unknown pathways/processes) of antioxidant defence/repair mechanisms.

6 Conclusion

Here we have attempted to summarise the principal mechanisms of antimalarial drug resistance for the major groups of drugs currently deployed in malaria-endemic countries. The protozoan parasite has been shown to deploy an array of mechanisms of escapism including (1) reducing the concentration of intracellular drug concentration by the action of altered or increased transport away from target sites, (2) altering the target site to reduce drug binding and (3) alteration of ring-stage development and up-regulation/scale-up of antioxidant/repair mechanisms. Our understanding of these mechanisms has been radically improved by pioneering genetic and biochemical advances notwithstanding the completion of the malaria genome project. Understanding the mechanisms underpinning drug resistance remains paramount for the development of effective global malaria chemotherapy; however there are many other factors which can reduce the efficacy of antimalarial drugs. These include drug pharmacodynamics, host immunity, malaria transmission and drug effectiveness (e.g. compliance). It may transpire that only a holistic approach will win the race against antimalarial drug resistance and pacify this terrible disease affecting poor countries.

Acknowledgements GAB and SAW acknowledge the support of the Wellcome Trust and the MRC.

References

1. WHO. World Malaria Report 2014. World Health Organisation; 2014.
2. Snow RW, Trape JF, Marsh K. The past, present and future of childhood malaria mortality in Africa. Trends Parasitol. 2001;17(12):593–7.
3. Wongsrichanalai C, et al. Epidemiology of drug-resistant malaria. Lancet Infect Dis. 2002;2(4):209–18.
4. Wootton JC, et al. Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. Nature. 2002;418(6895):320–3.
5. Chen N, et al. pfcrt Allelic types with two novel amino acid mutations in chloroquine-resistant Plasmodium falciparum isolates from the Philippines. Antimicrob Agents Chemother. 2003;47(11):3500–5.
6. White NJ. Antimalarial drug resistance: the pace quickens. J Antimicrob Chemother. 1992;30(5):571–85.
7. Sibley CH, et al. Pyrimethamine-sulfadoxine resistance in Plasmodium falciparum: what next? Trends Parasitol. 2001;17(12):582–8.
8. Glew RH, et al. Multidrug-resistant strain of Plasmodium falciparum from eastern Colombia. J Infect Dis. 1974;129(4):385–90.
9. Hall AP, et al. Amodiaquine resistant falciparum malaria in Thailand. Am J Trop Med Hyg. 1975;24(4):575–80.
10. Campbell CC, et al. Evaluation of amodiaquine treatment of chloroquine-resistant Plasmodium falciparum malaria on Zanzibar, 1982. Am J Trop Med Hyg. 1983;32(6):1216–20.
11. Childs GE, et al. A comparison of the in vitro activities of amodiaquine and desethylamodiaquine against isolates of Plasmodium falciparum. Am J Trop Med Hyg. 1989;40(1):7–11.
12. WHO. Global report on antimalarial drug efficacy and drug resistance: 2000–2010. 2010.
13. Sa JM, et al. Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proc Natl Acad Sci U S A. 2009;106(45):18883–9.
14. Fontanet AL, et al. High prevalence of mefloquine-resistant falciparum malaria in eastern Thailand. Bull World Health Organ. 1993;71(3–4):377–83.
15. Noedl H, et al. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med. 2008;359(24):2619–20.
16. Dondorp AM, et al. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med. 2009;361(5):455–67.
17. WHO. Status report on artemisinin resistance. 2014.
18. Perrin D. Dissociation constants of organic bases in aqueous solution. London: Butterworth; 1965.
19. Yayon A, Cabantchik ZI, Ginsburg H. Susceptibility of human malaria parasites to chloroquine is pH dependent. Proc Natl Acad Sci U S A. 1985;82(9):2784–8.
20. Krogstad DJ, Schlesinger PH, Gluzman IY. Antimalarials increase vesicle pH in Plasmodium falciparum. J Cell Biol. 1985;101(6):2302–9.

21. Bennett TN, et al. Drug resistance-associated pfCRT mutations confer decreased Plasmodium falciparum digestive vacuolar pH. Mol Biochem Parasitol. 2004;133(1):99–114.
22. Yayon A, Cabantchik ZI, Ginsburg H. Identification of the acidic compartment of Plasmodium falciparum-infected human erythrocytes as the target of the antimalarial drug chloroquine. EMBO J. 1984;3(11):2695–700.
23. Fitch CD, Kanjananggulpan P. The state of ferriprotoporphyrin IX in malaria pigment. J Biol Chem. 1987;262(32):15552–5.
24. Egan TJ, et al. Fate of haem iron in the malaria parasite Plasmodium falciparum. Biochem J. 2002;365(Pt 2):343–7.
25. Slater AF, Cerami A. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites [see comments]. Nature. 1992;355(6356):167–9.
26. Sullivan Jr DJ, et al. On the molecular mechanism of chloroquine's antimalarial action. Proc Natl Acad Sci U S A. 1996;93(21):11865–70.
27. Bray PG, et al. Access to hematin: the basis of chloroquine resistance. Mol Pharmacol. 1998;54(1):170–9.
28. Bray PG, et al. Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in Plasmodium falciparum. J Cell Biol. 1999;145(2):363–76.
29. Macomber PB, O'Brien RL, Hahn FE. Chloroquine: physiological basis of drug resistance in Plasmodium berghei. Science. 1966;152(727):1374–5.
30. Fitch CD. Plasmodium falciparum in owl monkeys: drug resistance and chloroquine binding capacity. Science. 1970;169(942):289–90.
31. Diribe CO, Warhurst DC. A study of the uptake of chloroquine in malaria-infected erythrocytes. High and low affinity uptake and the influence of glucose and its analogues. Biochem Pharmacol. 1985;34(17):3019–27.
32. Verdier F, et al. Chloroquine uptake by Plasmodium falciparum-infected human erythrocytes during in vitro culture and its relationship to chloroquine resistance. Antimicrob Agents Chemother. 1985;27(4):561–4.
33. Geary TG, Jensen JB, Ginsburg H. Uptake of [3H]chloroquine by drug-sensitive and -resistant strains of the human malaria parasite Plasmodium falciparum. Biochem Pharmacol. 1986;35(21):3805–12.
34. Gluzman IY, Schlesinger PH, Krogstad DJ. Inoculum effect with chloroquine and Plasmodium falciparum. Antimicrob Agents Chemother. 1987;31(1):32–6.
35. Kirk K, Saliba KJ. Chloroquine resistance and the pH of the malaria parasite's digestive vacuole. Drug Resist Updat. 2001;4(6):335–7.
36. Spiller DG, et al. The pH of the Plasmodium falciparum digestive vacuole: holy grail or dead-end trail? Trends Parasitol. 2002;18(10):441–4.
37. Bray PG, et al. Distribution of acridine orange fluorescence in Plasmodium falciparum-infected erythrocytes and its implications for the evaluation of digestive vacuole pH. Mol Biochem Parasitol. 2002;119(2):301–4. discussion 307–9, 311–3.
38. Ursos LM, Dzekunov SM, Roepe PD. The effects of chloroquine and verapamil on digestive vacuolar pH of P. falciparum either sensitive or resistant to chloroquine. Mol Biochem Parasitol. 2000;110(1):125–34.
39. Dzekunov SM, Ursos LM, Roepe PD. Digestive vacuolar pH of intact intraerythrocytic P. falciparum either sensitive or resistant to chloroquine. Mol Biochem Parasitol. 2000;110(1):107–24.
40. Wissing F, et al. Illumination of the malaria parasite Plasmodium falciparum alters intracellular pH. Implications for live cell imaging. J Biol Chem. 2002;277(40):37747–55.
41. Ursos LM, Roepe PD. Chloroquine resistance in the malarial parasite, Plasmodium falciparum. Med Res Rev. 2002;22(5):465–91.
42. Zhang J, Krugliak M, Ginsburg H. The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. Mol Biochem Parasitol. 1999;99(1):129–41.
43. Sanchez CP, Stein W, Lanzer M. Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in Plasmodium falciparum. Biochemistry. 2003;42(31):9383–94.
44. Johnson DJ, et al. Evidence for a central role for PfCRT in conferring Plasmodium falciparum resistance to diverse antimalarial agents. Mol Cell. 2004;15(6):867–77.
45. Sanchez CP, Wunsch S, Lanzer M. Identification of a chloroquine importer in Plasmodium falciparum. Differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. J Biol Chem. 1997;272(5):2652–8.
46. Wellems TE, et al. Chloroquine resistance not linked to mdr-like genes in a Plasmodium falciparum cross [see comments]. Nature. 1990;345(6272):253–5.
47. Wellems TE, Walker-Jonah A, Panton LJ. Genetic mapping of the chloroquine-resistance locus on Plasmodium falciparum chromosome 7. Proc Natl Acad Sci U S A. 1991;88(8):3382–6.
48. Walker-Jonah A, et al. An RFLP map of the Plasmodium falciparum genome, recombination rates and favored linkage groups in a genetic cross. Mol Biochem Parasitol. 1992;51(2):313–20.
49. Su X, et al. A genetic map and recombination parameters of the human malaria parasite plasmodium falciparum [In Process Citation]. Science. 1999;286(5443):1351–3.
50. Su X, et al. Complex polymorphisms in an approximately kDa protein are linked to chloroquine-resistant P. falciparum in Southeast Asia and Africa. Cell. 1997;91(5):593–603.
51. Fidock DA, et al. Allelic modifications of the cg2 and cg1 genes do not alter the chloroquine response of drug-resistant Plasmodium falciparum. Mol Biochem Parasitol. 2000;110(1):1–10.
52. Fidock DA, et al. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell. 2000;6(4):861–71.
53. Basco LK, Ringwald P. Analysis of the key pfcrt point mutation and in vitro and in vivo response to chloroquine in Yaounde, Cameroon. J Infect Dis. 2001;183(12):1828–31.
54. Chen N, et al. Sequence polymorphisms in pfcrt are strongly associated with chloroquine resistance in Plasmodium falciparum. J Infect Dis. 2001;183(10):1543–5.
55. Mehlotra RK, et al. Evolution of a unique Plasmodium falciparum chloroquine-resistance phenotype in association with pfcrt polymorphism in Papua New Guinea and South America. Proc Natl Acad Sci U S A. 2001;98(22):12689–94.
56. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science. 2002;298(5591):210–3.
57. Kuhn Y, et al. Trafficking of the phosphoprotein PfCRT to the digestive vacuolar membrane in Plasmodium falciparum. Traffic. 2010;11(2):236–49.
58. Ecker A, et al. PfCRT and its role in antimalarial drug resistance. Trends Parasitol. 2012;28(11):504–14.
59. Nomura T, et al. Evidence for different mechanisms of chloroquine resistance in 2 Plasmodium species that cause human malaria. J Infect Dis. 2001;183(11):1653–61.
60. Picot S, et al. A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. Malar J. 2009;8:89.
61. Ferdig MT, et al. Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol. 2004;52(4):985–97.
62. Kublin JG, et al. Reemergence of chloroquine-sensitive Plasmodium falciparum malaria after cessation of chloroquine use in Malawi. J Infect Dis. 2003;187(12):1870–5.
63. Wang X, et al. Decreased prevalence of the Plasmodium falciparum chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against P. falciparum malaria in

Hainan, People's Republic of China. Am J Trop Med Hyg. 2005;72(4):410–4.
64. Mwai L, et al. Chloroquine resistance before and after its withdrawal in Kenya. Malar J. 2009;8:106.
65. Zhang H, Howard EM, Roepe PD. Analysis of the antimalarial drug resistance protein Pfcrt expressed in yeast. J Biol Chem. 2002;277(51):49767–75.
66. Warhurst DC, Craig JC, Adagu IS. Lysosomes and drug resistance in malaria. Lancet. 2002;360(9345):1527–9.
67. Krogstad DJ, et al. Efflux of chloroquine from Plasmodium falciparum: mechanism of chloroquine resistance. Science. 1987;238(4831):1283–5.
68. Krogstad DJ, et al. Energy dependence of chloroquine accumulation and chloroquine efflux in Plasmodium falciparum. Biochem Pharmacol. 1992;43(1):57–62.
69. Ward S. Drug resistance mechanisms in malaria. Pharmacologist. 2002;44:A76.
70. Bray PG, et al. Defining the role of PfCRT in Plasmodium falciparum chloroquine resistance. Mol Microbiol. 2005;56(2):323–33.
71. Nessler S, et al. Evidence for activation of endogenous transporters in Xenopus laevis oocytes expressing the Plasmodium falciparum chloroquine resistance transporter, PfCRT. J Biol Chem. 2004;279(38):39438–46.
72. Bray PG, et al. PfCRT and the trans-vacuolar proton electrochemical gradient: regulating the access of chloroquine to ferriprotoporphyrin IX. Mol Microbiol. 2006;62(1):238–51.
73. Martin RE, Kirk K. The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. Mol Biol Evol. 2004;21(10):1938–49.
74. Tran CV, Saier Jr MH. The principal chloroquine resistance protein of Plasmodium falciparum is a member of the drug/metabolite transporter superfamily. Microbiology. 2004;150(Pt 1):1–3.
75. Bray PG, Ward SA. A comparison of the phenomenology and genetics of multidrug resistance in cancer cells and quinoline resistance in Plasmodium falciparum. Pharmacol Ther. 1998;77(1):1–28.
76. Martin RE, et al. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science. 2009;325(5948):1680–2.
77. Maughan SC, et al. Plant homologs of the Plasmodium falciparum chloroquine-resistance transporter, PfCRT, are required for glutathione homeostasis and stress responses. Proc Natl Acad Sci U S A. 2010;107(5):2331–6.
78. Patzewitz EM, et al. Glutathione transport: a new role for PfCRT in chloroquine resistance. Antioxid Redox Signal. 2013;19(7):683–95.
79. Lehane AM, et al. Degrees of chloroquine resistance in Plasmodium - is the redox system involved? Int J Parasitol Drugs Drug Resist. 2012;2:47–57.
80. Wilson CM, et al. Amplification of a gene related to mammalian mdr genes in drug-resistant Plasmodium falciparum. Science. 1989;244(4909):1184–6.
81. Zalis MG, et al. Characterization of the pfmdr2 gene for Plasmodium falciparum [published erratum appears in Mol Biochem Parasitol 1994 Feb;63(2):311]. Mol Biochem Parasitol. 1993;62(1):83–92.
82. Rubio JP, Cowman AF. Plasmodium falciparum: the pfmdr2 protein is not overexpressed in chloroquine-resistant isolates of the malaria parasite. Exp Parasitol. 1994;79(2):137–47.
83. Rubio JP, Thompson JK, Cowman AF. The var genes of Plasmodium falciparum are located in the subtelomeric region of most chromosomes. EMBO J. 1996;15(15):4069–77.
84. Foote SJ, et al. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum [see comments]. Nature. 1990;345(6272):255–8.
85. Wilson CM, et al. Amplification of pfmdr 1 associated with mefloquine and halofantrine resistance in Plasmodium falciparum from Thailand. Mol Biochem Parasitol. 1993;57(1):151–60.
86. Basco LK, et al. Analysis of pfmdr1 and drug susceptibility in fresh isolates of Plasmodium falciparum from sub-Saharan Africa. Mol Biochem Parasitol. 1995;74(2):157–66.
87. Basco LK, et al. Plasmodium falciparum: molecular characterization of multidrug-resistant Cambodian isolates. Exp Parasitol. 1996;82(2):97–103.
88. Cowman AF, et al. A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. J Cell Biol. 1991;113(5):1033–42.
89. Povoa MM, et al. Pfmdr1 Asn1042Asp and Asp1246Tyr polymorphisms, thought to be associated with chloroquine resistance, are present in chloroquine-resistant and -sensitive Brazilian field isolates of Plasmodium falciparum. Exp Parasitol. 1998;88(1):64–8.
90. Reed MB, et al. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature. 2000;403(6772):906–9.
91. Foote SJ, et al. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of P. falciparum. Cell. 1989;57(6):921–30.
92. Cowman AF, Galatis D, Thompson JK. Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. Proc Natl Acad Sci U S A. 1994;91(3):1143–7.
93. Price RN, et al. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet. 2004;364(9432):438–47.
94. Price RN, et al. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clin Infect Dis. 2006;42(11):1570–7.
95. Venkatesan M, et al. Polymorphisms in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for P. falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine. Am J Trop Med Hyg. 2014;91(4):833–43.
96. Davis TM, et al. Piperaquine: a resurgent antimalarial drug. Drugs. 2005;65(1):75–87.
97. Fan B, et al. In vitro sensitivity of Plasmodium falciparum to chloroquine, piperaquine, pyronaridine and artesunate in Yuxi prefecture of Yunnan province. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 1998;16(6):460–2.
98. Yang H, et al. Sensitivity of Plasmodium falciparum to seven antimalarials in China-Laos border. Chung Kuo Chi Sheng Chung Hsueh Yu Chi Sheng Chung Ping Tsa Chih. 1995;13(2):111–3.
99. Eastman RT, et al. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured Plasmodium falciparum parasites. Antimicrob Agents Chemother. 2011;55(8):3908–16.
100. Baraka V, et al. In vivo selection of Plasmodium falciparum Pfcrt and Pfmdr1 variants by artemether-lumefantrine and dihydroartemisinin-piperaquine in Burkina Faso. Antimicrob Agents Chemother. 2015;59(1):734–7.
101. Pascual A, et al. In vitro piperaquine susceptibility is not associated with the Plasmodium falciparum chloroquine resistance transporter gene. Malar J. 2013;12:431.
102. Pukrittayakamee S, et al. Quinine in severe falciparum malaria: evidence of declining efficacy in Thailand. Trans R Soc Trop Med Hyg. 1994;88(3):324–7.
103. Jelinek T, et al. Quinine resistant falciparum malaria acquired in east Africa. Trop Med Parasitol. 1995;46(1):38–40.
104. Segurado AA, di Santi SM, Shiroma M. In vivo and in vitro Plasmodium falciparum resistance to chloroquine, amodiaquine

and quinine in the Brazilian Amazon. Rev Inst Med Trop Sao Paulo. 1997;39(2):85–90.
105. Cooper RA, et al. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereo-specific quinine and quinidine responses in Plasmodium falciparum. Mol Pharmacol. 2002;61(1):35–42.
106. Menard D, et al. Global analysis of Plasmodium falciparum Na(+)/H(+) exchanger (pfnhe-1) allele polymorphism and its usefulness as a marker of in vitro resistance to quinine. Int J Parasitol Drugs Drug Resist. 2013;3:8–19.
107. Triglia T, Cowman AF. Primary structure and expression of the dihydropteroate synthetase gene of Plasmodium falciparum. Proc Natl Acad Sci U S A. 1994;91(15):7149–53.
108. Kasekarn W, et al. Molecular characterization of bifunctional hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase from Plasmodium falciparum. Mol Biochem Parasitol. 2004;137(1):43–53.
109. Bzik DJ, et al. Molecular cloning and sequence analysis of the Plasmodium falciparum dihydrofolate reductase-thymidylate synthase gene. Proc Natl Acad Sci U S A. 1987;84(23):8360–4.
110. Wang P, et al. Transfection studies to explore essential folate metabolism and antifolate drug synergy in the human malaria parasite Plasmodium falciparum. Mol Microbiol. 2004;51(5):1425–38.
111. Watkins WM, et al. Chlorproguanil/dapsone for the treatment of non-severe Plasmodium falciparum malaria in Kenya: a pilot study. Trans R Soc Trop Med Hyg. 1988;82(3):398–403.
112. Wootton DG, et al. Open-label comparative clinical study of chlorproguanil-dapsone fixed dose combination (Lapdap) alone or with three different doses of artesunate for uncomplicated Plasmodium falciparum malaria. PLoS One. 2008;3(3):e1779.
113. Winstanley P. Chlorproguanil-dapsone (LAPDAP) for uncomplicated falciparum malaria. Trop Med Int Health. 2001;6(11):952–4.
114. Brooks DR, et al. Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, Plasmodium falciparum, with differing resistance to sulfadoxine. Eur J Biochem. 1994;224(2):397–405.
115. Triglia T, et al. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in Plasmodium falciparum. Proc Natl Acad Sci U S A. 1997;94(25):13944–9.
116. Triglia T, et al. Allelic exchange at the endogenous genomic locus in Plasmodium falciparum proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. EMBO J. 1998;17(14):3807–15.
117. Triglia T, Cowman AF. The mechanism of resistance to sulfa drugs in Plasmodium falciparum. Drug Resist Updat. 1999;2(1):15–9.
118. Wang P, et al. Resistance to antifolates in Plasmodium falciparum monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. Mol Biochem Parasitol. 1997;89(2):161–77.
119. Wang P, et al. Sulfadoxine resistance in the human malaria parasite Plasmodium falciparum is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. Mol Microbiol. 1997;23(5):979–86.
120. Cowman AF, et al. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of Plasmodium falciparum. Proc Natl Acad Sci U S A. 1988;85(23):9109–13.
121. Peterson DS, Walliker D, Wellems TE. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci U S A. 1988;85(23):9114–8.
122. Wu Y, Kirkman LA, Wellems TE. Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci U S A. 1996;93(3):1130–4.
123. Yuthavong Y. Basis for antifolate action and resistance in malaria. Microbes Infect. 2002;4(2):175–82.
124. Baca AM, et al. Crystal structure of Mycobacterium tuberculosis 7,8-dihydropteroate synthase in complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. J Mol Biol. 2000;302(5):1193–212.
125. Sirawaraporn W, Yuthavong Y. Kinetic and molecular properties of dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant Plasmodium chabaudi. Mol Biochem Parasitol. 1984;10(3):355–67.
126. Snewin VA, et al. Characterisation of the dihydrofolate reductase-thymidylate synthetase gene from human malaria parasites highly resistant to pyrimethamine. Gene. 1989;76(1):41–52.
127. Yuvaniyama J, et al. Insights into antifolate resistance from malarial DHFR-TS structures. Nat Struct Biol. 2003;10(5):357–65.
128. Kublin JG, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of Plasmodium falciparum malaria. J Infect Dis. 2002;185(3):380–8.
129. WHO. WHO policy recommendation on intermittent preventive treatment during infancy with sulphadoxine-pyrimethamine (SP-IPTi) for Plasmodium falciparum malaria control in Africa. 2010.
130. WHO. World Health Organization; WHO Evidence Review Group Intermittent preventive treatment of malaria in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP). 2012.
131. Taylor SM, et al. Antenatal receipt of sulfadoxine-pyrimethamine does not exacerbate pregnancy-associated malaria despite the expansion of drug-resistant Plasmodium falciparum: clinical outcomes from the QuEERPAM study. Clin Infect Dis. 2012;55(1):42–50.
132. Cravo P, et al. Antimalarial drugs clear resistant parasites from partially immune hosts. Antimicrob Agents Chemother. 2001;45(10):2897–901.
133. Kinyanjui SM, et al. The antimalarial triazine WR99210 and the prodrug PS-15: folate reversal of in vitro activity against Plasmodium falciparum and a non-antifolate mode of action of the prodrug. Am J Trop Med Hyg. 1999;60(6):943–7.
134. van Hensbroek MB, et al. Iron, but not folic acid, combined with effective antimalarial therapy promotes haematological recovery in African children after acute falciparum malaria. Trans R Soc Trop Med Hyg. 1995;89(6):672–6.
135. Wang P, Sims PF, Hyde JE. A modified in vitro sulfadoxine susceptibility assay for Plasmodium falciparum suitable for investigating Fansidar resistance. Parasitology. 1997;115(Pt 3):223–30.
136. Nzila A, et al. Chemosensitization of Plasmodium falciparum by probenecid in vitro. Antimicrob Agents Chemother. 2003;47(7):2108–12.
137. Sowunmi A, et al. Open randomized study of pyrimethamine-sulphadoxine vs. pyrimethamine-sulphadoxine plus probenecid for the treatment of uncomplicated Plasmodium falciparum malaria in children. Trop Med Int Health. 2004;9(5):606–14.
138. Salcedo-Sora JE, et al. The molecular basis of folate salvage in Plasmodium falciparum: characterization of two folate transporters. J Biol Chem. 2011;286(52):44659–68.
139. Nirmalan N, et al. Transcriptional analysis of genes encoding enzymes of the folate pathway in the human malaria parasite Plasmodium falciparum. Mol Microbiol. 2002;46(1):179–90.
140. Nirmalan N, Sims PF, Hyde JE. Translational up-regulation of antifolate drug targets in the human malaria parasite Plasmodium falciparum upon challenge with inhibitors. Mol Biochem Parasitol. 2004;136(1):63–70.

141. Nixon GL, et al. Antimalarial pharmacology and therapeutics of atovaquone. J Antimicrob Chemother. 2013;68(5):977–85.
142. Fry M, Pudney M. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4- (4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). Biochem Pharmacol. 1992;43(7):1545–53.
143. Syafruddin D, Siregar JE, Marzuki S. Mutations in the cytochrome b gene of Plasmodium berghei conferring resistance to atovaquone [In Process Citation]. Mol Biochem Parasitol. 1999;104(2):185–94.
144. Birth D, Kao WC, Hunte C. Structural analysis of atovaquone-inhibited cytochrome bc1 complex reveals the molecular basis of antimalarial drug action. Nat Commun. 2014;5:4029.
145. Seymour KK, et al. dCTP levels are maintained in Plasmodium falciparum subjected to pyrimidine deficiency or excess. Ann Trop Med Parasitol. 1997;91(6):603–9.
146. Hammond DJ, Burchell JR, Pudney M. Inhibition of pyrimidine biosynthesis de novo in Plasmodium falciparum by 2-(4-t-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone in vitro. Mol Biochem Parasitol. 1985;14(1):97–109.
147. Painter HJ, et al. Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. Nature. 2007;446(7131):88–91.
148. Bulusu V, Jayaraman V, Balaram H. Metabolic fate of fumarate, a side product of the purine salvage pathway in the intraerythrocytic stages of Plasmodium falciparum. J Biol Chem. 2011;286(11):9236–45.
149. Biagini GA, et al. Generation of quinolone antimalarials targeting the Plasmodium falciparum mitochondrial respiratory chain for the treatment and prophylaxis of malaria. Proc Natl Acad Sci U S A. 2012;109(21):8298–303.
150. White NJ. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. Antimicrob Agents Chemother. 1997;41(7):1413–22.
151. Sanz LM, et al. P. falciparum in vitro killing rates allow to discriminate between different antimalarial mode-of-action. PLoS One. 2012;7(2):e30949.
152. Lalloo DG, Hill DR. Preventing malaria in travellers. BMJ. 2008;336(7657):1362–6.
153. Dembele L, et al. Towards an in vitro model of Plasmodium hypnozoites suitable for drug discovery. PLoS One. 2011;6(3):e18162.
154. Gassis S, Rathod PK. Frequency of drug resistance in Plasmodium falciparum: a nonsynergistic combination of 5-fluoroorotate and atovaquone suppresses in vitro resistance. Antimicrob Agents Chemother. 1996;40(4):914–9.
155. Rathod PK, McErlean T, Lee PC. Variations in frequencies of drug resistance in Plasmodium falciparum. Proc Natl Acad Sci U S A. 1997;94(17):9389–93.
156. Srivastava IK, et al. Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. Mol Microbiol. 1999;33(4):704–11.
157. Chiodini PL, et al. Evaluation of atovaquone in the treatment of patients with uncomplicated Plasmodium falciparum malaria. J Antimicrob Chemother. 1995;36(6):1073–8.
158. Looareesuwan S, et al. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am J Trop Med Hyg. 1996;54(1):62–6.
159. Looareesuwan S, et al. Efficacy and safety of atovaquone/proguanil compared with mefloquine for treatment of acute Plasmodium falciparum malaria in Thailand. Am J Trop Med Hyg. 1999;60(4):526–32.
160. Looareesuwan S, et al. Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Malarone Clinical Trials Study Group. Am J Trop Med Hyg. 1999;60(4):533–41.
161. Srivastava IK, Vaidya AB. A mechanism for the synergistic antimalarial action of atovaquone and proguanil. Antimicrob Agents Chemother. 1999;43(6):1334–9.
162. Korsinczky M, et al. Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob Agents Chemother. 2000;44(8):2100–8.
163. Fivelman QL, et al. Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. Malar J. 2002;1(1):1.
164. Schwartz E, Bujanover S, Kain KC. Genetic confirmation of atovaquone-proguanil-resistant Plasmodium falciparum malaria acquired by a nonimmune traveler to East Africa. Clin Infect Dis. 2003;37(3):450–1.
165. Fisher N, et al. Cytochrome b mutation Y268S conferring atovaquone resistance phenotype in malaria parasite results in reduced parasite bc(1) catalytic turnover and protein expression. J Biol Chem. 2012;287(13):9731–41.
166. Schwobel B, et al. Different mutation patterns of atovaquone resistance to Plasmodium falciparum in vitro and in vivo: rapid detection of codon 268 polymorphisms in the cytochrome b as potential in vivo resistance marker. Malar J. 2003;2(1):5.
167. Gil JP, et al. Detection of atovaquone and Malarone resistance conferring mutations in Plasmodium falciparum cytochrome b gene (cytb). Mol Cell Probes. 2003;17(2–3):85–9.
168. Wichmann O, et al. Malarone treatment failure not associated with previously described mutations in the cytochrome b gene. Malar J. 2004;3(1):14.
169. Smilkstein MJ, et al. A drug-selected Plasmodium falciparum lacking the need for conventional electron transport. Mol Biochem Parasitol. 2008;159(1):64–8.
170. Pesole G, et al. Nucleotide substitution rate of mammalian mitochondrial genomes. J Mol Evol. 1999;48(4):427–34.
171. Klayman DL. Qinghaosu (artemisinin): an antimalarial drug from China. Science. 1985;228(4703):1049–55.
172. O'Neill PM, Barton VE, Ward SA. The molecular mechanism of action of artemisinin--the debate continues. Molecules. 2010;15(3):1705–21.
173. Meshnick SR, et al. Iron-dependent free radical generation from the antimalarial agent artemisinin (qinghaosu). Antimicrob Agents Chemother. 1993;37(5):1108–14.
174. Meshnick SR, et al. Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. Mol Biochem Parasitol. 1991;49(2):181–9.
175. Posner GH, et al. Mechanism-based design, synthesis, and in vitro antimalarial testing of new 4-methylated trioxanes structurally related to artemisinin: the importance of a carbon-centered radical for antimalarial activity. J Med Chem. 1994;37(9):1256–8.
176. Posner GH, et al. Further evidence supporting the importance of and the restrictions on a carbon-centered radical for high antimalarial activity of 1,2,4-trioxanes like artemisinin. J Med Chem. 1995;38(13):2273–5.
177. Butler AR, et al. EPR evidence for the involvement of free radicals in the iron-catalysed decomposition of qinghaosu (artemisinin) and some derivatives; antimalarial action of some polycyclic endoperoxides. Free Radic Res. 1998;28(5):471–6.
178. O'Neill PM, et al. Biomimetic Fe(II)-mediated degradation of arteflene (Ro-42-1611). The first EPR spin-trapping evidence for the previously postulated secondary carbon-centered cyclohexyl radical. J Org Chem. 2000;65(5):1578–82.
179. Wu W-M, et al. Unified mechanistic framework for the Fe(II)-induced cleavage of Qinghaosu and derivatives/analogues. The first spin-trapping evidence for the previously postulated secondary C-4 radical. J Am Chem Soc. 1998;120(14):3316–25.
180. Haynes RK, et al. The Fe^{2+}-mediated decomposition, PfATP6 binding, and antimalarial activities of artemisone and other arte-

misinins: the unlikelihood of C-centered radicals as bioactive intermediates. ChemMedChem. 2007;2(10):1480–97.
181. Haynes RK, Pai HH-O, Voerste A. Ring opening of artemisinin (qinghaosu) and dihydroartemisinin and interception of the open hydroperoxides with Formation of N-oxides — a chemical model for antimalarial mode of action. Tetrahedron Lett. 1999;40(25):4715–8.
182. Haynes RK, et al. Interactions between artemisinins and other antimalarial drugs in relation to the cofactor model--a unifying proposal for drug action. ChemMedChem. 2012;7(12):2204–26.
183. Li W, et al. Yeast model uncovers dual roles of mitochondria in action of artemisinin. PLoS Genet. 2005;1(3):e36.
184. Wang J, et al. Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. PLoS One. 2010;5(3):e9582.
185. Olliaro P. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol Ther. 2001;89(2):207–19.
186. Golenser J, et al. Current perspectives on the mechanism of action of artemisinins. Int J Parasitol. 2006;36(14):1427–41.
187. Pandey AV, et al. Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite. J Biol Chem. 1999;274(27):19383–8.
188. Bhisutthibhan J, et al. The Plasmodium falciparum translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. J Biol Chem. 1998;273(26):16192–8.
189. Eckstein-Ludwig U, et al. Artemisinins target the SERCA of Plasmodium falciparum. Nature. 2003;424(6951):957–61.
190. Jiang JB, et al. Qinghaosu-induced changes in the morphology of Plasmodium inui. Am J Trop Med Hyg. 1985;34(3):424–8.
191. Kawai S, Kano S, Suzuki M. Morphologic effects of artemether on Plasmodium falciparum in Aotus trivirgatus. Am J Trop Med Hyg. 1993;49(6):812–8.
192. Maeno Y, et al. Morphologic effects of artemisinin in Plasmodium falciparum. Am J Trop Med Hyg. 1993;49(4):485–91.
193. Zhao Y, Hanton WK, Lee KH. Antimalarial agents, 2. Artesunate, an inhibitor of cytochrome oxidase activity in Plasmodium berghei. J Nat Prod. 1986;49(1):139–42.
194. Krungkrai J, et al. Mitochondrial oxygen consumption in asexual and sexual blood stages of the human malarial parasite, Plasmodium falciparum. Southeast Asian J Trop Med Public Health. 1999;30(4):636–42.
195. del Pilar Crespo M, et al. Artemisinin and a series of novel endoperoxide antimalarials exert early effects on digestive vacuole morphology. Antimicrob Agents Chemother. 2008;52(1):98–109.
196. Antoine T, et al. Rapid kill of malaria parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent depolarization of the membrane potential. J Antimicrob Chemother. 2014;69(4):1005–16.
197. Flegg JA, et al. Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. Malar J. 2011;10:339.
198. White NJ. The parasite clearance curve. Malar J. 2011;10:278.
199. Mu J, et al. Plasmodium falciparum genome-wide scans for positive selection, recombination hot spots and resistance to antimalarial drugs. Nat Genet. 2010;42(3):268–71.
200. Witkowski B, et al. Novel phenotypic assays for the detection of artemisinin-resistant Plasmodium falciparum malaria in Cambodia: in-vitro and ex-vivo drug-response studies. Lancet Infect Dis. 2013;13(12):1043–9.
201. Witkowski B, et al. Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia. Antimicrob Agents Chemother. 2013;57(2):914–23.
202. Ariey F, et al. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature. 2014;505(7481):50–5.
203. Cheeseman IH, et al. A major genome region underlying artemisinin resistance in malaria. Science. 2012;336(6077):79–82.
204. Takala-Harrison S, et al. Genetic loci associated with delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia. Proc Natl Acad Sci U S A. 2013;110(1):240–5.
205. Miotto O, et al. Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia. Nat Genet. 2013;45(6):648–55.
206. Amaratunga C, et al. Artemisinin resistance in Plasmodium falciparum. Lancet Infect Dis. 2014;14(6):449–50.
207. Straimer J, et al. Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates. Science. 2015;347(6220):428–31.
208. Mbengue A, et al. A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. Nature. 2015;520:683–7.
209. Tucker MS, et al. Phenotypic and genotypic analysis of in vitro-selected artemisinin-resistant progeny of Plasmodium falciparum. Antimicrob Agents Chemother. 2012;56(1):302–14.
210. Teuscher F, et al. Phenotypic changes in artemisinin-resistant Plasmodium falciparum lines in vitro: evidence for decreased sensitivity to dormancy and growth inhibition. Antimicrob Agents Chemother. 2012;56(1):428–31.
211. Klonis N, et al. Artemisinin activity against Plasmodium falciparum requires hemoglobin uptake and digestion. Proc Natl Acad Sci U S A. 2011;108(28):11405–10.
212. Mok S, et al. Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science. 2015;347(6220):431–5.
213. WHO. Chemotherapy of malaria. Report of a WHO scientific group. Geneva, World Health Organization (WHO Technical Report Series, No. 375); 1967.
214. Bruce-Chwatt LJ, et al. Chemotherapy of malaria. Rev. 2nd ed. Geneva: World Health Organization; 1986.
215. Solmaz SR, Hunte C. Structure of complex III with bound cytochrome c in reduced state and definition of a minimal core interface for electron transfer. J Biol Chem. 2008;283(25):17542–9.

Drug Resistance in *Leishmania*

42

Goutam Mandal, Vaidya Govindarajan, Mansi Sharma, Hiranmoy Bhattacharjee, and Rita Mukhopadhyay

1 Introduction

Leishmaniasis is a parasitic disease caused by the obligate intracellular protozoa of the genus *Leishmania*. At least 21 of the 30 species of *Leishmania* are known to be infectious to humans. The parasite exists in two forms. The promastigote form of the parasite resides in the intestinal tract of the insect vector and appears as a slender, spindle-shaped structure with an anterior flagellum. The amastigote forms of the parasite are small, oval-shaped structures that reside in macrophages and other mononuclear phagocytes in the mammalian host. The female phlebotomine sand flies are solely responsible for the transmission of *Leishmania* parasites among vertebrate hosts. Transmission of leishmaniasis could be anthroponotic, that is, transmission from human to human through the sand fly vector, in which humans are the sole reservoir hosts. The disease can also spread from animals to humans (zoonosis); in these cases, domestic animals (dogs) and wild animals (foxes, jackals, rodents, hyraxes) serve as the reservoir hosts.

Leishmaniasis is endemic in regions of 88 countries across 5 continents—the majority of the affected countries are in the tropics and subtropics. Approximately 12 million people worldwide are affected by leishmaniasis, while a total of 350 million people are at risk of contracting the disease (http://www.who.int/tdr/diseases/leish/). The disease in humans has been classified into three different forms, each having a broad range of clinical manifestations.

Visceral leishmaniasis (VL) is the most severe form of the disease and is fatal if left untreated. VL is caused by *Leishmania donovani*, *L. infantum*, or *L. chagasi* and is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia. Approximately 90 % of the 500,000 new cases of VL reported annually occur in Bangladesh, Brazil, India, Nepal, and Sudan.

Cutaneous leishmaniasis (CL) is caused by a variety of species including *L. major*, *L. tropica*, *L. mexicana*, *L. braziliensis*, and *L. panamensis*. CL, characterized by skin lesions on exposed parts of the body, such as the face, arms, and legs, may cause serious disabilities and permanently scar patients. It is the most common form of the disease with 1–1.5 million new cases reported annually worldwide, and 90 % of all CL cases are reported from Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria.

Mucocutaneous leishmaniasis (MCL) results from infiltration of *L. braziliensis* infection into nasopharynx. This infiltration produces extensive and destructive lesions of mucous membranes of the nose, mouth, and throat cavities. More than 90 % of MCL cases occur in Bolivia, Brazil, and Peru.

The last decade has recorded a sharp increase in leishmaniasis along with a significant expansion of *Leishmania*-endemic regions (http://www.who.int/gho/neglected_diseases/leishmaniasis/en/). This geographical spread is due to several factors. Widespread rural-urban migrations for business ventures bring nonimmune urban dwellers into endemic rural areas. Projects with considerable environmental impact, like dams and irrigation systems, as well as extensive deforestation contribute to the spread of the disease. Civil wars and regional conflicts leading to mass exodus, accompanied by the collapse of public health considerations, have also increased the number of *Leishmania*-infected patients. Additionally, *Leishmania*/HIV coinfection is currently emerging as an extremely serious comorbid medical condition and is considered a real threat in various parts of the world. VL has been widely recognized as an opportunistic infection among persons who are immunosuppressed, particularly in patients infected with human immunodeficiency virus [1–3].

G. Mandal, Ph.D. • V. Govindarajan • M. Sharma, Ph.D.
H. Bhattacharjee, Ph.D. • R. Mukhopadhyay, Ph.D. (✉)
Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, 11200 SW 8th Street, Miami, FL 33199, USA
e-mail: rmukhop@fiu.edu

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_42

The first-line compounds against all forms of leishmaniasis are the two pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) (Fig. 42.1). However, clinical resistance to this treatment is becoming prevalent [4–6]. In fact, more than 50 % of VL cases in Northeast India are resistant to Pentostam [7]. *Leishmania* resistant to trivalent antimony has also been reported [8]. The second line of anti-leishmanial drugs includes amphotericin B and pentamidine (Fig. 42.1). Alkyl-lysophospholipids (ALP), such as miltefosine (Fig. 42.1) and edelfosine, originally developed as anticancer drugs, have shown significant antiproliferative activity against *Leishmania* [9]. Miltefosine is the first oral drug that has been used against VL in India, including antimony-resistant cases [10]. Other drugs in various stages of clinical trials include allopurinol, atovaquone, fluconazole, paromomycin, and sitamaquine (Fig. 42.1).

Clinical- and/or laboratory-induced drug resistance has been observed with many of these drugs. Consequently, prevention and circumvention of resistance are important medical priorities. Understanding the mechanism of drug resistance will help in the development of tools towards recognition of resistance early in the infection process. This in turn will enable clinicians to start alternate or combination

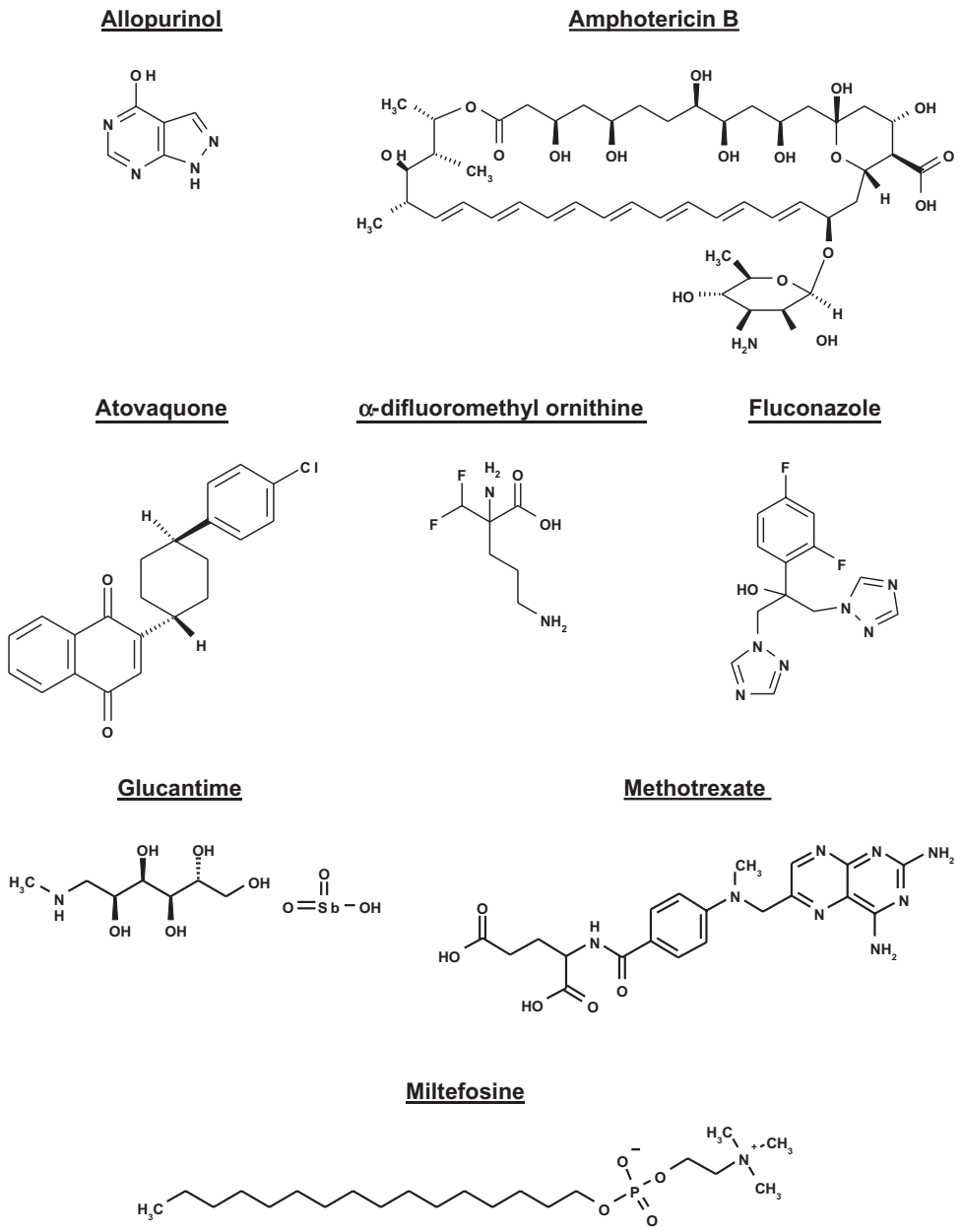


Fig. 42.1 Chemical structure of anti-leishmanial agents

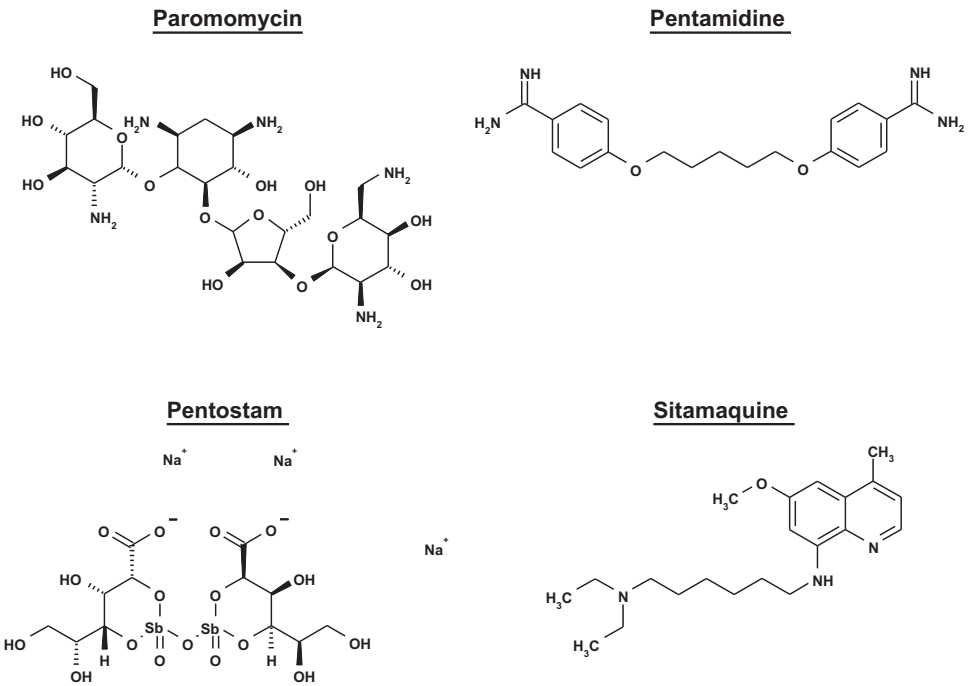


Fig. 42.1 (continued)

therapies at earlier stages of infection and to minimize the development of resistance. Additionally, identification of intracellular drug targets and parasite defense mechanisms will lead to rational drug design, thereby providing more effective treatment of the disease. Multiple biochemical mechanisms have been employed by *Leishmania* in conferring drug resistance [11]. These include (1) downregulation of the uptake system(s) for the drug, (2) intracellular sequestration, (3) drug inactivation or modification, (4) modification of the drug target to prevent binding of the drug or overproduction of the target so that drug concentration becomes limiting, (5) more efficient repair of drug damage, and (6) bypassing a blocked target.

2 Mechanisms of Drug Action and Resistance

2.1 Antimonials

Pentavalent antimonials have been used for the treatment of leishmaniasis for over half a century. The recommended regimen consists of daily injection of 20 mg/kg (maximum 850 mg) of either sodium stibogluconate or meglumine antimonate for 20–28 days [12].

2.1.1 Mechanisms of Action

Despite being used for several decades, the mode of action of pentavalent antimonials is poorly understood. The possibility of in vivo metabolic conversion of pentavalent [Sb(V)] to trivalent [Sb(III)] was suggested more than 50 years ago [13]. This hypothesis was supported by the observation that hamsters infected with *Leishmania garnhami*, and then treated with Glucantime [Sb(V)], showed similar serum concentrations of Sb(III) and Sb(V) [14]. Reduction of Sb(V) to Sb(III) was suggested to be associated with decreasing size and healing of the leishmanial ulcers [14]. Several investigators have shown that Sb(III) is more toxic than Sb(V) to either the promastigote or the amastigote forms of different *Leishmania* species [15–17]. Sereno et al. demonstrated that axenically grown amastigotes of *L. infantum* were more susceptible to Sb(III) than to Sb(V) [17]. However, these amastigotes were found to be poorly responsive to meglumine [Sb(V)] as compared to amastigotes grown in human macrophages [18]. These results strongly suggested a putative reductase residing within the macrophage, which catalyzes the conversion of Sb(V) to Sb(III).

Since arsenic and antimony are related metalloids, and arsenical-resistant *Leishmania* strains are frequently cross-resistant to antimonials, we considered the possibility that Sb(V) is reduced by a leishmanial As(V) reductase. The *Saccharomyces cerevisiae* arsenate reductase (ScAcr2p) sequence [19] was used to identify and clone the *L. major* homologue, LmACR2 [20]. LmACR2 was able to complement the arsenate-sensitive phenotype of either *Escherichia coli* or *S. cerevisiae* arsenate reductase-disrupted strains. Transfection of *Leishmania infantum* with *LmACR2* augmented Pentostam sensitivity in intracellular amastigotes. LmACR2 was purified and shown to reduce both As(V) and Sb(V) in vitro. We propose that LmACR2 is responsible for

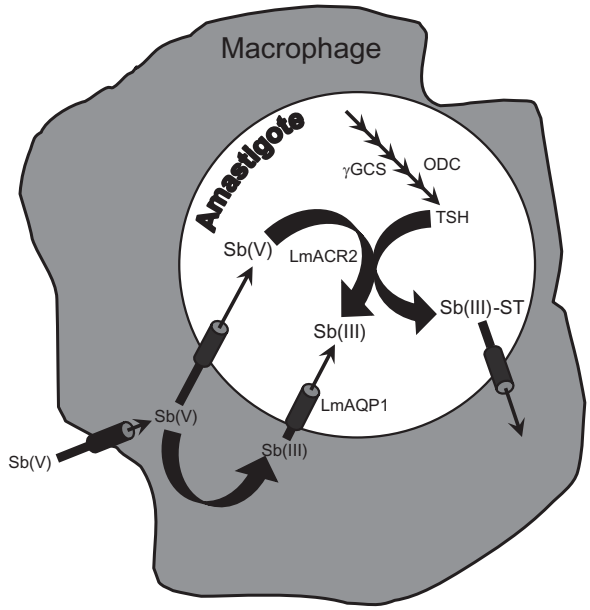


Fig. 42.2 Model of Pentostam uptake and resistance in macrophage-associated amastigotes of *Leishmania*. Sb(V) is taken up by macrophages, and a portion is reduced to Sb(III), which is then transported into the amastigote by LmAQP1. The other portion of the Sb(V) is taken into the amastigote and reduced to Sb(III) by LmACR2 and perhaps other enzymes such as thiol-dependent reductase (TDR1). Trypanothione (TSH) is overproduced in *Leishmania* by the higher activity of the rate-limiting enzymes γ-glutamyl cysteine synthetase (γGCS) and ornithine decarboxylase (ODC). Resistance is conferred when the plasma membrane pump extrudes As/Sb-TSH complex along with sequestration inside intracellular vesicles by an MRP homologue PGPA. The relative contributions of the two pathways to drug action would depend on the relative rates and expression of their respective components in both the human host and parasite. This could be different in different strains of *Leishmania*, as well as in different infected individuals, leading to variability in drug response

the reduction of pentavalent antimony in Pentostam to the active trivalent form of the drug in *Leishmania* [20] (Fig. 42.2). Denton et al. [21] identified and characterized a thiol-dependent reductase (TDR1) from *L. major* that can catalyze the reduction of pentavalent antimonials to the trivalent form using glutathione as a reductant. TDR1 is a trimer of two-domain monomers—each domain having some similarity to omega glutathione transferases. Higher abundance of the enzyme in mammalian stages of the parasite might explain the greater susceptibility of this parasite form to the drug (30).

Ultrastructural changes in *Leishmania tropica* within human macrophages exposed in vitro to Pentostam have been reported by Langreth et al. [22]. Pentostam-treated macrophages demonstrated loss of membrane definition. It was suggested that impaired macrophage membrane function may contribute to the effect of this drug against macrophage-contained *Leishmania*. To understand the antileishmanial effects of antimonial agents, Roberts et al. synthesized complexes of tri- and pentavalent antimony with mannan [16]. They observed that macrophages accumulated antimony after a 4-h exposure with potassium antimony tartrate, trivalent antimony-mannan, or pentavalent antimony-mannan, which was retained intracellularly for at least 3 days. Amastigotes inside macrophages had higher antimony content 6 days after a single 4-h treatment, suggesting that macrophages serve as reservoirs and prolong parasite exposure to antimonial agents.

Berman et al. [23] have shown that the viability of *Leishmania mexicana* promastigotes and amastigotes was decreased by 40–61 % following a 4-h exposure to 500 μg/ml of sodium stibogluconate. Such an exposure also resulted in a 56–65 % decrease in incorporation of label into purine nucleoside triphosphate along with a 34–60 % increase in incorporation of label into purine nucleoside monophosphate and diphosphate. Further experiments suggested that inhibition of glycolysis and the citric acid cycle might be partly responsible for the inability to phosphorylate ADP. An apparent decrease in ATP and GTP synthesis was therefore proposed to contribute to decreased macromolecular synthesis and to decreased *Leishmania* viability.

Chakraborty and Majumder [24] reported that one possible mode of action of antimonials may be in its ability to inhibit *Leishmania* topoisomerase I. These authors demonstrated that *L. donovani* topoisomerase I catalyzed relaxation of supercoiled plasmid pBR322. This catalysis was inhibited by sodium stibogluconate. Dose-dependent inhibition suggested that antimonials interact with *Leishmania* topoisomerase I rather than the DNA. However, calf thymus topoisomerase I and *E. coli* DNA gyrase were not inhibited by Sb(V).

Demicheli et al. (25) investigated the ability of pentavalent antimonials to form complexes with adenine nucleosides and deoxynucleosides in aqueous solution [25]. Circular dichroism (CD) titration suggested that adenosine and adenosine monophosphate but not 2′-deoxyadenosine form 1:2 Sb(V)-nucleoside complexes. NMR analysis indicated that Sb(V) binds to the sugar moiety at the 2′ position. Upon incubation of meglumine antimonate with adenosine, transfer of Sb(V) from its original ligand to the nucleoside molecule was observed at an acidic pH. Similar formation of Sb(V)-nucleoside complexes within the phagolysosome of *Leishmania*-bearing macrophages was proposed as a possible mechanism of anti-leishmanial activity of antimonials.

Mehta and Shaha [26] demonstrated that antimony caused mitochondrial membrane depolarization and concomitant thiol loss. Mitochondrial depolarization leads to lesser production of ATP and generates oxidative burst in an iron-dependent manner, finally culminating in cell death. In addition, antimonial compounds can generate oxidative burst within phagolysosomes of macrophages via phosphorylation of phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) Ras, and extracellular-signal-regulated kinase (ERK) [27].

Inhibition of these proteins or addition of a freeradical scavenger like *N*-acetylcysteine inhibited antimony-mediated killing of intracellular amastigotes—emphasized the contribution of oxidative burst within phagolysosomes of infected macrophages to antimony-mediated parasiticidal activity [27].

Wyllie et al. [28] have demonstrated that Sb(III) disrupts the trypanothione metabolism of Leishmania by two distinct mechanisms. First, Sb(III) induces rapid efflux of intracellular trypanothione and glutathione in approximately equimolar amounts, thus compromising the thiol-buffering capacity of the cell. Second, Sb(III) inhibits trypanothione reductase in intact cells, resulting in accumulation of the disulfide forms of trypanothione and glutathione. These two mechanisms combine to profoundly compromise the normal thiol redox state in both amastigote and promastigote stages.

Antimony has reportedly failed to act in immunocompromised hosts, such as patients suffering from AIDS or receiving immunosuppressive agents [29, 30], nude mice [31], and severe combined immunodeficient (SCID) mice [32]. Endogenous interleukin-2 (IL-2) [33], IL-4 [34], and IL-12 [35] also influence the efficacy of pentavalent antimony as an anti-leishmanial agent. These suggest that antimony can act through activation of the host immune system and requires a functional T-cell response.

2.1.2 Mechanisms of Resistance

Mechanisms of antimonial resistance in *Leishmania* has been an area of intense research for several decades. These investigations clearly established that antimonial resistance is multifactorial in *Leishmania*. Parasites use one or several mechanisms to become unresponsive against antimonials. Collectively, antimony-resistant *Leishmania* showed modifications primarily at the levels of (1) drug entry and (2) drug metabolism, efflux and/or sequestration.

Modulations at the Level of Drug Entry

Microbes often become resistant to drugs by mutation or downregulation of uptake systems. In bacteria [36], yeast [37], and mammals [38], aquaglyceroporins have been shown to be the uptake systems for trivalent metalloids. We have reported on the identification and characterization of aquaglyceroporins from *L. major* (LmAQP1) and *L. tarentolae* (LtAQP1), respectively [39] (Fig. 42.2). These *Leishmania* aquaglyceroporins have the conserved signature motifs of mammalian aquaglyceroporins [40]. *LmAQP1* was transfected into three different species of *Leishmania*—*L. tarentolae*, *L. infantum*, and *L. major*. Each transfectant became hypersensitive to both As(III) and Sb(III). We have also shown that the drug-resistant parasites, with different mechanisms of resistance, became hypersensitive to both metalloids after overexpression of LmAQP1. Increased rates of uptake of either As(III) or Sb(III) correlated with metalloid sensitivity of the wild-type and drug-resistant transfectants [39]. We have constructed a heterozygous knockout of *L. major* by disruption of one of the two alleles. This single knockout is tenfold more resistant to Sb(III) than the homozygous wild-type parent [39]. This supports our hypothesis that the amount of AQP1 in the plasma membrane is the rate-limiting step in the uptake of the activated form of the antimonial drugs. A single-point mutation of Glu152 of AQP1 abolished the metalloid conductance without affecting the physiological functions of AQP1 [41]. Another point mutation at Ala163 also inhibited metalloid accumulation [42]. These findings strengthen the argument that point mutations in the *AQP1* gene could give rise to clinical resistance. It was reported that the AQP1 mRNA levels are downregulated in promastigotes of several *Leishmania* species resistant to antimonials [43]. Recently, we found that antimony sensitivity of *Leishmania* is species specific: CL-causing species are 5–46 times more sensitive against antimonials compared to VL-causing species. The level of sensitivity was primarily governed by the expression levels of AQP1. Steady-state levels of AQP1 mRNA were 13–56-fold higher in CL-causing species compared to VL-causing species. Stability of AQP1 mRNA was determined by the nature of 3′UTR of the respective species (Mukhopadhyay, unpublished data). Several studies involving clinical resistant isolates also reported the downregulation of AQP1 mRNA [44–46]. We also reported that *Leishmania* mitogen-activated protein kinase 2 (MAPK2) positively regulates AQP1 protein stability by phosphorylating at Thr197 of AQP1. This phosphorylation also influenced the redistribution of AQP1 from flagella to pelicular membrane. Increased stability and redistribution of AQP1 reflected higher sensitivity against antimonials in AQP1 and MAPK2 co-overexpressing cells [47]. We discussed earlier that macrophages contribute significantly in reduction of pro-drug Sb(V) to active Sb(III). This reinstated the role of AQP1 in antimony sensitivity as AQP1 is the only reported facilitator of Sb(III) in *Leishmania*. In conclusion, irrespective of the status of other antimony-resistant factors (discussed below), alteration of AQP1 function can regulate antimony sensitivity profile of *Leishmania*. Thus, further studies are warranted to identify the arsenal of both posttranscriptional and posttranslational regulators of AQP1 to combat against antimonial resistance in *Leishmania*.

Modulations at the Level of Drug Metabolism and Efflux

As stated earlier, Sb(V) is reduced to Sb(III) to become antileishmanial [48]. ICP-MS measurements indicated that Sb(V) was reduced to highly toxic Sb(III) in *L. donovani* amastigotes. Furthermore, one of the Pentostam-resistant mutants lacked this reducing activity, suggesting a novel mechanism of Sb(V) resistance [48]. Earlier we discussed LmACR2. LmACR2 also confers Pentostam hypersensitivity to *Leishmania* amastigotes sensitive and resistant to Pentostam [20]. Why should

Leishmania evolve a specific metalloid reductase when it is known to be unfavorable for the pathogen? We have recently shown that, in addition to displaying arsenate and antimonate reductase activity, LmACR2 exhibits protein tyrosine phosphatase activity [49]. Most likely, the physiological function of LmACR2 is to dephosphorylate phosphotyrosine residues in leishmanial proteins, and not detoxification of metalloids. Another reductase TDR1 activity in *L. major* was ten times higher in amastigotes compared to promastigotes [50]. However, the role of these enzymes in antimonial resistance in clinical isolates is yet to be determined.

The ATP-binding cassette (ABC) protein PGPA (aka MRPA), which belongs to the ABCC subfamily (ABCC3), has been suggested to play a major role on metal resistance in *Leishmania* [51]. PGPA is a member of the multidrug resistance protein (MRP) family, a large family of ABC transporters, several of which are implicated in drug resistance [52]. The *PGPA* gene is frequently amplified in *Leishmania* cells selected for resistance to arsenite- or antimony-containing drugs [53–58], and its overexpression or disruption prove that PGPA is involved in metal resistance [57, 59–61]. We reported that arsenite-resistant *L. tarentolae* promastigotes overproduce trypanothione (TSH), a unique glutathione-spermidine conjugate found only in trypanosomatids [62]. We have also shown that two rate-limiting enzymes in the polyamine and glutathione biosynthetic pathways—ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (γ-GCS)—mediate the trypanothione overproduction [63, 64] (Fig. 42.2). However, increased TSH level alone was not sufficient to generate metal resistance. Downregulation of TSH levels by specific inhibitors of γ-GCS or ODC could revert the resistance [64]. Co-transfection of the *GSH1* gene that codes for the heavy subunit of γ-glutamylcysteine synthetase (γ-GCS) or *ODC* and *PGPA* genes in partial revertants, but not in wild-type cells, leads to synergistic levels of resistance, strongly suggesting that PGPA recognizes metals conjugated to TSH [63, 64]. PGPA is localized in small vesicles near flagellar pockets. These PGPA-containing vesicles sequester arsenic/antimony–thiol conjugates and are later disposed outside the cell [65].

Transport experiments in arsenite-resistant *L. tarentolae* mutants indicated the presence of an active efflux system that did not correlate with *PGPA* gene amplification [66, 67]. An analysis of *L. tarentolae PGPA* transfectants did not show a marked difference in the steady-state accumulation of arsenite [61], although a decrease in the uptake of antimony was proposed to explain the resistance in *L. major PGPA* transfectants [68].

Amplification of other members of the ABCC subfamily (ABCC4, ABCC5, and ABCC7) can also confer resistance to antimonials [69]. An ABC half-transporter ABCI4—localized both on mitochondria and plasma membrane—is also involved in efflux of Sb(III)-thiol conjugate. Overexpression of ABCI4 confers antimony resistance in *L. major* [70].

Several studies found that clinical antimonial resistant isolates also upregulated one or many of these genes [71–73]. Indian *L. donovani* clinical resistant isolates showed that higher TSH levels protect them from Sb(III)-induced oxidative burst. These resistant isolates also showed amplification in thiol biosynthetic machinery that outpaced the Sb(III)-mediated thiol loss [74]. Amplification of thiol-dependent antioxidant system and nonclassical MRP1 type activity was also reported in Indian antimony-resistant *L. donovani* clinical isolates [75, 76].

Brochu et al. [77] quantified the accumulation of Sb(V) and Sb(III) in *Leishmania* by using inductively coupled plasma mass spectrometry (ICP-MS). The accumulation was studied in three *Leishmania* species at various life stages that were either sensitive or resistant to antimony. Both the promastigote and amastigote forms of the parasites accumulated Sb(III) and Sb(V). Competition experiments with arsenite indicated that the routes of entry of Sb(V) and Sb(III) into the parasites were most likely different. However, the level of accumulation of either Sb(III) or Sb(V) did not correlate with the susceptibility of wild-type *Leishmania* cells to antimony. In contrast to metal susceptibility, resistance to Sb(III) correlated well with decreased antimony accumulation. This phenotype was energy dependent and highlighted the importance of transport systems in drug resistance of this protozoan parasite.

Several other novel antimony resistance mechanisms have been reported recently. A novel resistance protein (LinJ34.0570) belongs to the superfamily of leucine-rich repeat (LRR) proteins involved in antimonial resistance in *L. infantum* [78]. Antimony-resistant *L. donovani* can modulate macrophage gene expression differently compared to antimony-sensitive strains. Antimony-resistant *L. donovani* express a unique glycan with *N*-acetylgalactosamine as a terminal sugar. This unique sugar induces IL10 expression, which in turn upregulates MDR1 expression of the infected macrophages. MDR1 overexpression of macrophages can lead to antimony resistance [79, 80]. Proliferating cell nuclear antigen (PCNA) overexpressed in antimony-resistant *L. donovani* clinical isolates. Overexpression of PCNA reduced the SAG-induced NO production and DNA fragmentation, thereby contributing to the generation of SAG-resistant phenotype [81]. Arsenic is known to induce cross-resistance to antimony. The passage of antimony-sensitive *L. donovani* strains through mice chronically exposed to arsenic may become refractory to antimony. Thus, higher incidence rates of antimony unresponsiveness in India could be attributed to chronic exposure to high levels of arsenic through drinking water [82]. Downregulation of mitogen-activated protein kinase 1 (MAPK1) was also reported in clinical antimony-resistant isolates. Overexpression of MAPK1 reverted the resistant phenotype [83]. Upregulation of parasite surface antigen-2 (PSA-2) in

L. donovani clinical isolates confers resistance to antimony [84]. By using a functional cloning strategy, Schäfer et al. identified a novel antimony resistance marker ARM58 from *L. braziliensis* and *L. infantum* that protects the parasites against antimonial drugs [85].

2.2 Amphotericin B

Amphotericin B has often been used as a second line of treatment against leishmaniasis in India. In an uncontrolled study, a 93 % cure rate was observed in antimonial unresponsive patients treated with short-course regimens of amphotericin B fat emulsion (5 alternate-day infusions of 2 mg/kg) [86]. Although the drug is effective, its use is limited by toxicity complications, including renal impairment, anemia, fever, malaise, and hypokalemia. Liposomes have been proposed as an effective way to target drugs at macrophages. Amphotericin B incorporated into liposomes is highly effective against experimental leishmaniasis with low toxicity. Davidson et al. reported the successful treatment of a patient with multiple-drug-resistant visceral leishmaniasis using a commercially prepared formulation of liposomal amphotericin B [87].

2.2.1 Mechanisms of Action

Amphotericin B is an antifungal polyene antibiotic isolated from *Streptomyces nodosus*. Its anti-leishmanial activity was first shown in the early 1960s and was attributed to its selective affinity for 24 substituted sterols, namely ergosterol vis-à-vis cholesterol, the primary sterol counterpart in mammalian cells, eventually helping to increase drug selectivity towards the microorganism. Interference with membrane sterols results in loss of the permeability barrier to small metabolites that disrupt the integral protein function and solute equilibrium, ultimately leading to cell death [88, 89]. Uptake of [^{14}C] glucose was inhibited quickly while inhibition of respiration by the drug was a comparatively slower process [88]. Besides direct parasiticidal activity, amphotericin B can also modulate immune response towards a protective Th1 response [90]. Amphotericin B can also prevent entry of *Leishmania* into macrophages. Binding of amphotericin B with membrane cholesterol interferes with binding of promastigotes to macrophage membrane [91].

Toxic effects of amphotericin B deoxycholate have been largely ameliorated with the advent of lipid formulations of amphotericin B. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity and facilitating its preferential uptake by reticuloendothelial cells, achieving targeted drug delivery to the parasite, resulting in increasing efficacy and reduced toxicity [92]. Three such lipid formulations of amphotericin are (1) liposomal amphotericin B or AmBisome, (2) amphotericin B lipid complex or Abelcet (ABLC), and (3) amphotericin B colloidal dispersion or Amphocil. Liposomal amphoterin B is considered by many experts as the best existing drug against VL [93]. This formulation has also been successfully used against HIV-VL coinfection [94]. Recently, a nanoparticle delivery system to administer amphotericin B has been developed, which showed a higher efficacy compared to free amphotericin B deoxycholate in an experimental CL model [95]. Amphotericin B-γ cyclodextrin formulation has been shown to have promising anti-leishmanial activity. γ-Cyclodextrin showed a synergistic effect with amphotericin B for membrane destabilization. The formulation was administered as a topical gel and yielded six to eight times greater efficacy against experimental CL than amphotericin B alone [96].

2.2.2 Mechanisms of Resistance

To date, there is no clear-cut evidence of amphotericin B resistance in clinical settings; the increasing use and longer half-lives of amphotericin B in lipid formulations have the potential to cause emergence of unresponsiveness. However, to study the resistance mechanisms, amphotericin B-resistant *L. donovani* promastigotes were selected by increasing the drug pressure [97]. The resistant cells had 2.5 times longer generation time, decreased uptake, and increased efflux of the drug. The drug-resistant promastigotes showed increased membrane fluidity. Analysis of lipid composition showed that saturated fatty acids were prevalent in resistant cells, with stearic acid as the major fatty acid, and the major sterol was an ergosterol precursor—cholesta-5, 7, 24-trien-3β-ol—not ergosterol, as in the amphotericin B-sensitive strain [97].

Singh et al. [98] reported stepwise selection of two amphotericin B-resistant *L. tarentolae* cells. One of the mutants was also cross-resistant to ketoconazole. DNA amplification was observed in both mutants. Gene transfection experiments indicated that the link between the locus amplified, the resistance levels were not straightforward, and several mutations were possibly responsible for amphotericin B resistance.

Recently, Equbal et al. reported a positive correlation between trypanothione synthase upregulation and amphotericin B resistance in *L. donovani* promastigotes [99].

2.3 Pentamidine

Pentamidine isothienate, an aromatic diamidine, has been used as a second-line anti-leishmanial drug in antimony-unresponsive visceral leishmaniasis patients. The recommended dosage is 4 mg/kg by intramuscular route on alternate days for 6 weeks [86, 100, 101]. The drug achieves poor response rates (around 75 %) and is associated with side effects such as myalgia, nausea, headache, and hypoglycemia, with an exceptionally high risk of developing irreversible diabetes [86].

However, pentamidine has been observed to be very effective, and is the drug of choice for treatment of leishmaniasis in Guyana, where 90 % of leishmaniasis cases are due to *L. guyanensis.* Pentamidine, nonetheless, is considered a safer drug with regard to developing resistance, as drug's half-life is shorter [102].

2.3.1 Mechanisms of Action

The mechanism of action of pentamidine is poorly understood. The uptake process for pentamidine in *L. donovani* and *L. amazonensis* promastigotes and axenic amastigotes is saturable, carrier mediated, and energy dependent [103]. Pentamidine was found to be a competitive inhibitor of arginine transport [104, 105] in *L. donovani*, and a noncompetitive inhibitor of putrescine and spermidine transport in *L. infantum* [106], *L. donovani*, and *L. mexicana* [107]. The physiological roles of the carrier proteins that accumulate pentamidine are still unknown. When treated with low concentrations of pentamidine for 24 h, both *L. donovani* and *L. amazonensis* cells showed significant decreases in ornithine decarboxylase activity, the rate-limiting enzyme in the polyamine biosynthetic pathway [108]. Therefore, the polyamine biosynthesis pathway may be a target of pentamidine in *Leishmania*.

The mitochondrion has also been implicated in the action of pentamidine against leishmania [109]. *L. tropica* amastigotes exposed in vitro to pentamidine demonstrated swollen kinetoplasts and fragmentation of the kinetoplast DNA core [22, 110]. A rapid collapse of the mitochondrial inner membrane potential of *L. donovani* promastigotes was also observed upon treatment of these parasites with the drug [111]. Pentamidine may also disrupt mitochondrial activity by interfering with mitochondrial DNA topoisomerase II, much as antimonials do to nuclear DNA [112].

2.3.2 Mechanisms of Resistance

Resistance to pentamidine has been described for *L. donovani* and other *Leishmania* species [109]. The mechanism of resistance to pentamidine is not well understood. Coelho et al. [113] used a genetic strategy to search for loci able to mediate pentamidine resistance (PEN^r) when overexpressed in *L. major*. A shuttle cosmid library containing genomic DNA inserts was transfected into wild-type promastigotes and screened for PEN^r transfectants. Two different cosmids identifying the same locus were found, which differed from other known *Leishmania* drug resistance genes. The *PENr* gene was mapped by deletion and transposon mutagenesis to an open reading frame belonging to the P-glycoprotein/MRP ATP-binding cassette transporter superfamily and was named pentamidine resistance protein 1 (PRP1). PRP1-mediated pentamidine resistance could be reversed by verapamil and overexpression of PRP1 showed cross-resistance to Sb(III) but not to Sb(V). Although a 1.7–3.7-fold pentamidine resistance was observed, this may be significant in clinical drug resistance given the marginal efficacy of the drug against *Leishmania*.

Basselin et al. [109] compared the uptake of [^{3}H]pentamidine into wild-type and drug-resistant strains of *L. mexicana.* A substantial decrease in accumulation of the drug accompanied the resistance phenotype, although the apparent affinity for pentamidine by its carrier was not altered. These experiments indicated that diamidine drugs accumulate in the *Leishmania* mitochondrion. The development of resistance phenotype is accompanied by lack of mitochondrial accumulation of the drug and its exclusion from the parasites.

2.4 Alternate Agents

Alternate agents are used mostly in antimony-unresponsive cases. Miltefosine, allopurinol, atovaquone, and paromomycin are commonly used either alone or in combination with antimonials against different forms of leishmaniasis. Natural extracts have also been contemplated for use as antileishmanials due to the growing rate of resistance to traditional antileishmanial agents, greater efficacy, and lower toxicity.

2.4.1 Miltefosine

Miltefosine (hexadecylphosphocholine) is the first drug approved for the oral treatment of VL. Miltefosine was originally developed as an antineoplastic agent and later found to be highly active against *Leishmania* in vitro and in animal models [114, 115]. The recommended dose of miltefosine for the treatment of VL is approximately 2.5 mg/kg per day for 4 weeks [116]. Initial clinical trials showed miltefosine to be approximately 90 % effective in combating childhood VL [10, 117]. Although a cure rate that exceeded 90 % was achieved against CL in Iran [118], miltefosine was not successful against diffused CL as many patients relapse after cure [119]. Miltefosine is currently marketed as Impavido for use in treating CL and VL in Nepal, India, Bangladesh, Argentina, Bolivia, Colombia, Ecuador, Germany (mainly as anticancer), Honduras, Mexico, Pakistan, Paraguay, and Peru [120]. Major limitations of miltefosine are (1) low therapeutic window, (2) gastrointestinal disturbances, (3) renal toxicity, and (4) teratogenic, for which it is contraindicated in pregnancy and women of child-bearing age [92].

Mechanisms of Action

The molecular mechanisms that contribute to the antileishmanial activity of miltefosine are still unknown, but it is likely that miltefosine operates via carrier-mediated transport [120]. Preliminary studies on *Leishmania mexicana* promastigotes suggested that miltefosine may cause perturbation of ether-lipid metabolism, glycosylphosphatidylinositol

(GPI) anchor biosynthesis, and leishmanial signal transduction [121, 122]. Later, Lux et al. reported that miltefosine inhibits the glycosomal alkyl-specific acyl-CoA acyl transferase in a dose-dependent manner [123]. However, this is unlikely to be the primary target as high concentrations of the drug are required (IC_{50} value 50 μM) to inhibit the enzyme. Miltefosine-resistant strains of *L. donovani* exhibit low membrane sterol counts and higher concentrations of unsaturated hydrocarbons in the phospholipid bilayer, suggesting that miltefosine binds with membrane sterols and disrupts the bilayer, as does amphotericin B [120]. It was proposed that a perturbation of ether-lipid remodeling may be responsible for the anti-leishmanial activity of miltefosine [121]. Zufferey and Mamoun [124] reported that choline transport into *Leishmania* is inhibited by miltefosine. Miltefosine has also been reported to induce an apoptosis-like death in *L. donovani* promastigotes and amastigotes (both intra- and extracellular), and in *L. amazonensis* and *L. infantum* promastigotes [120, 125]. Interferon-γ deficiency compromised anti-leishmanial activity of miltefosine, suggesting that the drug requires host immune support. In addition, miltefosine induced interferon-γ deficiency expression and IL-12 production in infected macrophages to restore Th1/Th2 balance of infected macrophages [120].

Mechanisms of Resistance

Miltefosine-resistant *L. donovani* strains have been raised in vitro [126]. The promastigotes were cross-resistant to edelfosine but not to standard anti-leishmanial drugs. The resistant mutants were found to be deficient (>95 %) in their ability to take up [^{14}C] miltefosine. Binding of the drug to the plasma membrane and efflux from the cells were similar in the resistant and sensitive lines. The resistant promastigotes were also unable to take up other short-chain phospholipid analogues, independently of their polar head group, even though endocytosis remained unaltered. This suggested that a short-chain phospholipid translocase might be downregulated or mutated in the resistant promastigotes [126].

A putative miltefosine transporter (LdMT) has been cloned by functional rescue using a resistant *L. donovani* strain defective in the inward-directed translocation of both miltefosine and glycerophospholipids. LdMT is a novel P-type ATPase belonging to the partially characterized aminophospholipid translocase (APT) subfamily. Resistant parasites transfected with LdMT regained their sensitivity to miltefosine and also the ability to normally take up [^{14}C] miltefosine and fluorescent-labeled glycerophospholipids. LdMT was shown to localize to the plasma membrane, and its overexpression in *L. tarentolae*, a species nonsensitive to miltefosine, significantly increased the uptake of [^{14}C] miltefosine, strongly suggesting that this protein behaves as a true translocase. Both *LdMT*-resistant alleles contained single but distinct point mutations, each of which impaired the transport function, thereby explaining the resistant phenotype. These results clearly demonstrate the direct involvement of LdMT in miltefosine and phospholipid translocation in *Leishmania* [126]. It has also been observed that *L. tropica* cells overexpressing a P-glycoprotein-like transporter are cross-resistant to alkyl-lysophospholipids, as are miltefosine and edelfosine [127]. Thus, greater efflux of the drug by MDR-like transporters may be another mechanism by which *Leishmania* cells become resistant to miltefosine. Overexpression of a member of ATP-binding cassette (ABC) subfamily G (ABCG)-like transporter, LiABCG4, which is localized mainly in the parasite plasma membrane of Leishmania, reduced accumulation of phosphatidylcholine analogues and conferred resistance to miltefosine [128]. *Leishmania* can also become resistant against miltefosine by preventing drug-induced apoptotic cell death [129].

2.4.2 Allopurinol

Allopurinol (20 mg/kg/day) in combination with antimonials has been used with some efficacy against VL [130–132]. Das et al. reported a randomized clinical trial of a combination of pentamidine (half dose) and allopurinol (15 mg/kg) in the treatment of antimony-unresponsive cases of VL [101]. The combination therapy was found to be more effective in achieving ultimate cure with an added advantage of reduced toxicity in unresponsive cases as compared to full doses of pentamidine. Allopurinol was also used as a combination therapy with a meglumine antimoniate (Glucantime) and yielded a 74 % cure rate. Notably, the cure rate for allopurinol alone was 80 %, indicating that Allopurinol can be used as monotherapy [133].

Allopurinol riboside is not phosphorylated by the kinases normally found in mammalian cells and shows little or no toxicity. *Leishmania* on the other hand have a nucleoside phosphotransferase that can catalyze conversion of allopurinol riboside to its 5′-monophosphate. Subsequently, allopurinol riboside 5′-monophosphate is sequentially acted upon by adenylosuccinate synthetase and lyase to form the corresponding adenosine nucleotide analogues, which are incorporated into RNA, thereby conferring anti-leishmanial activity [134, 135]. Clinical resistance has not been reported since this drug has not been used widely.

Despite the lack of resistance, widespread use of Allopurinol is hindered by a group of rare, but potentially fatal, side effects collectively called allopurinol hypersensitivity syndrome (AHS). The syndrome is characterized by fever, leukocytosis, and hepatitis. AHS also causes toxic epidermal necrolysis (TEN)—fatal loss of the epidermis, erosion of the mouth and nasal mucous membranes, and blindness caused by ocular lesions and kidney failure [136].

2.4.3 Atovaquone

Atovaquone, a hydroxynaphthoquinone, has been shown to have anti-leishmanial effects in murine models and has been suggested for use as an adjunct to conventional antimony treatment in VL [137, 138].

The mechanism of action of atovaquone against *Leishmania* is not known. In *Plasmodium*, atovaquone appears to act by selectively affecting mitochondrial electron transport, resulting in inhibition of nucleic acid and ATP synthesis [139, 140]. Atovaquone-resistant *L. infantum* promastigotes were selected in vitro by stepwise drug pressure, and showed no cross-resistance to other anti-leishmanial drugs [141]. The resistant promastigotes showed decreased ergosterol biosynthesis (five times less than susceptible strains), increased membrane cholesterol content, and decreased membrane fluidity, all of which were likely responsible for blocking the passage of atovaquone through the membrane [141].

2.4.4 Paromomycin

Paromomycin (identical to aminosidine), obtained from cultures of *Streptomyces rimosus*, belongs to the class of aminocyclitol aminoglycosides and possesses both antibacterial and antiprotozoal activity. Although developed in the 1960s as an anti-leishmanial agent, it remained neglected until the 1980s when topical formulations were found to be effective in CL and a parenteral formulation for VL was also developed. Paromomycin has been used either alone or in combination with Sb(V) for the treatment of VL, and was first reported by Chunge et al. [142], albeit in a small number of patients. Its superiority in combination with Sb(V) compared to Sb(V) alone has clearly been demonstrated in several studies from India [143, 144]. A study from Sudan also demonstrated that when combined with Sb(V), it was possible to reduce the duration of treatment from 30 days to 17 days, with superior efficacy and decreased mortality [145]. Recently, a Phase III clinical trial in India reported that, similar to amphotericin, injectable paromomycin alone is equally effective against Indian VL [146, 147]. Another recent in vitro study reported the synergistic effect of Sb(V) and paromomycin cotreatment against antimony-resistant *L. tropica* [148]. A recent Phase II trial of topical application of paromomycin in combination with gentamicin as a treatment for CL by *L. panamensis* showed an 87 % cure rate [149].

Little is known about the mechanism of action of paromomycin. The drug has been shown to affect the RNA synthesis and modify membrane polar lipids and membrane fluidity in *L. donovani* promastigotes [150]. Paromomycin also inhibits protein synthesis by binding to the parasite's ribosomes and disrupting the mitochondrial membrane potential [151].

Although no clinical evidence of paromomycin resistance has been reported, several studies used laboratory-generated resistant strains to study the resistance mechanisms. *L. donovani* promastigotes resistant to 800 μM of paromomycin were selected by exposing them to gradual increments of the drug [152]. These promastigotes did not acquire multidrug resistance. Paromomycin resistance was stable in the absence of the drug in the culture and also remained stable in amastigotes isolated after passing through mice. The major mechanism of resistance seemed to be due to decreased drug uptake, probably as a consequence of altered membrane composition [152]. Laboratory-raised paromomycin-resistant *L. donovani* strain showed decreased intracellular drug accumulation, increased membrane fluidity, and expression of MDR1 and protein phosphatase 2A. The resistant strain was also resistant against nitrosative stress and stimulated host interleukin-10 expression. The susceptibility of paromomycin-resistant strain against other antileishmanial agents (sodium antimony gluconate and miltefosine) remained unchanged [153]. Proteomic analysis revealed that paromomycin-resistant *L. donovani* strains showed upregulation of proteins involved in vesicular trafficking, ribosomal proteins, glycolytic enzymes, and stress proteins [154].

2.4.5 Other Drugs

Fluconazole

Treatment with fluconazole (200 mg daily for 6 weeks), an orally active antifungal azole, has been found to be safe and effective in treating CL caused by *L. major* [155]. In a clinical trial in CL caused by *L. braziliensis*, a 75–100 % efficacy rate was shown depending on the dose of fluconazole used [156]. Another clinical trial in CL caused by *L. major* reported an 80 % cure rate using 400 mg of oral fluconazole [157]. The mechanism of action of fluconazole against *Leishmania* is not known. In *Trypanosoma cruzi*, fluconazole inhibits the cytochrome P450 enzyme, sterol 14α-demethylase, with consequent loss of normal sterols and accumulation of 14α-methyl sterols [158].

Sitamaquine

Sitamaquine (1 mg/kg/day for 2 weeks) has shown promise as an orally effective agent for the treatment of VL [159]. In Phase II clinical trials conducted in India [160] and Kenya [161] a ≥80 % cure rate was shown in both locations. An earlier Phase II clinical trial in Brazil against *L. chagasi* produced similar results [162]. But, a topical application against CL caused by *L. major* in experimental animal model failed to clear the infection [163]. Sitamaquine has significant side effects, namely methemoglobinema and nephrotoxicity [164]. Entry of sitamaquine into the cell does not require transport proteins. Rather, the drug directly interacts with the lipid bilayer and accumulates in acidocalcisome. The drug alkalizes the acidocalcisome after accumulation. However, the level of accumulation does not directly correlate with the cytotoxic activity and the mechanisms of actions are not well understood [164–166]. *L. tropica*

amastigotes showed cytoplasmic condensation when exposed to sitamaquine [22] and oxidation of hemoglobin has also been reported [167]. The cytosol and the mitochondria are apparent targets, as cytosol corrosion and compromised mitochondrial membrane integrity were observed in sitamaquine-affected cells [164]. To date, there are no reports of clinical unresponsiveness to sitamaquine. Also, sitamaquine possesses a short elimination half-life, which reduces the potential of resistance. Laboratory-raised resistant strains have the ability to efflux the drug from their cytoplasm using an unknown energy-dependent process [168]. A recent study reported that sitamaquine-resistance *L. donovani* accumulated fivefold less drug compared to wild type, and reduced accumulation was not related to a modification of the drug uptake system. In resistant strain, phosphatidylethanolamine-*N*-methyl-transferase was partially inactivated which strongly affected sterol and phospholipid metabolisms. However, the author failed to demonstrate whether reduced phosphatidylethanolamine-*N*-methyl-transferase activity has a role in generation of resistant phenotypes [169].

α-Difluoromethyl Ornithine

It has been observed that modulation of the polyamine biosynthetic pathway by inhibitors like α-difluoromethyl ornithine (DFMO) may be leishmanicidal [170, 171]. DFMO is a suicide inhibitor of ornithine decarboxylase, the rate-limiting enzyme in the polyamine biosynthetic pathway [172]. Although DFMO was used successfully against sleeping sickness [173], it has never been used clinically against leishmaniasis. In vitro resistance to this ornithine analogue has been reported [171, 174]. DFMO resistance has been shown to be associated with increased ornithine decarboxylase activity [174] and unstable amplification of two extrachromosomal elements [175]. *L. donovani* strains resistant to difluoromethyl ornithine have been shown to upregulate proteins associated with free radical detoxification and polyamine metabolism [176]. Bis-benzyl polyamine analogues such as MDL27695 that condense DNA were able to inhibit *L. donovani* promastigote growth in vitro [177]. Thus, drugs that manipulate the polyamine biosynthetic pathway may be used as alternate therapies for leishmaniasis.

Antifolates

Although antifolates are not used clinically against leishmaniasis, in vitro study shows that an anticancer drug like methotrexate (MTX) has considerable potential against leishmaniasis. Also, new drug targets have been identified by studying MTX resistance in vitro.

Mechanisms of Action

Antifolates (like MTX) are specific inhibitors of dihydrofolate reductase (DHFR). DHFR is the key enzyme for providing reduced folates to the cell. Reduced folates are utilized as cofactors in a variety of one-carbon transfer reactions such as in the synthesis of thymidylate. Furthermore, reduction of folate production is instrumental in the prevention of DNA and RNA production in the parasites. In plants and protozoans, DHFR is fused to thymidylate synthase (TS), resulting in a bifunctional DHFR-TS enzyme [178, 179]. DHFR reduces DHF to tetrahydrofolate (THF), which is then converted to dTMP by TS. MTX is an analogue of dihydrofolate (DHF). Competitive inhibition of DHFR by MTX allows TS to run continuously, depleting the cells of THF, thereby inhibiting growth.

Pteridine reductase (PTR1) of *Leishmania* reduces pteridines such as biopterin and folate [180], and it has the potential to act as a bypass and/or modulator of DHFR inhibition under physiological conditions. This suggests a reason that may explain why antifolate chemotherapy has not been very successful in leishmaniasis. Therefore, successful antifolate chemotherapy in *Leishmania* will have to target both DHFR and PTR1.

Mechanisms of Resistance

The mechanisms of MTX resistance have been studied by analyzing MTX-resistant strains raised by stepwise selection in vitro [179, 181]. It has been observed that *L. major* promastigotes resistant to structurally unrelated drugs like primaquine or terbinafine that produce H-region amplification are highly cross-resistant to MTX [182]. A second MTX-resistant *L. donovani* strain was cross-resistant to aminopterin, but just as sensitive to pyrimethamine, trimethoprim, and cytotoxic purine and pyrimidine analogues [183]. The first mutation observed was the amplification of the DHFR-TS gene as a part of the R-locus [184]. Later, another locus was found to be amplified in response to MTX resistance, which was named the H locus [185]. PTR1 was present in the H locus; when overexpressed, it may reduce DHF to THF, thereby providing resistance to MTX. PGPA overexpression has also been observed in *Leishmania* [186]. Another gene *ORF G* was identified by functional cloning in the LD1/CD1 genomic locus that is frequently amplified in several *Leishmania* strains. Overexpression of *ORF G* provided MTX resistance by increasing the uptake of pterins and selectively increasing the uptake of folic acid but not MTX. This compensated for the mutations in the high-affinity folate/MTX transporter of the resistant mutants. Amplification of DHFR-TS in MTX-resistant *Leishmania* was only observed in *L. major* but not in any other species. On the other hand, the PTR1 amplification and reduced uptake were observed in all species selected for MTX resistance in vitro [187]. The reason for this discrepancy is unknown.

It has also been shown that *Leishmania* has several folate transporter genes as it is a folate auxotroph. FT5 is a high-affinity folate transporter, and MTX transport in a resistant mutant (FT5 null) is inhibited only at low substrate

concentrations (50 nM) [188]. At other times, it has been observed that MTX-resistant *L. donovani* is genetically deficient in other folate-MTX transporter(s) [183]. Polyglutamylation of folates and MTX is an important determinant of MTX susceptibility. Modulation of the folylpolyglutamate synthase (FPGS) responsible for polyglutamylation has recently been found to be responsible for MTX resistance in *Leishmania*. However, FPGS transfectants were much more sensitive to MTX in folate-deficient medium [189]. It has also been observed that presence of shorter glutamate chains on MTX correlated with resistance [189]. Apurinic/apyrimidinic (AP) endonuclease is a component of DNA base excision repair pathway and also plays a role in antioxidant system. *L. major* cells overexpressing AP endonuclease showed resistance against MTX [190].

Methotrexate resistance has also been tied to the presence of two proteins, methionine adenosyltransferase and *S*-adenosylmethionine, and their anti-methotrexate functions. According to Drummelsmith, *L. major* methionine adenosyltransferase levels spiked after preliminary administration of methotrexate, leading to detoxification. Furthermore, cellular *S*-adenosylmethionine levels increased, and the strong correlation between increased *S*-adenosylmethionine production and folate regeneration was noted [191].

Modification of drug targets by point mutations has been implicated in MTX resistance. MTX-resistant *L. major* exhibits an amplification of DHFR-TS along with structurally altered DHFR-TS [192]. The altered DHFR revealed an Met53-to-Arg substitution. This resulted in a 30-fold increase in the K_i for MTX in the mutant enzyme when compared to the wild type.

3 Spread of Resistance

Leishmaniasis is primarily a zoonotic disease with dogs and wild canids, carnivores, rodents, sloths, and anteaters as reservoir hosts. However, VL caused by *L. donovani* in India, China, and East Africa, as well as the CL caused by *L. tropica* in the Mediterranean countries, is anthroponotic. There are no known animal reservoir hosts other than man for these two parasite species. Therefore, when the parasite becomes resistant to a drug, the resistance spreads quickly and efficiently.

Pentavalent antimonial unresponsiveness is an emerging problem in endemic areas. Therefore, information on factors which may modulate the transmission of drug-resistant phenotypes and parasites during the life cycle is necessary. Bhattacharyya et al. [193] reported that wild-type parasites isolated from VL patients, who were clinically cured after treatment with Sb(V), were a mixture of resistant and sensitive cells. The resistant promastigotes were also resistant as amastigotes in vivo. It was further observed that Sb(V)-sensitive parasites could be made resistant to the drug by repeated passages in experimental animals followed by incomplete treatment with suboptimal doses of the drug. These results suggested that the steady rise in Sb(V) unresponsiveness of VL patients in India is due to infection with resistant parasites, generated as a result of irregular and often incomplete treatment of the patients [193].

Using a continuous drug pressure protocol, Sereno et al. [194] induced pentamidine-resistant *L. mexicana* amastigotes. Two clones with different levels of resistance to pentamidine were selected in vitro—LmPENT5 resistant to 5 μM and LmPENT20 resistant to 20 μM of pentamidine. During in vitro infectivity experiments, axenically grown LmPENT20 amastigotes remained pentamidine resistant, whereas LmPENT5 amastigotes lost their ability to resist pentamidine. These results indicate that the level of pentamidine tolerated by resistant amastigotes after the life cycle was dependent on the induced level of resistance. This factor may be significant in the in vivo transmission of drug-resistant parasites by sand flies. These observations demonstrate that different factors may modulate the transmission of *Leishmania* drug resistance during the parasite's life cycle.

Recently, it has been observed that *Leishmania* species develop much of their resistance through the possession of multiple chromosomes. Although possession of multiple chromosome copies is typically detrimental, *Leishmania* possesses an innate core of chromosomes throughout a given species. The ability to have several variations of genes within a single organism allows for rapid selection of ideal genetics, particularly in the asexually reproducing *Leishmania.*

Plasmids play a tremendous role in leishmanial drug resistance. Each plasmid contains several genes that the parasite may "turn on" in order to respond to stressors. However, the plasmids are typically lost after 100–200 cellular divisions. As such, the plasmids themselves have evolved to compartmentalize at least one plasmid each time the parasite cell divides, thus conserving the genotype and enabling rapid evolution and resistance to pharmaceutical stressors [195].

Primarily, drug resistance in *Leishmania* is a result of adaptation to drug stress. Target specificity in leishmanicidal drugs causes the parasite to induce rapid selectivity and develop countermeasures to aid in survival. This can be seen in the previously discussed amphotericin B (AmB) resistance in *Leishmania*. AmB targets and binds to the sterols (mainly ergosterol) present in the cellular membrane. The subsequent disruption of the membrane allows AmB to form pores and small metabolites to pass through the pores. However, *Leishmania* have adapted to AmB by reducing their membrane sterol counts and integrating more unsaturated fatty acids versus saturated fatty acids into the bilayer in order to increase membrane fluidity. *Leishmania* will use these adaptations to enact broad-spectrum resistance to other drugs with similar cellular targets.

Another possibility for drug resistance spread in *Leishmania* is the half-lives of the anti-leishmanials themselves. Leishmanicidals such as miltefosine possess extraordinarily long half-lives; however, the lengthy duration of the drug in the patient's body also allows the parasites to have a longer exposure time to the drug as well. This grants *Leishmania* the ability to respond to selective pressure for a longer time period, thus developing a genome resistant to the drug.

Basic fitness distinctions between drug-stressed and wild-type specimens may also play a significant role in the spread of drug resistance in *Leishmania.* Fitness with regard to pathogens is defined as the ability of a pathogen to thrive, reproduce, and transmit itself into new hosts. Fitness becomes a significant factor in resistance communication due to the so-called metabolic price the parasite must pay in return for resistance to a drug. However, this fact is not evident in the parasite. This may be attributed to its ability to thrive in the vector's (sand fly) midgut, a relatively hostile environment. As such, the parasite has developed the uncanny ability to successfully develop resistance to a variety of stressors without a marked drop in fitness within a given population. In a recent mathematical study it was observed that *L. donovani* possessed a higher degree of fitness over drug-sensitive wild-type varieties [196].

Leishmania has also developed resistance by increasing its virulence. Resistant strains have been shown to exhibit a higher cell density and a higher metacyclic cell count, increasing their infectivity. Field isolates of *L. donovani* exhibit increased virulence in comparison to their vitro counterparts. This is due to an increased number of surface glycoconjugates, which are associated with the generation of the metacyclic parasites [197].

The induction of apoptotic cell death is a common effector in the control of pathogens, particularly in the control of *Leishmania*. However, it has been observed that strains resistant to front-line anti-leishmanial drugs exhibit a resistance to the associated apoptosis. Furthermore, leishmanial resistance to apoptosis induced by one drug allows the parasite to acquire resistance to apoptosis induced by other drugs and hence spreading resistance [198].

References

1. Choi CM, Lerner EA. Leishmaniasis: recognition and management with a focus on the immunocompromised patient. Am J Clin Dermatol. 2002;3(2):91–105.
2. Silva ES, et al. Visceral leishmaniasis caused by *Leishmania (Viannia) braziliensis* in a patient infected with human immunodeficiency virus. Rev Inst Med Trop Sao Paulo. 2002;44(3):145–9.
3. Monge-Maillo B, et al. Visceral leishmaniasis and HIV coinfection in the Mediterranean region. PLoS Negl Trop Dis. 2014;8(8):e3021.
4. Faraut-Gambarelli F, et al. In vitro and in vivo resistance of *Leishmania infantum* to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. Antimicrob Agents Chemother. 1997;41(4):827–30.
5. Jackson JE, et al. Quantitative in vitro drug potency and drug susceptibility evaluation of *Leishmania spp*. from patients unresponsive to pentavalent antimony therapy. Am J Trop Med Hyg. 1990;43(5):464–80.
6. Ashutosh, Sundar S, Goyal N. Molecular mechanisms of antimony resistance in *Leishmania*. J Med Microbiol. 2007;56(Pt 2):143–53.
7. Sundar S, et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis. 2000;31(4):1104–7.
8. Ouellette M, et al. Amplification of ABC transporter gene pgpA and of other heavy metal resistance genes in *Leishmania tarentolae* and their study by gene transfection and gene disruption. Methods Enzymol. 1998;292:182–93.
9. Urbina JA. Lipid biosynthesis pathways as chemotherapeutic targets in kinetoplastid parasites. Parasitology. 1997;114(Suppl):S91–9.
10. Sundar S, et al. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med. 2002;347(22):1739–46.
11. Borst P, Ouellette M. New mechanisms of drug resistance in parasitic protozoa. Annu Rev Microbiol. 1995;49:427–60.
12. Herwaldt BL. Leishmaniasis. Lancet. 1999;354(9185):1191–9.
13. Goodwin LG. Pentostam (sodium stibogluconate); a 50-year personal reminiscence. Trans R Soc Trop Med Hyg. 1995;89(3):339–41.
14. Lugo de Yarbuh A, et al. Antimony determination in tissues and serum of hamsters infected with *Leishmania garnhami* and treated with meglumine antimoniate. Ann Trop Med Parasitol. 1994;88(1):37–41.
15. Mottram JC, Coombs GH. *Leishmania mexicana*: enzyme activities of amastigotes and promastigotes and their inhibition by antimonials and arsenicals. Exp Parasitol. 1985;59(2):151–60.
16. Roberts WL, Berman JD, Rainey PM. In vitro antileishmanial properties of tri- and pentavalent antimonial preparations. Antimicrob Agents Chemother. 1995;39(6):1234–9.
17. Sereno D, Lemesre JL. Axenically cultured amastigote forms as an in vitro model for investigation of antileishmanial agents. Antimicrob Agents Chemother. 1997;41(5):972–6.
18. Sereno D, et al. Axenically grown amastigotes of *Leishmania infantum* used as an in vitro model to investigate the pentavalent antimony mode of action. Antimicrob Agents Chemother. 1998;42(12):3097–102.
19. Mukhopadhyay R, Shi J, Rosen BP. Purification and characterization of Acr2p, the *Saccharomyces cerevisiae* arsenate reductase. J Biol Chem. 2000;275(28):21149–57.
20. Zhou Y, et al. *Leishmania major* LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug pentostam. J Biol Chem. 2004;279(36):37445–51.
21. Denton H, McGregor JC, Coombs GH. Reduction of antileishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. Biochem J. 2004;381(Pt 2):405–12.
22. Langreth SG, et al. Fine-structural alterations in *Leishmania tropica* within human macrophages exposed to antileishmanial drugs in vitro. J Protozool. 1983;30(3):555–61.
23. Berman JD, Waddell D, Hanson BD. Biochemical mechanisms of the antileishmanial activity of sodium stibogluconate. Antimicrob Agents Chemother. 1985;27(6):916–20.
24. Chakraborty AK, Majumder HK. Mode of action of pentavalent antimonials: specific inhibition of type I DNA topoisomerase of *Leishmania donovani*. Biochem Biophys Res Commun. 1988;152(2):605–11.

25. Demicheli C, et al. Antimony(V) complex formation with adenine nucleosides in aqueous solution. Biochim Biophys Acta. 2002;1570(3):192–8.
26. Mehta A, Shaha C. Mechanism of metalloid-induced death in *Leishmania spp*. role of iron, reactive oxygen species, Ca2+, and glutathione. Free Radic Biol Med. 2006;40(10):1857–68.
27. Mookerjee Basu J, et al. Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. Antimicrob Agents Chemother. 2006;50(5):1788–97.
28. Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. J Biol Chem. 2004;279(38):39925–32.
29. Ritmeijer K, et al. Ethiopian visceral leishmaniasis: generic and proprietary sodium stibogluconate are equivalent; HIV co-infected patients have a poor outcome. Trans R Soc Trop Med Hyg. 2001;95(6):668–72.
30. Fernandez-Guerrero ML, et al. Visceral leishmaniasis in immunocompromised hosts. Am J Med. 1987;83(6):1098–102.
31. Murray HW, et al. Requirement for T cells and effect of lymphokines in successful chemotherapy for an intracellular infection. Experimental visceral leishmaniasis. J Clin Invest. 1989;83(4):1253–7.
32. Escobar P, Yardley V, Croft SL. Activities of hexadecylphosphocholine (miltefosine), Am Bisome, and sodium stibogluconate (Pentostam) against Leishmania donovani in immunodeficient scid mice. Antimicrob Agents Chemother. 2001;45(6):1872–5.
33. Murray HW, et al. Role and effect of IL-2 in experimental visceral leishmaniasis. J Immunol. 1993;151(2):929–38.
34. Alexander J, et al. Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur J Immunol. 2000;30(10):2935–43.
35. Murray HW, et al. Interleukin-12 regulates the response to chemotherapy in experimental visceral leishmaniasis. J Infect Dis. 2000;182(5):1497–502.
36. Sanders OI, et al. Antimonite is accumulated by the glycerol facilitator GlpF in *Escherichia coli*. J Bacteriol. 1997;179(10):3365–7.
37. Wysocki R, et al. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. Mol Microbiol. 2001;40(6):1391–401.
38. Liu Z, et al. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci U S A. 2002;99(9):6053–8.
39. Gourbal B, et al. Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. J Biol Chem. 2004;279(30):31010–7.
40. Agre P, et al. Aquaporin water channels--from atomic structure to clinical medicine. J Physiol. 2002;542(Pt 1):3–16.
41. Uzcategui NL, et al. Alteration in glycerol and metalloid permeability by a single mutation in the extracellular C-loop of *Leishmania major* aquaglyceroporin LmAQP1. Mol Microbiol. 2008;70(6):1477–86.
42. Mukhopadhyay R, et al. The role of alanine 163 in solute permeability of *Leishmania major* aquaglyceroporin LmAQP1. Mol Biochem Parasitol. 2011;175(1):83–90.
43. Marquis N, et al. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant *Leishmania*. Mol Microbiol. 2005;57(6):1690–9.
44. Mandal S, et al. Assessing aquaglyceroporin gene status and expression profile in antimony-susceptible and -resistant clinical isolates of *Leishmania donovani* from India. J Antimicrob Chemother. 2010;65(3):496–507.
45. Maharjan M, et al. Role of aquaglyceroporin (AQP1) gene and drug uptake in antimony-resistant clinical isolates of *Leishmania donovani*. Am J Trop Med Hyg. 2008;79(1):69–75.
46. Decuypere S, et al. Gene expression analysis of the mechanism of natural Sb(V) resistance in *Leishmania donovani* isolates from Nepal. Antimicrob Agents Chemother. 2005;49(11):4616–21.
47. Mandal G, et al. Modulation of *Leishmania major* aquaglyceroporin activity by a mitogen-activated protein kinase. Mol Microbiol. 2012;85(6):1204–18.
48. Shaked-Mishan P, et al. Novel intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. J Biol Chem. 2001;276(6):3971–6.
49. Zhou Y, Bhattacharjee H, Mukhopadhyay R. Bifunctional role of the leishmanial antimonate reductase LmACR2 as a protein tyrosine phosphatase. Mol Biochem Parasitol. 2006;148(2):161–8.
50. Jeddi F, Piarroux R, Mary C. Antimony resistance in *Leishmania*, focusing on experimental research. J Trop Med. 2011:695382.
51. Ouellette M, et al. ABC transporters in *Leishmania* and their role in drug resistance. Drug Resist Updat. 1998;1:43–8.
52. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol. 1992;8:67–113.
53. Detke S, Katakura K, Chang KP. DNA amplification in arsenite-resistant *Leishmania*. Exp Cell Res. 1989;180(1):161–70.
54. Ouellette M, et al. Direct and inverted DNA repeats associated with P-glycoprotein gene amplification in drug resistant *Leishmania*. EMBO J. 1991;10(4):1009–16.
55. Grondin K, Papadopoulou B, Ouellette M. Homologous recombination between direct repeat sequences yields P-glycoprotein containing amplicons in arsenite resistant *Leishmania*. Nucleic Acids Res. 1993;21(8):1895–901.
56. Ferreira-Pinto KC, et al. *Leishmania (V.) guyanensis*: isolation and characterization of glucantime-resistant cell lines. Can J Microbiol. 1996;42(9):944–9.
57. Legare D, et al. Efflux systems and increased trypanothione levels in arsenite-resistant *Leishmania*. Exp Parasitol. 1997;87(3):275–82.
58. Haimeur A, et al. Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. Mol Biochem Parasitol. 2000;108(1):131–5.
59. Callahan HL, Beverley SM. Heavy metal resistance: a new role for P-glycoproteins in *Leishmania*. J Biol Chem. 1991;266(28):18427–30.
60. Papadopoulou B, et al. Gene disruption of the P-glycoprotein related gene pgpa of *Leishmania tarentolae*. Biochem Biophys Res Commun. 1996;224(3):772–8.
61. Papadopoulou B, et al. Contribution of the *Leishmania* P-glycoprotein-related gene ltpgpA to oxyanion resistance. J Biol Chem. 1994;269(16):11980–6.
62. Mukhopadhyay R, et al. Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. Proc Natl Acad Sci U S A. 1996;93(19):10383–7.
63. Grondin K, et al. Co-amplification of the gamma-glutamylcysteine synthetase gene gsh1 and of the ABC transporter gene pgpA in arsenite-resistant *Leishmania tarentolae*. EMBO J. 1997;16(11):3057–65.
64. Haimeur A, et al. Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant *Leishmania*. Mol Microbiol. 1999;34(4):726–35.
65. Legare D, et al. The Leishmania ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. J Biol Chem. 2001;276(28):26301–7.
66. Dey S, et al. High level arsenite resistance in *Leishmania tarentolae* is mediated by an active extrusion system. Mol Biochem Parasitol. 1994;67(1):49–57.
67. Dey S, et al. An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. Proc Natl Acad Sci U S A. 1996;93(5):2192–7.
68. Callahan HL, et al. The PGPA gene of *Leishmania major* mediates antimony (SbIII) resistance by decreasing influx and not by increasing efflux. Mol Biochem Parasitol. 1994;68(1):145–9.

69. Leprohon P, Legare D, Ouellette M. Intracellular localization of the ABCC proteins of *Leishmania* and their role in resistance to antimonials. Antimicrob Agents Chemother. 2009;53(6):2646–9.
70. Manzano JI, et al. A new ABC half-transporter in *Leishmania major* is involved in resistance to antimony. Antimicrob Agents Chemother. 2013;57(8):3719–30.
71. Kumar D, et al. Biomarkers of antimony resistance: need for expression analysis of multiple genes to distinguish resistance phenotype in clinical isolates of *Leishmania donovani*. Parasitol Res. 2012;111(1):223–30.
72. Mittal MK, et al. Characterization of natural antimony resistance in *Leishmania donovani* isolates. Am J Trop Med Hyg. 2007;76(4):681–8.
73. Adaui V, et al. Comparison of gene expression patterns among *Leishmania braziliensis* clinical isolates showing a different in vitro susceptibility to pentavalent antimony. Parasitology. 2011;138(2):183–93.
74. Mandal G, et al. Increased levels of thiols protect antimony unresponsive *Leishmania donovani* field isolates against reactive oxygen species generated by trivalent antimony. Parasitology. 2007;134(Pt 12):1679–87.
75. Mandal G, et al. Functionality of drug efflux pumps in antimonial resistant *Leishmania donovani* field isolates. Indian J Biochem Biophys. 2009;46(1):86–92.
76. Wyllie S, et al. Elevated levels of tryparedoxin peroxidase in antimony unresponsive *Leishmania donovani* field isolates. Mol Biochem Parasitol. 2010;173(2):162–4.
77. Brochu C, et al. Antimony uptake systems in the protozoan parasite *Leishmania* and accumulation differences in antimony-resistant parasites. Antimicrob Agents Chemother. 2003;47(10):3073–9.
78. Genest PA, et al. A protein of the leucine-rich repeats (LRRs) superfamily is implicated in antimony resistance in *Leishmania infantum* amastigotes. Mol Biochem Parasitol. 2008;158(1):95–9.
79. Mukherjee B, et al. Antimony-resistant but not antimony-sensitive *Leishmania donovani* up-regulates host IL-10 to overexpress multidrug-resistant protein 1. Proc Natl Acad Sci U S A. 2013;110(7):E575–82.
80. Mukhopadhyay R, et al. Characterisation of antimony-resistant *Leishmania donovani* isolates: biochemical and biophysical studies and interaction with host cells. Int J Parasitol. 2011;41(13–14):1311–21.
81. Tandon R, et al. Characterization of the proliferating cell nuclear antigen of *Leishmania donovani* clinical isolates and its association with antimony resistance. Antimicrob Agents Chemother. 2014;58(6):2997–3007.
82. Perry MR, et al. Chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs in a mouse model of visceral leishmaniasis. Proc Natl Acad Sci U S A. 2013;110(49):19932–7.
83. Ashutosh, et al. Downregulation of mitogen-activated protein kinase 1 of *Leishmania donovani* field isolates is associated with antimony resistance. Antimicrob Agents Chemother. 2012;56(1):518–25.
84. Bhandari V, et al. Increased parasite surface antigen-2 expression in clinical isolates of *Leishmania donovani* augments antimony resistance. Biochem Biophys Res Commun. 2013;440(4):646–51.
85. Schafer C, et al. Reduced antimony accumulation in ARM58-overexpressing *Leishmania infantum*. Antimicrob Agents Chemother. 2014;58(3):1565–74.
86. Rosenthal E, Marty P. Recent understanding in the treatment of visceral leishmaniasis. J Postgrad Med. 2003;49(1):61–8.
87. Davidson RN, et al. Liposomal amphotericin B in drug-resistant visceral leishmaniasis. Lancet. 1991;337(8749):1061–2.
88. Saha AK, Mukherjee T, Bhaduri A. Mechanism of action of amphotericin B on *Leishmania donovani* promastigotes. Mol Biochem Parasitol. 1986;19(3):195–200.
89. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev. 2006;19(1):111–26.
90. Cuna WR, et al. Enhancement of a TH1 immune response in amphotericin B-treated mucocutaneous leishmaniasis. J Biomed Biotechnol. 2007;2007(5):96410.
91. Paila YD, Saha B, Chattopadhyay A. Amphotericin B inhibits entry of *Leishmania donovani* into primary macrophages. Biochem Biophys Res Commun. 2010;399(3):429–33.
92. Sundar S, Chatterjee M. Visceral leishmaniasis - current therapeutic modalities. Indian J Med Res. 2006;123(3):345–52.
93. Chappuis F, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol. 2007;5(11):873–82.
94. Sundar S, Chakravarty J. Liposomal amphotericin B and leishmaniasis: dose and response. J Glob Infect Dis. 2010;2(2):159–66.
95. de Carvalho RF, et al. Leishmanicidal activity of amphotericin B encapsulated in PLGA-DMSA nanoparticles to treat cutaneous leishmaniasis in C57BL/6 mice. Exp Parasitol. 2013;135(2):217–22.
96. Ruiz HK, et al. New amphotericin B-gamma cyclodextrin formulation for topical use with synergistic activity against diverse fungal species and *Leishmania spp*. Int J Pharm. 2014;473(1–2):148–57.
97. Mbongo N, et al. Mechanism of amphotericin B resistance in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother. 1998;42(2):352–7.
98. Singh AK, Papadopoulou B, Ouellette M. Gene amplification in amphotericin B-resistant *Leishmania tarentolae*. Exp Parasitol. 2001;99(3):141–7.
99. Equbal A, et al. Stage-dependent expression and up-regulation of trypanothione synthetase in amphotericin B resistant *Leishmania donovani*. PLoS One. 2014;9(6):e97600.
100. Mishra M, et al. Amphotericin versus pentamidine in antimony-unresponsive kala-azar. Lancet. 1992;340(8830):1256–7.
101. Das VN, et al. A randomized clinical trial of low dosage combination of pentamidine and allopurinol in the treatment of antimony unresponsive cases of visceral leishmaniasis. J Assoc Physicians India. 2001;49:609–13.
102. Masmoudi A, et al. Old World cutaneous leishmaniasis: diagnosis and treatment. J Dermatol Case Rep. 2013;7(2):31–41.
103. Basselin M, Lawrence F, Robert-Gero M. Pentamidine uptake in *Leishmania donovani* and *Leishmania amazonensis* promastigotes and axenic amastigotes. Biochem J. 1996;315(Pt 2):631–4.
104. Kandpal M, et al. Correlation between inhibition of growth and arginine transport of *Leishmania donovani* promastigotes in vitro by diamidines. Life Sci. 1996;59(7):PL75–80.
105. Kandpal M, Tekwani BL. Polyamine transport systems of *Leishmania donovani* promastigotes. Life Sci. 1997;60(20):1793–801.
106. Reguera R, et al. Putrescine uptake inhibition by aromatic diamidines in *Leishmania infantum* promastigotes. Biochem Pharmacol. 1994;47(10):1859–66.
107. Basselin M, Coombs GH, Barrett MP. Putrescine and spermidine transport in *Leishmania*. Mol Biochem Parasitol. 2000;109(1):37–46.
108. Basselin M, et al. Effects of pentamidine on polyamine level and biosynthesis in wild-type, pentamidine-treated, and pentamidine-resistant *Leishmania*. Exp Parasitol. 1997;85(3):274–82.
109. Basselin M, et al. Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. Antimicrob Agents Chemother. 2002;46(12):3731–8.
110. Hentzer B, Kobayasi T. The ultrastructural changes of *Leishmania tropica* after treatment with pentamidine. Ann Trop Med Parasitol. 1977;71(2):157–66.
111. Vercesi AE, Docampo R. Ca2+ transport by digitonin-permeabilized *Leishmania donovani*. Effects of Ca2+, pentamidine and WR-6026 on mitochondrial membrane potential in situ. Biochem J. 1992;284(Pt 2):463–7.
112. Wong IL, et al. Quinacrine and a novel apigenin dimer can synergistically increase the pentamidine susceptibility of the protozoan

parasite *Leishmania*. J Antimicrob Chemother. 2009;63(6):1179–90.
113. Coelho AC, Beverley SM, Cotrim PC. Functional genetic identification of PRP1, an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*. Mol Biochem Parasitol. 2003;130(2):83–90.
114. Croft SL, et al. The activity of alkyl phosphorylcholines and related derivatives against *Leishmania donovani*. Biochem Pharmacol. 1987;36(16):2633–6.
115. Kuhlencord A, et al. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrob Agents Chemother. 1992;36(8):1630–4.
116. Jha TK, et al. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N Engl J Med. 1999;341(24):1795–800.
117. Sundar S, et al. Oral miltefosine treatment in children with mild to moderate Indian visceral leishmaniasis. Pediatr Infect Dis J. 2003;22(5):434–8.
118. Mohebali M, et al. Comparison of miltefosine and meglumine antimoniate for the treatment of zoonotic cutaneous leishmaniasis (ZCL) by a randomized clinical trial in Iran. Acta Trop. 2007;103(1):33–40.
119. Zerpa O, et al. Diffuse cutaneous leishmaniasis responds to miltefosine but then relapses. Br J Dermatol. 2007;156(6):1328–35.
120. Dorlo TP, et al. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. J Antimicrob Chemother. 2012;67(11):2576–97.
121. Lux H, et al. Ether lipid metabolism, GPI anchor biosynthesis, and signal transduction are putative targets for anti-leishmanial alkyl phospholipid analogues. Adv Exp Med Biol. 1996;416:201–11.
122. Croft SL, Seifert K, Duchene M. Antiprotozoal activities of phospholipid analogues. Mol Biochem Parasitol. 2003;126(2):165–72.
123. Lux H, et al. Ether--lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether--lipid analogues in *Leishmania*. Mol Biochem Parasitol. 2000;111(1):1–14.
124. Zufferey R, Mamoun CB. Choline transport in *Leishmania major* promastigotes and its inhibition by choline and phosphocholine analogs. Mol Biochem Parasitol. 2002;125(1–2):127–34.
125. Paris C, et al. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother. 2004;48(3):852–9.
126. Perez-Victoria FJ, Castanys S, Gamarro F. *Leishmania donovani* resistance to miltefosine involves a defective inward translocation of the drug. Antimicrob Agents Chemother. 2003;47(8):2397–403.
127. Perez-Victoria JM, et al. Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. Antimicrob Agents Chemother. 2001;45(9):2468–74.
128. Castanys-Munoz E, et al. A novel ATP-binding cassette transporter from *Leishmania* is involved in transport of phosphatidylcholine analogues and resistance to alkyl-phospholipids. Mol Microbiol. 2007;64(5):1141–53.
129. Mishra J, Singh S. Miltefosine resistance in *Leishmania donovani* involves suppression of oxidative stress-induced programmed cell death. Exp Parasitol. 2013;135(2):397–406.
130. Kager PA, et al. Allopurinol in the treatment of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 1981;75(4):556–9.
131. Chunge CN, et al. Visceral leishmaniasis unresponsive to antimonial drugs. III. Successful treatment using a combination of sodium stibogluconate plus allopurinol. Trans R Soc Trop Med Hyg. 1985;79(5):715–8.
132. Singh NK, et al. Combination therapy in Kala-azar. J Assoc Physicians India. 1995;43(5):319–20.
133. Martinez S, Marr JJ. Allopurinol in the treatment of American cutaneous leishmaniasis. N Engl J Med. 1992;326(11):741–4.
134. Nelson DJ, et al. Allopurinol ribonucleoside as an antileishmanial agent. Biological effects, metabolism, and enzymatic phosphorylation. J Biol Chem. 1979;254(22):11544–9.
135. Rainey P, Santi DV. Metabolism and mechanism of action of formycin B in *Leishmania*. Proc Natl Acad Sci U S A. 1983;80(1):288–92.
136. Dalbeth N, Stamp L. Allopurinol dosing in renal impairment: walking the tightrope between adequate urate lowering and adverse events. Semin Dial. 2007;20(5):391–5.
137. Jernigan JA, et al. In vitro activity of atovaquone against *Leishmania chagasi* promastigotes. Antimicrob Agents Chemother. 1996;40(4):1064.
138. Murray HW, Hariprashad J. Activity of oral atovaquone alone and in combination with antimony in experimental visceral leishmaniasis. Antimicrob Agents Chemother. 1996;40(3):586–7.
139. Fry M, Beesley JE. Mitochondria of mammalian Plasmodium spp. Parasitology. 1991;102(Pt 1):17–26.
140. Fry M, Pudney M. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). Biochem Pharmacol. 1992;43(7):1545–53.
141. Cauchetier E, et al. Characterisation of atovaquone resistance in *Leishmania infantum* promastigotes. Int J Parasitol. 2002;32(8):1043–51.
142. Chunge CN, et al. Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. Trans R Soc Trop Med Hyg. 1990;84(2):221–5.
143. Jha TK, et al. Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for treating visceral leishmaniasis in North Bihar, India. BMJ. 1998;316(7139):1200–5.
144. Thakur CP, et al. Aminosidine plus sodium stibogluconate for the treatment of Indian kala-azar: a randomized dose-finding clinical trial. Trans R Soc Trop Med Hyg. 1995;89(2):219–23.
145. Melaku Y, et al. Treatment of kala-azar in southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. Am J Trop Med Hyg. 2007;77(1):89–94.
146. Sundar S, et al. Injectable paromomycin for Visceral leishmaniasis in India. N Engl J Med. 2007;356(25):2571–81.
147. Thakur CP, et al. Treatment of visceral leishmaniasis with injectable paromomycin (aminosidine). An open-label randomized phase-II clinical study. Trans R Soc Trop Med Hyg. 2000;94(4):432–3.
148. Shakya N, Bajpai P, Gupta S. Therapeutic switching in leishmania chemotherapy: a distinct approach towards unsatisfied treatment needs. J Parasit Dis. 2011;35(2):104–12.
149. Sosa N, et al. Randomized, double-blinded, phase 2 trial of WR 279,396 (paromomycin and gentamicin) for cutaneous leishmaniasis in Panama. Am J Trop Med Hyg. 2013;89(3):557–63.
150. Maarouf M, et al. Biochemical alterations in paromomycin-treated *Leishmania donovani* promastigotes. Parasitol Res. 1997;83(2):198–202.
151. Jhingran A, et al. Paromomycin: uptake and resistance in *Leishmania donovani*. Mol Biochem Parasitol. 2009;164(2):111–7.
152. Maarouf M, et al. Development and characterization of paromomycin-resistant *Leishmania donovani* promastigotes. Parasite. 1998;5(2):167–73.
153. Bhandari V, et al. Elucidation of cellular mechanisms involved in experimental paromomycin resistance in *Leishmania donovani*. Antimicrob Agents Chemother. 2014;58(5):2580–5.
154. Chawla B, et al. Paromomycin affects translation and vesicle-mediated trafficking as revealed by proteomics of paromomycin -susceptible -resistant *Leishmania donovani*. PLoS One. 2011;6(10), e26660.
155. Alrajhi AA, et al. Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. N Engl J Med. 2002;346(12):891–5.

156. Sousa AQ, et al. High-dose oral fluconazole therapy effective for cutaneous leishmaniasis due to *Leishmania (Vianna) braziliensis*. Clin Infect Dis. 2011;53(7):693–5.
157. Emad M, et al. Superior efficacy of oral fluconazole 400 mg daily versus oral fluconazole 200 mg daily in the treatment of cutaneous *Leishmania major* infection: a randomized clinical trial. J Am Acad Dermatol. 2011;64(3):606–8.
158. Goad LJ, et al. The activity of ketoconazole and other azoles against *Trypanosoma cruzi*: biochemistry and chemotherapeutic action in vitro. Mol Biochem Parasitol. 1989;32(2–3):179–89.
159. Sherwood JA, et al. Phase 2 efficacy trial of an oral 8-aminoquinoline (WR6026) for treatment of visceral leishmaniasis. Clin Infect Dis. 1994;19(6):1034–9.
160. Jha TK, et al. A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. Am J Trop Med Hyg. 2005;73(6):1005–11.
161. Wasunna MK, et al. A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. Am J Trop Med Hyg. 2005;73(5):871–6.
162. Dietze R, et al. Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by *Leishmania chagasi*. Am J Trop Med Hyg. 2001;65(6):685–9.
163. Garnier T, et al. In-vitro and in-vivo studies on a topical formulation of sitamaquine dihydrochloride for cutaneous leishmaniasis. J Pharm Pharmacol. 2006;58(8):1043–54.
164. Loiseau PM, Cojean S, Schrevel J. Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. Parasite. 2011;18(2):115–9.
165. Singh N, Kumar M, Singh RK. Leishmaniasis: current status of available drugs and new potential drug targets. Asian Pac J Trop Med. 2012;5(6):485–97.
166. Lopez-Martin C, et al. Sitamaquine sensitivity in *Leishmania* species is not mediated by drug accumulation in acidocalcisomes. Antimicrob Agents Chemother. 2008;52(11):4030–6.
167. Steinhaus RK, et al. Formation of methemoglobin and metmyoglobin using 8-aminoquinoline derivatives or sodium nitrite and subsequent reaction with cyanide. J Appl Toxicol. 1990;10(5):345–51.
168. Coimbra ES, et al. Mechanism of interaction of sitamaquine with *Leishmania donovani*. J Antimicrob Chemother. 2010;65(12):2548–55.
169. Imbert L, et al. Sitamaquine-resistance in *Leishmania donovani* affects drug accumulation and lipid metabolism. Biomed Pharmacother. 2014;68(7):893–7.
170. Kaur K, et al. Effects of DL-alpha-difluoromethylornithine on *Leishmania donovani* promastigotes. J Protozool. 1986;33(4):518–21.
171. Mukhopadhyay R, Kapoor P, Madhubala R. Characterization of alpha-difluoromethylornithine resistant *Leishmania donovani* and its susceptibility to other inhibitors of the polyamine biosynthetic pathway. Pharmacol Res. 1996;34(1–2):43–6.
172. Poulin R, et al. Mechanism of the irreversible inactivation of mouse ornithine decarboxylase by alpha-difluoromethylornithine. Characterization of sequences at the inhibitor and coenzyme binding sites. J Biol Chem. 1992;267(1):150–8.
173. Docampo R, Moreno SN. Current chemotherapy of human African trypanosomiasis. Parasitol Res. 2003;90(Supp 1):S10–3.
174. Coons T, et al. Alpha-difluoromethylornithine resistance in *Leishmania donovani* is associated with increased ornithine decarboxylase activity. Mol Biochem Parasitol. 1990;39(1):77–89.
175. Hanson S, et al. Unstable amplification of two extrachromosomal elements in alpha-difluoromethylornithine-resistant *Leishmania donovani*. Mol Cell Biol. 1992;12(12):5499–507.
176. Singh AK, et al. A quantitative proteomic screen to identify potential drug resistance mechanism in alpha-difluoromethylornithine (DFMO) resistant *Leishmania donovani*. J Proteomics. 2014;102:44–59.
177. Mukhopadhyay R, Madhubala R. Effects of bis(benzyl)polyamine analogs on *Leishmania donovani* promastigotes. Exp Parasitol. 1995;81(1):39–46.
178. Ivanetich KM, Santi DV. Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. FASEB J. 1990;4(6):1591–7.
179. Nare B, et al. New approaches to *Leishmania* chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. Parasitology. 1997;114(Suppl):S101–10.
180. Bello AR, et al. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci U S A. 1994;91(24):11442–6.
181. Ouellette M, et al. In: Rosen B.P.a.M.S., editor. Resolving the antibiotic paradox. New York: Plenum Publishing Corporation; 1998. p. 99–113.
182. Ellenberger TE, Beverley SM. Multiple drug resistance and conservative amplification of the H region in *Leishmania major*. J Biol Chem. 1989;264(25):15094–103.
183. Kaur K, et al. Methotrexate-resistant *Leishmania donovani* genetically deficient in the folate-methotrexate transporter. J Biol Chem. 1988;263(15):7020–8.
184. Coderre JA, et al. Overproduction of a bifunctional thymidylate synthetase-dihydrofolate reductase and DNA amplification in methotrexate-resistant *Leishmania tropica*. Proc Natl Acad Sci U S A. 1983;80(8):2132–6.
185. Beverley SM, et al. Unstable DNA amplifications in methotrexate-resistant *Leishmania* consist of extrachromosomal circles which relocalize during stabilization. Cell. 1984;38(2):431–9.
186. Gamarro F, et al. P-glycoprotein overexpression in methotrexate-resistant *Leishmania tropica*. Biochem Pharmacol. 1994;47(11):1939–47.
187. Kundig C, et al. Role of the locus and of the resistance gene on gene amplification frequency in methotrexate resistant *Leishmania tarentolae*. Nucleic Acids Res. 1999;27(18):3653–9.
188. Richard D, Kundig C, Ouellette M. A new type of high affinity folic acid transporter in the protozoan parasite *Leishmania* and deletion of its gene in methotrexate-resistant cells. J Biol Chem. 2002;277(33):29460–7.
189. El-Fadili A, et al. Effect of polyglutamylation of methotrexate on its accumulation and the development of resistance in the protozoan parasite *Leishmania*. Biochem Pharmacol. 2003;66(6):999–1008.
190. Gallego C, et al. Overexpression of AP endonuclease protects *Leishmania major* cells against methotrexate induced DNA fragmentation and hydrogen peroxide. Mol Biochem Parasitol. 2005;141(2):191–7.
191. Drummelsmith J, et al. Differential protein expression analysis of *Leishmania major* reveals novel roles for methionine adenosyltransferase and S-adenosylmethionine in methotrexate resistance. J Biol Chem. 2004;279(32):33273–80.
192. Arrebola R, et al. Isolation and characterization of a mutant dihydrofolate reductase-thymidylate synthase from methotrexate-resistant *Leishmania* cells. J Biol Chem. 1994;269(14):10590–6.
193. Bhattacharyya A, Mukherjee M, Duttagupta S. Studies on stibanate unresponsive isolates of *Leishmania donovani*. J Biosci. 2002;27(5):503–8.
194. Sereno D, Lemesre JL. In vitro life cycle of pentamidine-resistant amastigotes: stability of the chemoresistant phenotypes is dependent on the level of resistance induced. Antimicrob Agents Chemother. 1997;41(9):1898–903.
195. Boucher N, et al. The ribosomal RNA gene promoter and adjacent cis-acting DNA sequences govern plasmid DNA partitioning and stable inheritance in the parasitic protozoan *Leishmania*. Nucleic Acids Res. 2004;32(9):2925–36.
196. Vanaerschot M, et al. Drug resistance in vectorborne parasites: multiple actors and scenarios for an evolutionary arms race. FEMS Microbiol Rev. 2014;38(1):41–55.
197. Kaur G, Rajput B. Comparative analysis of the omics technologies used to study antimonial, amphotericin B, and pentamidine resistance in *Leishmania*. J Parasitol Res. 2014;2014:726328.
198. Moreira W, Leprohon P, Ouellette M. Tolerance to drug-induced cell death favours the acquisition of multidrug resistance in *Leishmania*. Cell Death Dis. 2011;2:e201.

Drug Resistance in *Trypanosoma brucei*

43

Fabrice E. Graf and Pascal Mäser

1 Introduction

Experimenting with trypanosomes, Paul Ehrlich (1854–1915) observed that he could select for resistance by sublethal exposure to drugs, and he went on to define complementary groups based on patterns of cross-resistance. Ehrlich proposed to use these laboratory-selected drug-resistant lines to phenotypically classify newly identified trypanocides [1]. Since these pioneering studies, African trypanosomes have remained model organisms to study the mechanisms of drug action and drug resistance. After over 100 years of research on the mechanisms of drug resistance in African trypanosomes, reduction of drug import has crystallized as the predominant cause of resistance. This was recognized by classical studies in the 1930s and substantiated by recent approaches implementing next-generation sequencing and reverse genetics. More recently, the amenability of *Trypanosoma brucei brucei* to reverse genetic engineering has proven how nutrient transporters play key roles in the uptake of—and susceptibility to—clinically used trypanocides. These include aquaglyceroporin 2 and the adenosine transporter 1 for melarsoprol and pentamidine, and the amino acid permease 6 for eflornithine. Loss-of-function mutations in such transporters are viable because of the high degree of redundancy in the nutrient import machinery of *T. brucei* bloodstream forms. Thus *T. brucei* are, on the one hand, fascinating organisms that continue to provide new insights into the biology of eukaryotes; on the other hand, they are lethal pathogens of tropical Africa.

Sleeping sickness, also known as African Human trypanosomiasis (HAT), is caused by the two human-pathogenic subspecies of *T. brucei*: *T. b. rhodesiense* in East Africa and *T. b. gambiense* in West Africa. To date, 98 % of cases are due to *T. b. gambiense* [2]. Infections by *T. b. rhodesiense* lead to a more acute form of the disease which progresses within weeks to months to the second stage, when the trypanosomes have infected the central nervous system (CNS). *T. b. gambiense* infections are more chronic and can take years until involvement of the CNS. Symptoms during the hemolymphatic first stage include fever, swollen lymph glands, muscle and joint pains, and headaches. In stage two, neurological symptoms occur including change of personality, confusion, slurred speech, seizures, difficulties in walking and talking, and alteration of the circadian rhythm leading to disrupted sleeping patterns and coma. Untreated HAT, East or West African form, is a fatal disease [3].

Trypanosomes of the *brucei* complex do not naturally occur out of Africa since they strictly depend on the tsetse fly (*Glossina* spp.) for transmission. Tsetse flies are viviparous with a low reproductive number, which explains why they have never invaded other continents. Given its confinement to tropical Africa, HAT does not represent an attractive market for drug development. Nevertheless there is reason for optimism that new drugs for HAT will soon be available [4]. Thanks to public-private partnerships and product-development partnerships such as the Drugs for Neglected Diseases Initiative DNDi, new drugs are being tested in clinical trials and further candidates are in development. Better and safer drugs for the treatment of HAT are needed urgently since the current armory is limited; the drugs are also toxic and have unfavorable pharmacological properties.

2 Drugs for HAT

The treatment of HAT fully relies on chemotherapy. A vaccine is not available and the prospects for a vaccine are dim due to the fact that the bloodstream-form trypanosomes constantly undergo antigenic variation. The drugs presently used for the treatment of sleeping sickness are pentamidine and suramin for the first, hemolymphatic stage of the disease

F.E. Graf, Ph.D. • P. Mäser, Ph.D. (✉)
Swiss Tropical and Public Health Institute,
Parasite Chemotherapy Unit, Socinstrasse 57,
4051 Basel, Switzerland
e-mail: fabrice.graf@unibas.ch; pascal.maeser@unibas.ch

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_43

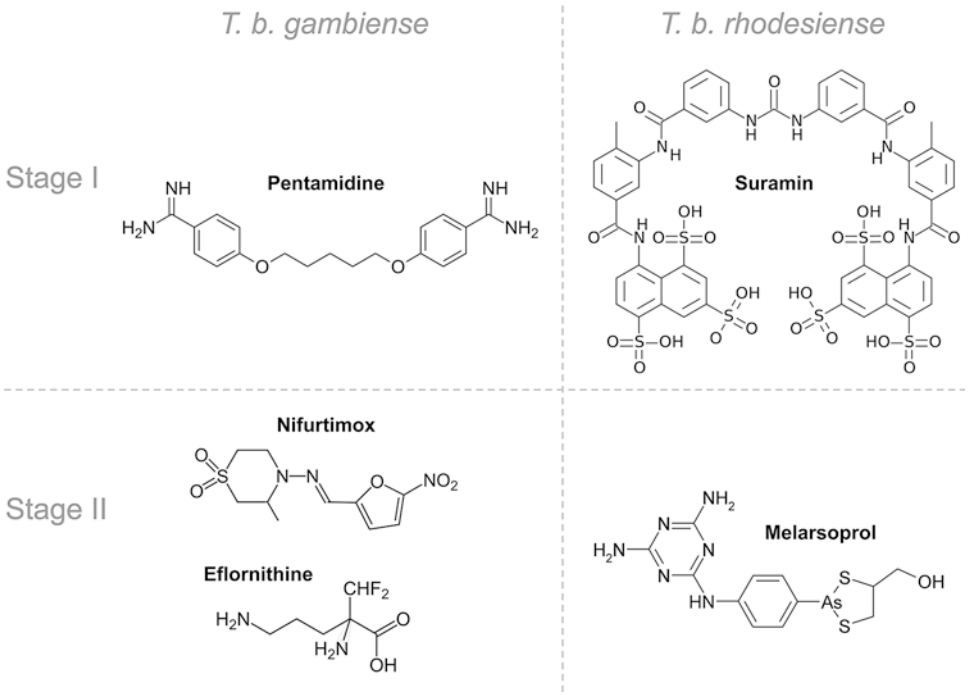


Fig. 43.1 Structures of the currently used drugs and their field of application in the treatment of HAT (pictures are from Wikimedia Commons)

(Fig. 43.1), and melarsoprol and nifurtimox/eflornithine combination therapy (NECT) for the second stage (Fig. 43.1), when the trypanosomes have invaded the cerebrospinal fluid. These drugs are dated, impractical, and toxic. In particular the chemotherapy of late-stage sleeping sickness is problematic. Melarsoprol can cause severe and frequently fatal encephalopathies whereas the less toxic eflornithine must be administered intravenously by infusion, a logistic nightmare in the remote and crisis-shaken areas that are afflicted by sleeping sickness. Besides good tolerability, the target-product profile for a new drug therefore includes oral bioavailability, heat stability, brain permeability, and ideally single-dose cure. Except for the last, these criteria are fulfilled by the two molecules that are currently in clinical development: fexinidazole in phase II/III and SCY-7158 in phase II. Both are untypical molecules for modern drug candidates with moderate in vitro activity, and yet they are highly effective in vivo. Fexinidazole is a nitroimidazole that was originally developed by Hoechst as an antibiotic [5]. It has a positive Ames test but was later on shown not to be mutagenic to mammalian cells [6]. Fexinidazole and its active metabolite fexinidazole sulfone have in vitro IC_{50} of around 1 μM against *T. brucei spp*. [7]. Fexinidazole given twice daily at 100 mg/kg for 5 days cures the chronic *T. brucei mouse* model [7]. To humans it is administered by daily oral dose over 10 consecutive days. SCY-7158 is a benzoxaborole from Anacor Pharmaceuticals (Palo Alto, CA), a company specialized in organic boron chemistry. SCY-7158 cures the chronic *T. brucei* mouse model at a daily oral dose of 25 mg/kg for 7 consecutive days [8, 9]. Further drug development projects are under way and the pipeline for HAT is, finally, in good shape [10]. However, until new drugs will be available the current ones need to be used in a sustainable way. This requires an understanding of the molecular mechanisms underlying drug resistance.

3 New Technologies to Study Drug Resistance

The non human-pathogenic *Trypanosoma brucei brucei* has become a model organism for eukaryote microbiology. The trypanosomes can be cloned and cultivated axenically, and they are amenable to reverse genetic manipulation such as gene knockout by homologous recombination or gene knockdown by RNAi-mediated silencing [11]. Furthermore, *T. brucei* genes do not contain introns and all mRNA carry a conserved 5′ leader sequence spliced in-trans [12], which facilitates applications such as full-length cDNA cloning or transcriptional start prediction. The first *T. brucei* ssp. genome was sequenced in 2005 [13] and by now several are available (www.tritrypdb.org). In addition to the general breakthroughs in DNA sequencing and omics technologies, a number of new approaches were developed that are targeted specifically towards *T. brucei*. These include the spliced leader-trapping protocol for RNA-Seq [14] and genome-wide RNAi libraries for inducible expression of small interfering RNAs in the trypanosomes themselves [15, 16]. Screening of such RNAi libraries enhanced the understanding of trypanosome biology [17, 18] and proved to be

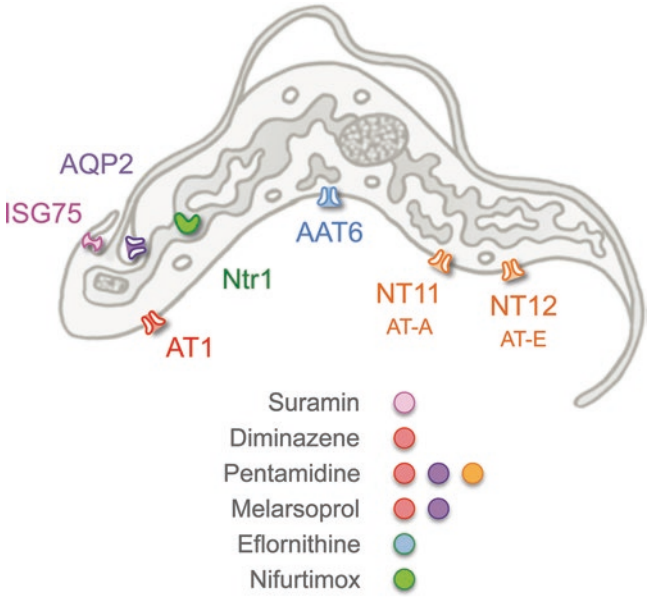


Fig. 43.2 Schematic overview of the gene products that mediate drug resistance in *T. brucei* bloodstream forms

particularly useful to study drug action, by selecting small interfering RNAs that cause drug resistance by downregulating a gene product necessary for drug activity [15, 16, 19–21]. These technologies combined with next-generation sequencing approaches have greatly enhanced our understanding of the molecular mechanisms of drug resistance in *T. brucei* (Fig. 43.2).

4 Mechanisms of Drug Resistance

In the following, we shall briefly introduce the drugs that are presently used to treat HAT (Fig. 43.1) and concentrate on the molecular mechanisms of drug resistance as they have been investigated in laboratory strains of *T. b. brucei*.

4.1 Melarsoprol–Pentamidine Cross-Resistance

Pentamidine, in clinical use since the 1930s, is an aromatic diamidine mainly used to treat first-stage *T. b. gambiense* infections (Fig. 43.1). Melarsoprol is a melaminophenyl arsenical and the only drug that cures *T. b. gambiense* as well as *T. b. rhodesiense* in second-stage infections (Fig. 43.1). Organic arsenicals and diamidines are among the oldest classes of trypanocides. The mechanisms of resistance to these agents were studied in classical experiments by Frank Hawking (1905–1986; father of the physicist Stephen Hawking) and coworkers, who used bioassays with post-incubation media, chemical quantification of arsenite in trypanosomes, and intrinsic fluorescence of the diamidine stilbamidine to measure drug uptake in bloodstream-form trypanosomes. These experiments demonstrated that resistant trypanosomes absorbed less drugs than susceptible ones [22–24]. A second important piece of information to the puzzle of trypanocide resistance was delivered by Williamson and Rollo, who discovered the phenomenon of cross-resistance between melamine-based arsenicals and diamidines [25, 26]. Melarsoprol–pentamidine cross-resistance (MPXR) has since been described by several different labs [27–31], and it has become the most extensively studied case of drug resistance in *T. brucei*. Melarsoprol and pentamidine have only little structural similarity (Fig. 43.1), and yet the two drugs share common transport systems for uptake into trypanosomes. The first such transport system to be identified was a purine permease termed P2 that imports adenine, adenosine, melamine-based arsenicals, and diamidines (Table 43.1). P2 was found to be absent in MPXR *T. brucei* [32, 33]. The gene encoding P2 was subsequently identified in a functional screen for growth on adenosine in a purine-auxotrophic mutant of *Saccharomyces cerevisiae* and named *TbAT1* for adenosine transporter 1 [34]. Mutations in *TbAT1* were detected in MPXR *T. brucei*, in lab-selected lines as well as in field isolates [34–37]. Substrate-binding motifs were mapped based on the functional characterization of P2 in *T. brucei* [38, 39]. Two additional nucleoside/nucleobase transporters [40, 41], TbNT11 (also named AT-A) and TbNT12 (also named AT-E), were shown to transport pentamidine when expressed in *Xenopus laevis* oocytes or in *Leishmania* mutants that were deficient in nucleobase or nucleoside uptake [42]. Melarsoprol inhibited adenine uptake via TbNT11 but was not a substrate itself [42]. To what proportion these two transporters contribute to pentamidine uptake in bloodstream-form *T. brucei*, and whether functional loss of either transporter leads to pentamidine resistance, remains to be investigated.

A second transporter involved in MPXR has recently been identified, the aquaglyceroporin TbAPQ2. This was achieved with RNAi library screens for melarsoprol or pentamidine resistance [20, 21, 43]. Aquaporins are water channels and were discovered in the early 1990s in human red blood cells [44] and later found in all kingdoms of life [45]. *T. brucei* possess three aquaglyceroporins (TbAQP1-3) which transport, in addition to water, glycerol and other small neutral solutes [46]. Homozygous deletion of *TbAPQ2* in *T. brucei* bloodstream forms caused 2-fold resistance to melarsoprol and 16-fold resistance to pentamidine; reintroduction of *TbAQP2* into the knockout cells restored sensitivity [43]. Expression of *TbAQP2* in *Leishmania mexicana* promastigotes increased their sensitivity to pentamidine and melarsoprol by factors of 40 and 1000, respectively [47]. An involvement of aquaglyceroporins in resistance to arsenite and antimonite had also been shown for tumor cells [48],

Table 43.1 Transporters of *T. brucei* involved in drug import and their physiological substrates (selected)

Transporter	TriTrypDB	GenBank	Physiological substrate	Toxic substrate
TbAT1/P2	Tb927.5.286b	AAD45278	Adenosine Adenine	Melarsoprol Pentamidine Diminazene DB75 Cordycepin Tubercidin
TbNT11.1 (AT-A)	Tb927.9.15980	XM_822640.1	Adenine Hypoxanthine Xanthine	Pentamidine
TbNT12.1 (AT-E)	Tb927.3.590	XM_838562.1	Adenine	Pentamidine
AQP2	Tb927.10.14170	XM_822804.1	Water Dihydroxyacetone Glycerol Urea	Melarsoprol Pentamidine Sb III As III
TbAAT6	Tb927.8.5450	XM_842282.1	Proline Other neutral amino acids	Eflornithine

Arabidopsis thaliana [49, 50], and *Leishmania* [51–53]. However, while TbAQP2 also transports As(III) and Sb(III) [54], its role in MPXR [47] is, to our knowledge, the first demonstration of an aquaglyceroporin involved in transport of larger organic molecules. By now mutations in *TbAQP2* have been detected in many MPXR lines of *T. brucei* ssp., lab strains selected in vitro or in vivo [47] as well as field isolates [31].

Another candidate gene for melarsoprol resistance is *TbMRPA* (multidrug-resistance-associated protein A), a member of subfamily C of the superfamily of ATP-binding cassette (ABC) transporters. In tumor cells MRP proteins can mediate multidrug resistance by actively exporting drug-glutathione conjugates [55]. TbMRPA is thought to export MelT, the conjugate of melarsoprol to trypanothione [56]. Trypanothione is a biochemical peculiarity of trypanosomatids: two glutathione tripeptides covalently linked via the polyamine spermidine [57, 58]. While the role of MRPA orthologues in drug efflux has been extensively studied in *Leishmania* [59–63], their function in *T. brucei* is less clear. Overexpression of *TbMRPA* in *T. brucei* led to an about tenfold increase in melarsoprol resistance in vitro [64] but not in vivo [65]. Overexpression of *TbMRPA* in *tbat1*$^{-/-}$ cells showed that the two resistance mechanisms, reduced drug influx and increased drug efflux, were strictly additive [66].

4.2 Suramin Resistance

The sulfated naphthylamine suramin (Fig. 43.1) is the product of what was one of the first medicinal chemistry programs. Starting from the dyes trypan red and trypan blue, which Paul Ehrlich had shown to be trypanocidal, suramin ("Bayer 205") was synthesized as a colorless, antitrypanosomal derivative by Bayer in 1916. It is the oldest drug in use against HAT and still the drug of choice against first-stage *T. b. rhodesiense* infections. Suramin is an intriguing molecule of manifold applications. Besides HAT, suramin has been tested also for river blindness [67], various cancers [68], candidiasis [69], autism [70], AIDS [71], and as an experimental compound in developmental biology [72]—and yet its modes of action are not fully understood. Suramin is negatively charged at physiological pH which prevents it from crossing the plasma membrane by passive diffusion, as well as from crossing the blood-brain barrier. The molecule is very large for a drug (1297 Da). Over 99 % is bound to plasma proteins, and the half-life of elimination is extremely long (44–54 days in human plasma). Suramin uptake into trypanosomes was proposed to occur via receptor-mediated endocytosis after binding to the low-density lipoprotein (LDL) [73].

Genome-wide RNAi screening for suramin resistance in *T. brucei* confirmed the endocytotic uptake route as several genes from this pathway emerged as hits [21]. One of the main determinants required for suramin activity was the invariant surface glycoprotein ISG75, supporting the model that suramin is imported via endocytosis and that ISG75 is the suramin receptor on the trypanosomes' surface [74]. Other downstream genes such as the four subunits of the adaptin complex-1 (AP-1), the Golgi/lysosomal protein-1 (GLP-1), the endosomal membrane protein 70 (EMP70), the major glycosomal protein p67, cathepsin-L, and the major facilitator superfamily transporter (MFST) were all linked to the endocytotic pathway. RNAi-mediated knockdown in bloodstream-form *T. brucei* of these genes led to a reduced suramin sensitivity in vitro, with the highest resistance factor (>10) for MFST. In addition, ubiquitin hydrolase (UBH1) was identified as a hit in the RNAi screen and knockdown led to reduced suramin sensitivity. This was likely an indirect effect as ISG75 is a transmembrane protein whose internalization relies on the ubiquitination of cytoplasmic lysine

residues [75]. Two *T. brucei* lines that had been independently selected for suramin resistance in vitro subsequently lost their resistance phenotype when transformed into the insect (procyclic) stage [29]. This is in agreement with an involvement of ISG75 in suramin susceptibility because expression of ISG75 is bloodstream-form specific [76].

4.3 Nifurtimox Resistance

Nifurtimox is orally bioavailable and one of the two frontline drugs for Chagas disease, in use for more than 40 years. Since 2009, nifurtimox—although not very active alone—is also applied against human African trypanosomiasis in combination with eflornithine [77, 78]. Nifurtimox-eflornithine combination therapy (NECT) for HAT has been included in the WHO's Model List of Essential Medicines. Nifurtimox is a nitrofuran (Fig. 43.1) that functions as a prodrug. The activation is enzymatically mediated by reduction of the nitro group. In *T. brucei* and *T. cruzi*, a bacterial-like type I nitroreductase (NTR) activates the prodrug by two consecutive reductions [79]. NTR is an NADH-dependent enzyme localized in the mitochondrion. Mammalian genomes do not contain an NTR orthologue. Resistance to nifurtimox and other nitroheterocyclic drugs has been attributed to a reduction of NTR activity [79]. *T. cruzi* epimastigotes selected for nifurtimox resistance in vitro lost a copy of NTR [79, 80]. Gene knockout experiments confirmed the crucial role of NTR in nifurtimox resistance as well as cross-resistance to benznidazole, a related nitroimidazole and the second drug in clinical use against *T. cruzi* infections. Heterozygous deletion of the *NTR* gene in *T. brucei* rendered the cells nifurtimox resistant whereas ectopic overexpression of *NTR* caused hypersensitivity [79]. Homozygous deletion of *NTR* in *T. brucei* was only achieved when the expression of the ectopic copy had been induced, indicating that NTR is essential for bloodstream-form *T. brucei* [79]. Alsford and Horn's RNAi screen [21] also confirmed the importance of NTR; in addition to NTR, it identified a putative flavokinase plus four genes involved in the biosynthesis of ubiquinone [15, 21]. Flavokinase converts riboflavin to flavin-mononucleotide, which is an essential cofactor of NTR, while ubiquinone functions as electron acceptor from NADH mediated by NTR. Cross-resistance was also observed between nifurtimox and fexinidazole. *T. brucei* selected in vitro for nifurtimox resistance (8×) were also resistant (27×) to fexinidazole [81]. These trypanosomes were infective to mice and the cross-resistance phenotype was also manifest in vivo. Trypanosomes that had been selected with fexinidazole were also cross-resistant to nifurtimox [81]. The resistance factor was 10× to either drug, indicating that the underlying mechanism of resistance might be somewhat different. The *NTR* genes were unaltered in those lines but reduced expression level could not be ruled out [81].

4.4 Eflornithine Resistance

Eflornithine or difluoromethyl-ornithine (DFMO) was synthesized in 1978 and initially developed as an anticancer drug [82]. It is the newest drug applied for HAT and the only treatment for stage 2 of the disease in case of melarsoprol treatment failure [83]. Since 2009 eflornithine is used in combination therapy with nifurtimox (NECT). This is now the recommended treatment option for second-stage *T. b. gambiense* infections as NECT is far less toxic than melarsoprol. However, eflornithine is less active against *T. b. rhodesiense* [84] and NECT is not being implemented for the treatment of East African sleeping sickness. Eflornithine is one of the very few trypanocidal drugs whose target is known. It covalently binds to, and irreversibly inhibits, the enzyme ornithine decarboxylase (ODC), blocking polyamine synthesis and subsequently trypanothione production [85–87]. The molecular mechanism of eflornithine resistance remained elusive and no genetic marker was identified until recently. Vincent and coworkers have selected two *T. b. brucei* lines independently for eflornithine resistance in vitro. ODC activity was unaltered and there was no change in the levels of metabolites of the polyamine biosynthetic pathway [88]. When the lines were probed by PCR for all known *T. brucei* amino acid permeases (because eflornithine is itself an amino acid and likely taken up by such a transporter) it turned out that both lines had lost the gene encoding the *T. brucei* amino acid transporter 6 (*TbAAT6*). RNAi-mediated knockdown of *TbAAT6* expression in sensitive *T. brucei* confirmed the role of *TbAAT6* in eflornithine resistance while reintroduction of *TbAAT6* rescued the drug-sensitive phenotype of the resistant mutants [88]. Furthermore, two genome-wide loss of function screens using RNAi libraries independently linked the silencing of *TbAAT6* expression to eflornithine resistance [15, 16].

5 Drug Resistance in the Clinics

In contrast to the livestock-pathogenic trypanosomes where drug resistance is widespread, the situation is less critical regarding the treatment of first-stage HAT patients. Suramin, for instance, is generally efficacious against first-stage *T. b. rhodesiense* infections, in spite of its old age and the fact that resistance can be selected for in the lab [29, 89]. However, suramin resistance is a problem in the management of *T. evansi* [90, 91], a non-tsetse-transmitted trypanosome that causes Surra in cattle, equines, and camelids and that is very closely related to *T. brucei* [92]. Similarly, pentamidine treatment failures are rare and the reported relapsing patients were likely in many cases early stage 2 infections, indicative of misdiagnosis rather than drug resistance [93]. At the same time, diminazene resistance is jeopardizing the treatment of

Nagana [94, 95]. For *T. brucei* ssp., this discrepancy has been attributed to the fact that diminazene is imported into the trypanosomes via a single transporter, TbAT1, whereas pentamidine has at least two additional routes of import, referred to as HAPT and LAPT for high- and low-affinity pentamidine transporters, respectively [96]; TbAQP2 does not transport diminazene and corresponds to HAPT [47]. Thus differences in the redundancy of import routes may provide an explanation for the higher prevalence of drug resistance in livestock trypanosomoses compared to human trypanosomiasis, lowering the probability of emergence in the latter. For *T. congolense* the situation is less clear. *T. congolense* possess an adenosine transporter gene, *TcoAT1*, that has been implicated in diminazene resistance based on molecular epidemiology [97]. However, TcoAT1 is not the direct orthologue of TbAT1 and it does not transport diminazene when expressed in *T. b. brucei* [98]. A more obvious effect than a lower probability of emergence might be the lower probability of the spread of drug resistance in the human-pathogenic trypanosomes, arising from the fact that the treated patients are hospitalized and not accessible for tsetse flies.

Patients relapsing from eflornithine monotherapy have been reported from the Democratic Republic of the Congo (up to 27 % [99]) and from Angola (8.1 % [100]). Whether this was caused by drug-resistant parasites or other factors is unclear. Combination therapy with nifurtimox may improve the treatment success of eflornithine and delay the possible emergence of drug resistance. Nifurtimox resistance has so far not been encountered for African trypanosomes in the clinics, but was reported for *T. cruzi* [101, 102]. However, eflornithine and nifurtimox resistance is readily selected for in vitro [79, 88], and the efficacy of NECT will decline if eflornithine-resistant parasites emerge because nifurtimox by itself is not very potent.

HAT treatment failures have been most critical with melarsoprol. Relapse rates of 5–8 % are considered normal for melarsoprol treatment [103]. Beginning in the 1990s, much higher rates of melarsoprol treatment failures have been reported from different areas, sometimes above 50 % [104–107]. After the discovery of the gene encoding the P2 transporter [34], clinical isolates were analyzed for mutations in this potential marker. Several studies correlated the occurrence of (nonfunctional) mutant alleles of *TbAT1* to a higher incidence of melarsoprol treatment failures in different HAT foci [35, 36, 108]. These correlations were usually significant but not absolutely conclusive, indicating the contribution of additional factors [109]. More recently, *T. b. gambiense* isolates from Mbuji-Mayi (Democratic Republic of the Congo), an area of high relapse rates after melarsoprol treatment [107, 110], have been found to carry mutations in the aquaglyceroporin tandem locus [31, 111]. The mutants had a deletion that led to the formation of a chimeric gene between the neighboring *AQP2* and *AQP3* and loss of either wild-type gene. These isolates were melarsoprol and pentamidine resistant in vitro [31] and had reduced melarsoprol sensitivity in vivo [111]. This represents the first clinical case of MPXR. Reintroduction of the wild-type *AQP2* gene into an MPXR-resistant *T. b. gambiense* isolate completely restored its drug susceptibility, demonstrating the loss of AQP2 function as the cause of drug resistance [112].

6 Conclusion

In summary, drug resistance in *T. brucei* is intriguingly linked to drug uptake. The predominant mechanism of resistance is reduced drug import caused by loss-of-function mutations in nonessential nutrient transporters that happen to import drugs in addition to their physiological substrates (Table 43.1). Other typical mechanisms of drug resistance such as overexpression or mutation of the target do not seem not to play a critical role in *T. brucei*. A possible explanation for this phenomenon is that drugs like the diamidines, arsenicals, or suramin have multiple intracellular targets; so it is very difficult for a cell to withstand once the drug has been taken up. But even for eflornithine, which has a clearly defined target enzyme, loss of import turned out to be the resistance mechanism rather than mutations in ODC. Obviously RNAi screens are biased towards loss-of-function mutations and hence most effective to identify drug import pathways. However, the loss of drug import has also been confirmed in forward genetic experiments with laboratory-selected resistant mutants of *T. brucei* and even with drug-resistant field isolates.

We believe that loss of import as a mechanism of drug resistance is strongly favored by the high degree of redundancy within the nutrient uptake machinery of *T. brucei*. The *T. brucei* genome covers each of the main metabolite classes (i.e., purines, sugars, or amino acids) with dozens of transporter genes. The high degree of redundancy means that transporters can be lost without a fitness cost. This is in agreement with the fact that the drug-resistant *T. brucei* transporter mutants characterized so far did not exhibit a growth deficit. Furthermore, the transporter genes of *T. brucei* are often arranged in tandem clusters, which allows for loss of genetic material by homologous recombination between very similar genes. If indeed the redundancy of nutrient import routes is at the core of drug resistance in *T. brucei*, a main conclusion is that we should aim for novel drugs that are taken up by the trypanosomes either via essential transporters or via multiple transporters. Thus transport phenomena must not be neglected in drug R&D for African trypanosomes.

Acknowledgments We gratefully acknowledge financial support by the Swiss National Science Foundation, the Mathieu Foundation of the University of Basel, and the Freiwillige Akademische Gesellschaft Basel.

References

1. Ehrlich P. Chemotherapeutische Trypanosomen-Studien. Berlin Klin Wochenschr. 1907;11:310–4.
2. Franco JR, Simarro PP, Diarra A, Jannin JG. Epidemiology of human African trypanosomiasis. Clin Epidemiol. 2014;6:257–75.
3. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. Lancet. 2010;375:148–59.
4. Mäser P, Wittlin S, Rottmann M, Wenzler T, Kaiser M, Brun R. Antiparasitic agents: new drugs on the horizon. Curr Opin Pharmacol. 2012;12:562–6.
5. Torreele E, Bourdin Trunz B, Tweats D, Kaiser M, Brun R, Mazue G, Bray MA, Pecoul B. Fexinidazole--a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. PLoS Negl Trop Dis. 2011;4:e923.
6. Tweats D, Bourdin Trunz B, Torreele E. Genotoxicity profile of fexinidazole–a drug candidate in clinical development for human African trypanomiasis (sleeping sickness). Mutagenesis. 2012;27:523–32.
7. Kaiser M, Bray MA, Cal M, Bourdin Trunz B, Torreele E, Brun R. Antitrypanosomal activity of fexinidazole, a new oral nitroimidazole drug candidate for treatment of sleeping sickness. Antimicrob Agents Chemother. 2011;55:5602–8.
8. Nare B, Wring S, Bacchi C, Beaudet B, Bowling T, Brun R, et al. Discovery of novel orally bioavailable oxaborole 6-carboxamides that demonstrate cure in a murine model of late stage central nervous system African trypanosomiasis. Antimicrob Agents Chemother. 2010;54:4379–88.
9. Jacobs RT, Nare B, Wring SA, Orr MD, Chen D, Sligar JM, et al. SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human African trypanosomiasis. PLoS Negl Trop Dis. 2011;5:e1151.
10. Brun R, Don R, Jacobs RT, Wang MZ, Barrett MP. Development of novel drugs for human African trypanosomiasis. Future Microbiol. 2011;6:677–91.
11. Wirtz E, Leal S, Ochatt C, Cross GA. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei. Mol Biochem Parasitol. 1999;99:89–101.
12. Walder JA, Eder PS, Engman DM, Brentano ST, Walder RY, Knutzon DS, Dorfman DM, Donelson JE. The 35-nucleotide spliced leader sequence is common to all trypanosome messenger RNA's. Science. 1986;233:569–71.
13. Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, et al. The genome of the African trypanosome Trypanosoma brucei. Science. 2005;309:416–22.
14. Nilsson D, Gunasekera K, Mani J, Osteras M, Farinelli L, Baerlocher L, Roditi I, Ochsenreiter T. Spliced leader trapping reveals widespread alternative splicing patterns in the highly dynamic transcriptome of Trypanosoma brucei. PLoS Pathog. 2010;6:e1001037.
15. Baker N, Alsford S, Horn D. Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. Mol Biochem Parasitol. 2011;176:55–7.
16. Schumann Burkard G, Jutzi P, Roditi I. Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. Mol Biochem Parasitol. 2011;175:91–4.
17. Alsford S, Turner DJ, Obado SO, Sanchez-Flores A, Glover L, Berriman M, Hertz-Fowler C, Horn D. High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. Genome Res. 2011;21:915–24.
18. Mony BM, MacGregor P, Ivens A, Rojas F, Cowton A, Young J, Horn D, Matthews K. Genome-wide dissection of the quorum sensing signalling pathway in Trypanosoma brucei. Nature. 2014;505:681–5.
19. Gould MK, Bachmaier S, Ali JA, Alsford S, Tagoe DN, Munday JC, Schnaufer AC, Horn D, Boshart M, de Koning HP. Cyclic AMP effectors in African trypanosomes revealed by genome-scale RNA interference library screening for resistance to the phosphodiesterase inhibitor CpdA. Antimicrob Agents Chemother. 2013;57:4882–93.
20. Alsford S, Kelly JM, Baker N, Horn D. Genetic dissection of drug resistance in trypanosomes. Parasitology. 2013;140:1478–91.
21. Alsford S, Eckert S, Baker N, Glover L, Sanchez-Flores A, Leung KF, Turner DJ, Field MC, Berriman M, Horn D. High-throughput decoding of antitrypanosomal drug efficacy and resistance. Nature. 2012;482:232–6.
22. Yorke W, Murgatroyd F, Hawking F. Preliminary contribution on the nature of drug resistance. Ann Trop Med Parasitol. 1931;25:351–8.
23. Hawking F. The absorption of arsenical compounds and tartar emetic by normal and resistant trypanosomes and its relation to drug-resistance. J Pharmcol Exp Ther. 1936;59:123–56.
24. Hawking F, Smiles J. The distribution of 4:4′-diamidino stilbene in trypanosomes and mice as shown by fluorescence. Ann Trop Med Parasitol. 1941;35:45–50.
25. Rollo IM, Williamson J. Acquired resistance to 'melarsen', tryparsamide and amidines in pathogenic trypanosomes after treatment with 'melarsen' alone. Nature. 1951;167:147–8.
26. Williamson J, Rollo IM. Drug resistance in trapanososmes: cross-resitance analyses. Br J Pharmacol Chemother. 1959;14:423–30.
27. Frommel TO, Balber AE. Flow cytofluorimetric analysis of drug accumulation by multidrug-resistant *Trypanosoma brucei brucei* and *T. b. rhodesiense*. Mol Biochem Parasitol. 1987;26:183–92.
28. Pospichal H, Brun R, Kaminsky R, Jenni L. Induction of resistance to melarsenoxide cysteamine (Mel Cy) in *Trypanosoma brucei brucei*. Acta Trop. 1994;58:187–97.
29. Scott AG, Tait A, Turner MR. Characterisation of cloned lines of *Trypanosoma brucei* expressing stable resistance to MelCy and Suramin. Acta Trop. 1996;60:251–62.
30. Bernhard SC, Nerima B, Mäser P, Brun R. Melarsoprol- and pentamidine-resistant Trypanosoma brucei rhodesiense populations and their cross-resistance. Int J Parasitol. 2007;37:1443–8.
31. Graf FE, Ludin P, Wenzler T, Kaiser M, Brun R, Pyana PP, Büscher P, de Koning HP, Horn D, Mäser P. Aquaporin 2 mutations in Trypanosoma brucei gambiense field isolates correlate with decreased susceptibility to pentamidine and melarsoprol. PLoS Negl Trop Dis. 2013;7:e2475.
32. Carter NS, Fairlamb AH. Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature. 1993;361:173–5.
33. Carter NS, Berger BJ, Fairlamb AH. Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. J Biol Chem. 1995;270:28153–7.
34. Mäser P, Sütterlin C, Kralli A, Kaminsky R. A nucleoside transporter from Trypanosoma brucei involved in drug resistance. Science. 1999;285:242–4.
35. Matovu E, Geiser F, Schneider V, Mäser P, Enyaru JCK, Kaminsky R, Gallati S, Seebeck T. Genetic variants of the *TbAT1* adenosine transporter from African trypanosomes in relapse infections following melarsoprol therapy. Mol Biochem Parasitol. 2001;117:73–81.
36. Kazibwe AJ, Nerima B, de Koning HP, Mäser P, Barrett MP, Matovu E. Genotypic status of the TbAT1/P2 adenosine transporter of Trypanosoma brucei gambiense isolates from Northwestern Uganda following melarsoprol withdrawal. PLoS Negl Trop Dis. 2009;3:e523.
37. Stewart ML, Burchmore RJ, Clucas C, Hertz-Fowler C, Brooks K, Tait A, Macleod A, Turner CM, De Koning HP, Wong PE, Barrett MP. Multiple genetic mechanisms lead to loss of functional TbAT1 expression in drug-resistant trypanosomes. Eukaryot Cell. 2010;9:336–43.

38. Lüscher A, de Koning HP, Mäser P. Chemotherapeutic strategies against Trypanosoma brucei: drug targets vs. drug targeting. Curr Drug Targets. 2006;13:555–67.
39. de Koning HP, Jarvis SM. Adenosine transporters in bloodstream forms of Trypanosoma brucei brucei: substrate recognition motifs and affinity for trypanocidal drugs. Mol Pharmacol. 1999;56: 1162–70.
40. de Koning HP, Bridges DJ, Burchmore RJ. Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. FEMS Microbiol Rev. 2005;29:987–1020.
41. Al-Salabi MI, Wallace LJ, Luscher A, Mäser P, Candlish D, Rodenko B, Gould MK, Jabeen I, Ajith SN, de Koning HP. Molecular interactions underlying the unusually high adenosine affinity of a novel Trypanosoma brucei nucleoside transporter. Mol Pharmacol. 2007;71:921–9.
42. Ortiz D, Sanchez MA, Quecke P, Landfear SM. Two novel nucleobase/pentamidine transporters from Trypanosoma brucei. Mol Biochem Parasitol. 2009;163:67–76.
43. Baker N, Glover L, Munday JC, Aguinaga Andres D, Barrett MP, de Koning HP, Horn D. Aquaglyceroporin 2 controls susceptibility to melarsoprol and pentamidine in African trypanosomes. Proc Natl Acad Sci U S A. 2012;109:10996–1001.
44. Preston GM, Carroll TP, Guggino WB, Agre P. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science. 1992;256:385–7.
45. King LS, Kozono D, Agre P. From structure to disease: the evolving tale of aquaporin biology. Nat Rev Mol Cell Biol. 2004;5:687–98.
46. Uzcategui NL, Szallies A, Pavlovic-Djuranovic S, Palmada M, Figarella K, Boehmer C, Lang F, Beitz E, Duszenko M. Cloning, heterologous expression, and characterization of three aquaglyceroporins from Trypanosoma brucei. J Biol Chem. 2004;279:42669–76.
47. Munday JC, Eze AA, Baker N, Glover L, Clucas C, Aguinaga Andres D, et al. Trypanosoma brucei aquaglyceroporin 2 is a high-affinity transporter for pentamidine and melaminophenyl arsenic drugs and the main genetic determinant of resistance to these drugs. J Antimicrob Chemother. 2014;69:651–63.
48. Liu Z, Shen J, Carbrey JM, Mukhopadhyay R, Agre P, Rosen BP. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci U S A. 2002;99:6053–8.
49. Isayenkov SV, Maathuis FJ. The Arabidopsis thaliana aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. FEBS Lett. 2008;582:1625–8.
50. Kamiya T, Tanaka M, Mitani N, Ma JF, Maeshima M, Fujiwara T. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of Arabidopsis thaliana. J Biol Chem. 2009;284:2114–20.
51. Gourbal B, Sonuc N, Bhattacharjee H, Legare D, Sundar S, Ouellette M, Rosen BP, Mukhopadhyay R. Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin. J Biol Chem. 2004;279:31010–7.
52. Marquis N, Gourbal B, Rosen BP, Mukhopadhyay R, Ouellette M. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant Leishmania. Mol Microbiol. 2005;57:1690–9.
53. Mandal S, Maharjan M, Singh S, Chatterjee M, Madhubala R. Assessing aquaglyceroporin gene status and expression profile in antimony-susceptible and -resistant clinical isolates of Leishmania donovani from India. J Antimicrob Chemother. 2010;65:496–507.
54. Uzcategui NL, Figarella K, Bassarak B, Meza NW, Mukhopadhyay R, Ramirez JL, Duszenko M. Trypanosoma brucei aquaglyceroporins facilitate the uptake of arsenite and antimonite in a pH dependent way. Cell Physiol Biochem. 2013;32:880–8.
55. Keppler D, Leier I, Jedlitschky G. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. Biol Chem. 1997;378:787–91.
56. Fairlamb AH, Henderson GB, Cerami A. Trypanothione is the primary target for arsenical drugs against African trypanosomes. Proc Natl Acad Sci U S A. 1989;86:2607–11.
57. Fairlamb A, Blackburn P, Ulrich P, Chait B, Cerami A. Trypanothione: a novel bis (glutathionyl) spermidine cofactor for glutathione reductase in trypanosomatids. Science. 1985;22:1485–7.
58. Fairlamb AH, Cerami A. Metabolism and functions of trypanothione in the kinetoplastida. Annu Rev Microbiol. 1992;46: 695–729.
59. Leprohon P, Legare D, Ouellette M. Intracellular localization of the ABCC proteins of Leishmania and their role in resistance to antimonials. Antimicrob Agents Chemother. 2009;53:2646–9.
60. Mukherjee A, Padmanabhan PK, Singh S, Roy G, Girard I, Chatterjee M, Ouellette M, Madhubala R. Role of ABC transporter MRPA, gamma-glutamylcysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of Leishmania donovani. J Antimicrob Chemother. 2007;59:204–11.
61. Legare D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M. The Leishmania ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. J Biol Chem. 2001;276:26301–7.
62. Ouellette M, Fase-Fowler F, Borst P. The amplified H-circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein. EMBO J. 1990;9:1027–33.
63. Ouellette M, Haimeur A, Grondin K, Legare D, Papadopoulou B. Amplification of ABC transporter gene pgpA and of other heavy metal resistance genes in Leishmania tarentolae and their study by gene transfection and gene disruption. Methods Enzymol. 1998;292:182–93.
64. Shahi SK, Krauth-Siegel RL, Clayton CE. Overexpression of the putative thiol conjugate transporter TbMRPA causes melarsoprol resistance in Trypanosoma brucei. Mol Microbiol. 2002;43:1129–38.
65. Alibu VP, Richter C, Voncken F, Marti G, Shahi S, Renggli CK, Seebeck T, Brun R, Clayton C. The role of Trypanosoma brucei MRPA in melarsoprol susceptibility. Mol Biochem Parasitol. 2006;146:38–44.
66. Lüscher A, Nerima B, Mäser P. Combined contribution of TbAT1 and TbMRPA to drug resistance in Trypanosoma brucei. Mol Biochem Parasitol. 2006;150:364–6.
67. Anderson J, Fuglsang H. Further studies on the treatment of ocular onchocerciasis with diethylcarbamazine and suramin. Br J Ophthalmol. 1978;62:450–7.
68. McGeary RP, Bennett AJ, Tran QB, Cosgrove KL, Ross BP. Suramin: clinical uses and structure–activity relationships. Mini Rev Med Chem. 2008;8:1384–94.
69. Braga-Silva LA, dos Santos AL, Portela MB, Souto-Padron T, de Araujo Soares RM. Effect of suramin on the human pathogen Candida albicans: implications on the fungal development and virulence. FEMS Immunol Med Microbiol. 2007;51:399–406.
70. Naviaux RK, Zolkipli Z, Wang L, Nakayama T, Naviaux JC, Le TP, Schuchbauer MA, Rogac M, Tang Q, Dugan LL, Powell SB. Antipurinergic therapy corrects the autism-like features in the poly(IC) mouse model. PLoS One. 2013;8:e57380.
71. Agarwal HK, Doncel GF, Parang K. Synthesis and anti-HIV activities of suramin conjugates of 3′-fluoro- 2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine. Med Chem. 2012;8:193–7.
72. Oschwald R, Clement JH, Knochel W, Grunz H. Suramin prevents transcription of dorsal marker genes in Xenopus laevis embryos, isolated dorsal blastopore lips and activin A induced animal caps. Mech Dev. 1993;43:121–33.
73. Vansterkenburg ELM, Coppens I, Wilting J, Bos OJM, Fischer MJE, Janssen LHM, Opperdoes FR. The uptake of the trypanocidal drug suramin in combination with low-density lipoproteins by *Trypanosoma brucei* and its possible mode of action. Acta Trop. 1993;54:237–50.

74. Alsford S, Field MC, Horn D. Receptor-mediated endocytosis for drug delivery in African trypanosomes: fulfilling Paul Ehrlich's vision of chemotherapy. Trends Parasitol. 2013;29:207–12.
75. Leung KF, Riley FS, Carrington M, Field MC. Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes. Eukaryot Cell. 2011;10:916–31.
76. Ziegelbauer K, Multhaup G, Overath P. Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of Trypanosoma brucei. J Biol Chem. 1992;267:10797–803.
77. Priotto G, Kasparian S, Mutombo W, Ngouama D, Ghorashian S, Arnold U, et al. Nifurtimox-eflornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet. 2009;374:56–64.
78. Yun O, Priotto G, Tong J, Flevaud L, Chappuis F. NECT is next: implementing the new drug combination therapy for Trypanosoma brucei gambiense sleeping sickness. PLoS Negl Trop Dis. 2010;4:e720.
79. Wilkinson SR, Taylor MC, Horn D, Kelly JM, Cheeseman I. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. Proc Natl Acad Sci U S A. 2008;105:5022–7.
80. Mejia-Jaramillo AM, Fernandez GJ, Palacio L, Triana-Chavez O. Gene expression study using real-time PCR identifies an NTR gene as a major marker of resistance to benzonidazole in Trypanosoma cruzi. Parasit Vectors. 2011;4:169.
81. Sokolova AY, Wyllie S, Patterson S, Oza SL, Read KD, Fairlamb AH. Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. Antimicrob Agents Chemother. 2010;54:2893–900.
82. Bacchi CJ, Nathan HC, Hutner SH, McCann PP, Sjoerdsma A. Polyamine metabolism: a potential therapeutic target in trypanosomes. Science. 1980;210:332–4.
83. Burri C, Brun R. Eflornithine for the treatment of human African trypanosomiasis. Parasitol Res. 2003;90(Supp 1):S49–52.
84. Iten M, Matovu E, Brun R, Kaminsky R. Innate lack of susceptibility of Ugandan *Trypanosoma brucei rhodesiense* to DL-a-difluoromethylornithine (DFMO). Trop Med Parasitol. 1995;46:190–4.
85. Wang CC. A novel suicide inhibitor strategy for antiparasitic drug development. J Cell Biochem. 1991;45:49–53.
86. Phillips MA, Coffino P, Wang CC. Cloning and sequencing of the ornithine decarboxylase gene from *Trypanosoma brucei*. Implications for enzyme turnover and selective difluoromethylornithine inhibition. J Biol Chem. 1987;262:8721–7.
87. Fairlamb AH, Henderson GB, Bacchi CJ, Cerami A. In vivo effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of Trypanosoma brucei. Mol Biochem Parasitol. 1987;24:185–91.
88. Vincent IM, Creek D, Watson DG, Kamleh MA, Woods DJ, Wong PE, Burchmore RJ, Barrett MP. A molecular mechanism for eflornithine resistance in African trypanosomes. PLoS Pathog. 2010;6:e1001204.
89. Mutugi MW, Boid R, Luckins AG. Experimental induction of suramin-resistance in cloned and uncloned stocks of Trypanosoma evansi using immunosuppressed and immunocompetent mice. Trop Med Parasitol. 1994;45:232–6.
90. Brun R, Lun ZR. Drug sensitivity of Chinese Trypanosoma evansi and Trypanosoma equiperdum isolates. Vet Parasitol. 1994;52:37–46.
91. El Rayah IE, Kaminsky R, Schmid C, El Malik KH. Drug resistance in Sudanese Trypanosoma evansi. Vet Parasitol. 1999;80:281–7.
92. Carnes J, Anupama A, Balmer O, Jackson A, Lewis M, Brown R, et al. Genome and phylogenetic analyses of Trypanosoma evansi reveal extensive similarity to T. brucei and multiple independent origins for dyskinetoplasty. PLoS Negl Trop Dis. 2015;9:e3404.
93. Balasegaram M, Harris S, Checchi F, Hamel C, Karunakara U. Treatment outcomes and risk factors for relapse in patients with early-stage human African trypanosomiasis (HAT) in the Republic of the Congo. Bull World Health Organ. 2006;84:777–82.
94. Mungube EO, Vitouley HS, Allegye-Cudjoe E, Diall O, Boucoum Z, Diarra B, Sanogo Y, Randolph T, Bauer B, Zessin KH, Clausen PH. Detection of multiple drug-resistant Trypanosoma congolense populations in village cattle of south-east Mali. Parasit Vectors. 2012;5:155.
95. Moti Y, Fikru R, Van Den Abbeele J, Buscher P, Van den Bossche P, Duchateau L, Delespaux V. Ghibe river basin in Ethiopia: present situation of trypanocidal drug resistance in Trypanosoma congolense using tests in mice and PCR-RFLP. Vet Parasitol. 2012;189:197–203.
96. de Koning H. Uptake of pentamidine in Trypanosoma brucei is mediated by three distinct transporters: implications for cross-resistance with arsenicals. Mol Pharmacol. 2001;59:586–92.
97. Delespaux V, Chitanga S, Geysen D, Goethals A, van den Bossche P, Geerts S. SSCP analysis of the P2 purine transporter TcoAT1 gene of Trypanosoma congolense leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks. Acta Trop. 2006;100:96–102.
98. Munday JC, Rojas Lopez KE, Eze AA, Delespaux V, Van Den Abbeele J, Rowan T, Barrett MP, Morrison LJ, de Koning HP. Functional expression of TcoAT1 reveals it to be a P1-type nucleoside transporter with no capacity for diminazene uptake. Int J Parasitol Drugs Drug Resist. 2013;3:69–76.
99. Pepin J, Khonde N, Maiso F, Doua F, Jaffar S, Ngampo S, Mpia B, Mbulamberi D, Kuzoe F. Short-course eflornithine in Gambian trypanosomiasis: a multicentre randomized controlled trial. Bull World Health Organ. 2000;78:1284–95.
100. Balasegaram M, Harris S, Checchi F, Ghorashian S, Hamel C, Karunakara U. Melarsoprol versus eflornithine for treating late-stage Gambian trypanosomiasis in the Republic of the Congo. Bull World Health Organ. 2006;84:783–91.
101. Filardi LS, Brener Z. Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. Trans R Soc Trop Med Hyg. 1987;81:755–9.
102. Murta SM, Gazzinelli RT, Brener Z, Romanha AJ. Molecular characterization of susceptible and naturally resistant strains of Trypanosoma cruzi to benznidazole and nifurtimox. Mol Biochem Parasitol. 1998;93:203–14.
103. Pepin J, Milord F, Khonde A, Niyonsenga T, Loko L, Mpia B. Gambiense trypanosomiasis: frequency of, and risk factors for, failure of melarsoprol therapy. Trans R Soc Trop Med Hyg. 1994;88:447–52.
104. Legros D, Evans S, Maiso F, Enyaru JC, Mbulamberi D. Risk factors for treatment failure after melarsoprol for Trypanosoma brucei gambiense trypanosomiasis in Uganda. Trans R Soc Trop Med Hyg. 1999;93:439–42.
105. Stanghellini A, Josenando T. The situation of sleeping sickness in Angola: a calamity. Trop Med Int Health. 2001;6:330–4.
106. Robays J, Nyamowala G, Sese C, Betu Ku Mesu Kande V, Lutumba P, Van der Veken W, Boelaert M. High failure rates of melarsoprol for sleeping sickness, Democratic Republic of Congo. Emerg Infect Dis. 2008;14:966–7.
107. Mumba Ngoyi D, Lejon V, Pyana P, Boelaert M, Ilunga M, Menten J, Mulunda JP, Van Nieuwenhove S, Muyembe Tamfum JJ, Buscher P. How to shorten patient follow-up after treatment for Trypanosoma brucei gambiense sleeping sickness. J Infect Dis. 2010;201:453–63.
108. Nerima B, Matovu E, Lubega GW, Enyaru JC. Detection of mutant P2 adenosine transporter (TbAT1) gene in Trypanosoma brucei gambiense isolates from northwest Uganda using allele-specific polymerase chain reaction. Trop Med Int Health. 2007;12:1361–8.

109. Brun R, Schumacher R, Schmid C, Kunz C, Burri C. The phenomenon of treatment failures in Human African Trypanosomiasis. Trop Med Int Health. 2001;6:906–14.
110. Pyana PP, Ngay Lukusa I, Mumba Ngoyi D, Van Reet N, Kaiser M, Karhemere Bin Shamamba S, Buscher P. Isolation of Trypanosoma brucei gambiense from cured and relapsed sleeping sickness patients and adaptation to laboratory mice. PLoS Negl Trop Dis. 2011;5:e1025.
111. Pyana P, Van Reet N, Mumba Ngoyi D, Ngay Lukusa I, Karhemere Bin Shamamba S, Buscher P. Melarsoprol sensitivity profile of Trypanosoma brucei gambiense isolates from cured and relapsed sleeping sickness patients from the Democratic Republic of the Congo. PLoS Negl Trop Dis. 2014;8:e3212.
112. Graf FE, Baker N, Munday JC, de Koning HP, Horn D, Mäser P. Chimerization at the AQP2-AQP3 locus is the genetic basis of melarsoprol-pentamidine cross-resistance in clinical Trypanosoma brucei gambiense isolates. Int J Parasitol Drugs Drug Resist. 2015;5:65–8.

Mechanisms of Drug Resistance in *Toxoplasma gondii*

44

Alexandre Mzabi, Dominique Aubert, and Isabelle Villena

1 Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan with a worldwide distribution. Active drugs against *Toxoplasma gondii* are mainly represented by the inhibitors of folic acid synthesis: sulfonamides and pyrimethamine. These drugs are primarily active against the tachyzoites and have no effect on the cysts. In clinical practice, therapeutic failures and relapses are observed. The understanding of the failure mechanisms against these drugs in *Toxoplasma gondii* is essential due to the lack of effective and validated therapeutic alternatives. While the mechanisms of resistance are relatively well described in *Toxoplasma gondii* for the minor drugs (clindamycin and atovaquone), the mechanisms of resistance to the sulfadiazine (belonging to the sulfonamides class) are still not described. However, recent studies examine the role of enzymes involved in the folic acid synthesis, and the role of several transporters of xenobiotics, including the ATP-Binding Cassette transporters. These studies are mainly based on the analogy of drug resistance in the other *Apicomplexa*.

2 *Toxoplasma gondii:* Parasitic Cycle and Pathogenicity

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan parasite that infects more than one-third of the world's human population. The population type of *Toxoplasma gondii* consists of three main clonal lineages correlated with virulence expression in mice [1, 2]. Moreover, atypical strains were described as representing a separate group resulting from genetic recombination of major genotypes or from different genotypes. Recent studies, comparing more than 900 isolates from around the world, have regrouped the strains into 15 separate haplogroups. These haplogroups comprise the three main clonal lineages initially described (type I, II and III) and other haplogroups which cluster various atypical strains and new clonal lineages [3].

During its cycle, three stages are described in *Toxoplasma gondii* (Fig. 44.1): sporozoites in oocysts, tachyzoites, and bradyzoites in cysts [5]. Oocysts are only produced in the final hosts, members of Felidae family. When eliminated in faeces and after sporulation, they can be ingested by humans or other intermediate hosts, invading intestinal cells and then transformed into tachyzoites (Fig. 44.2), the rapidly multiplying form of *Toxoplasma gondii*. Tachyzoites disseminate through the organism and then settle in muscle tissues and in the central nervous system, transforming into cysts containing bradyzoites. This is thought to be a response to the host immune reaction. Ingestion of cysts, in contaminated meat, is also a source of infection, as bradyzoites transform back into tachyzoites while infecting a new host [7]. Most infections are asymptomatic in humans, but *Toxoplasma gondii* can cause severe clinical diseases such as encephalitis or a systemic infection in immunocompromised patients, more specifically individuals with HIV infection and in cases of congenital toxoplasmosis after mother-to-child transmission during pregnancy [8].

3 Available Therapies

The principal drugs known as active against *Toxoplasma gondii* are divided into two major groups: the inhibitors of folic acid synthesis and the macrolides [9]. These drugs are primarily active against the tachyzoites and have no effect on cysts.

A. Mzabi, M.D. (✉) • D. Aubert, Ph.D. • I. Villena, M.D.
Laboratory Parasitology-Mycology, Hospital Maison Blanche and EA 3800, University of Reims Champagne-Ardenne
Reims, France
e-mail: amzabi@chu-reims.fr

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_44

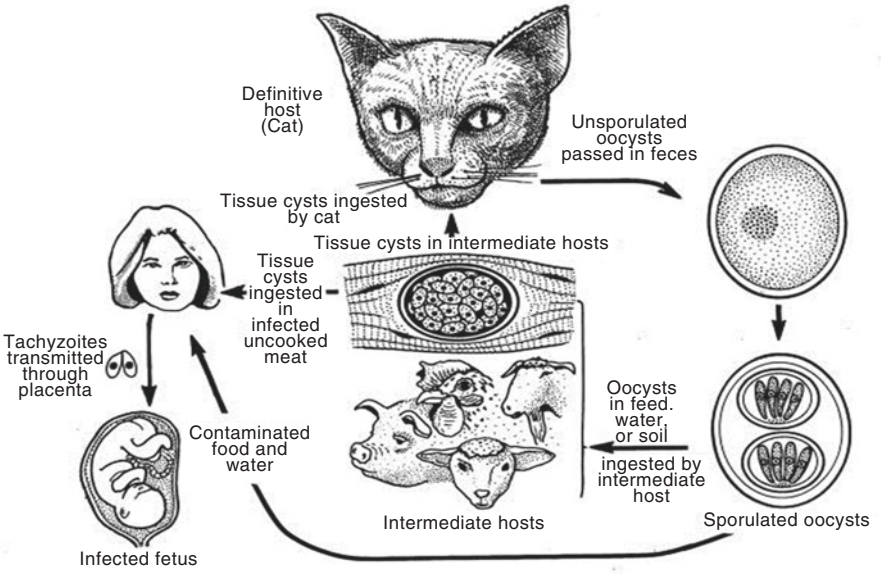


Fig. 44.1 *Toxoplasma gondii*: parasitic cycle [4]

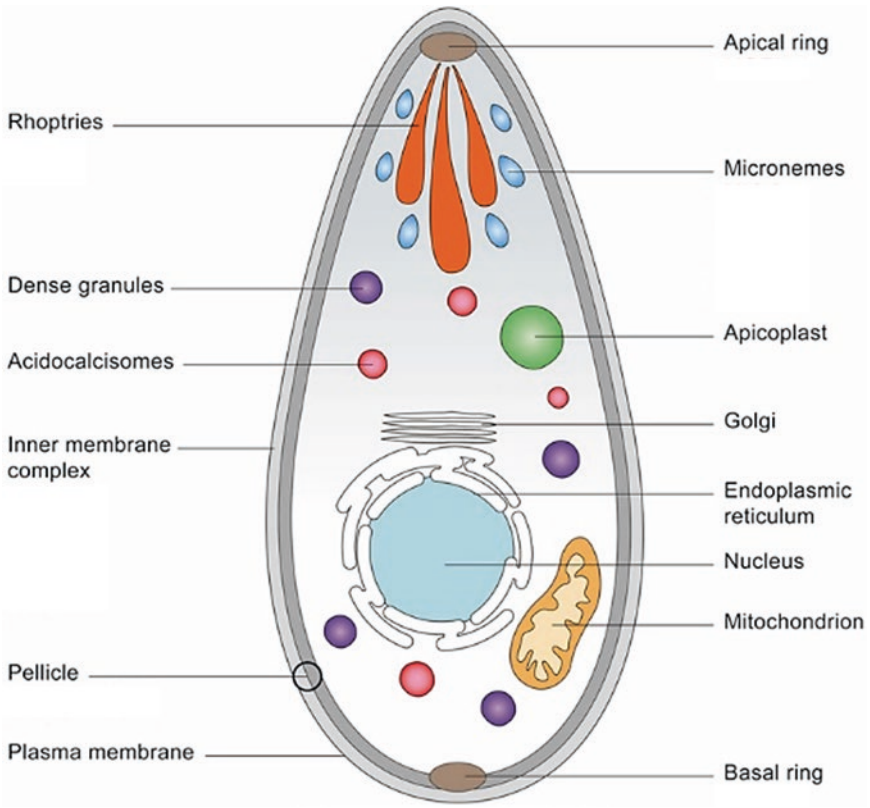


Fig. 44.2 Schematic representation of *Toxoplasma gondii* tachyzoite morphology [6]

3.1 Inhibitors of Folic Acid Synthesis

These drugs include dihydrofolate reductase (DHFR) inhibitors and dihydropteroate synthase (DHPS) inhibitors. These two anti-*Toxoplasma* drugs are used in combination because of a synergistic effect on two key enzymes of folic acid metabolism, DHPS and DHFR [10, 11].

These drugs act together by inhibiting the folic acid synthesis in *Toxoplasma gondii*, but also that of its host. As a consequence, they have a powerful anti-parasitic effect (changes of purine synthesis and parasitic division), which is associated with adverse haematological events (neutropenia, thrombocytopenia). These drugs diffuse well into the organism and cross the placental barrier.

Among the DHFR inhibitors, the most active drug is pyrimethamine, which has a parasiticide effect on *Toxoplasma gondii* tachyzoites at very low concentrations (50 % inhibitory concentration <0.2 mg/L, with a half-life of 92 h). To limit the adverse haematological events, pyrimethamine is administered with folinic acid. Trimethoprim is also active in *Toxoplasma gondii* at 50–100 times higher concentrations than pyrimethamine (50 % inhibitory concentration = 2 mg/L, with a half-life of 10–12 h).

For DHPS inhibitors, many sulfonamides are active against *Toxoplasma gondii* and their use is mainly guided by pharmacokinetics. Sulfadiazine (50 % inhibitory concentration = 2.5 mg/L) and sulfamethoxazole (50 % inhibitory concentration = 1 mg/L) have a short half-life (10–12 h), requiring daily administration, while the sulfadoxine (50 % inhibitory concentration = 25–50 mg/L) is a slow-release sulphonamide (half-life >100 h), less active than sulfadiazine, but can be administered weekly.

The most often prescribed active associations are pyrimethamine-sulfadiazine, pyrimethamine-sulfadoxine, or trimethoprim-sulfamethoxazole. These associations (except the pyrimethamine-sulfadoxine association) are used for the treatment and secondary prophylaxis of severe toxoplasmosis in immunocompromised patients. The pyrimethamine-sulfonamides association is used in congenital toxoplasmosis (prenatal and postnatal treatment).

As indicated above, in order to limit adverse haematological events, these treatments are administered with folinic acid. However, recent studies highlight the potential capacities of *Toxoplasma gondii* to recover the exogenous folate [12]. It has been shown that *Plasmodium falciparum* has two membrane proteins, PfFT1 and PfFT2, enabling the transport of exogenous folate [13]. After having analysed the ToxoDB database (http://toxodb.org/toxo/), it appears that *Toxoplasma gondii* also presents these two proteins. Another exogenous folate transporter family has also been described in *Toxoplasma gondii*, named the BT1 family [12]. The presence of these folate transporters in *Toxoplasma gondii* shows that this parasite could recover the exogenous folate for its survival. These observations raise the question about the systematic administration of folinic acid, which could potentially enhance the parasite's survival.

3.2 Macrolides

Macrolides include a wide variety of molecules. These antibiotics are only active at high concentrations (50 % inhibitory concentration = 1–100 mg/L) because of their parasitostatic effect, but the drugs' action is not well known. These drugs do not diffuse correctly in some organs, such as the brain and the eye, limiting their use in the treatment of severe toxoplasmosis. In contrast, macrolides diffuse well in the placenta and could reduce mother-to-child transmission of the parasite [14–16].

Spiramycin is the major macrolide used in the treatment of the benign form of acquired toxoplasmosis and during pregnancy in prevention of congenital toxoplasmosis. Other macrolides have better tissue concentrations, but are contraindicated during pregnancy.

Clindamycin is mainly used in association with pyrimethamine in the treatment of cerebral or eye toxoplasmosis, mainly in toxoplasmosis reactivation in immunocompromised patients. The action of the drug is well documented, and clindamycin appears to act by disrupting the protein synthesis of the parasite at the level of the cytoplasm and apicoplast [17, 18].

3.3 Other Drugs

Atovaquone, which acts by inhibiting the mitochondrial respiratory chain (cytochrome B1) of the parasite, is active on the tachyzoites and cysts of *Toxoplasma gondii*. However, the use of this drug is limited in the treatment of toxoplasmosis, because of its poor bioavailability.

4 Main Therapeutic Regimens

4.1 Acquired Toxoplasmosis

The asymptomatic forms do not justify treatment, and the benign forms can be treated by spiramycin. In severe forms with visceral injuries, a treatment with pyrimethamine-sulfonamides is effective.

4.2 Congenital Toxoplasmosis

The preventive treatment of the congenital toxoplasmosis in case of maternal seroconversion consists of administering spiramycin until childbirth, or until the result of the prenatal diagnosis, although its efficacy on the reduction of mother-to-child transmission is debatable [19, 20]. In the case of proven foetal contamination, prenatal treatment of congenital toxoplasmosis consists of administering pyrimethamine-sulfadiazine or pyrimethamine-sulfadoxine, with the concomitant administration of folinic acid. The efficacy of this treatment on foetal injuries seems to have been established by few studies [21, 22], even if the interest of prenatal treatment has not clearly been found in the meta-analysis by Thiébaut et al. [20] on 26 European cohorts. However, this data must be discussed in the absence of prospective studies [20, 23]. The postnatal treatment of congenital toxoplasmosis consists of the prolonged administration of the same drug association, and always with the folinic acid. This treatment is generally administered for at least 12 months, even 24 months depending on the research teams [24, 25]. A multicentre

French research programme is currently underway to compare the duration of treatment (a short period of 3 months vs. a long period of 12 months) with the occurrence of chorioretinitis at 5 years of age [26].

4.3 Toxoplasmosis in Immunocompromised Patients

The curative treatment of severe toxoplasmosis in immunocompromised patients consists of administering pyrimethamine-sulfadiazine [9, 27], with concomitant administration of folinic acid.

Other drugs can also be used such as a trimethoprim-sulfamethoxazole association or macrolides. Some clinical studies suggest the association of macrolides with pyrimethamine, especially in case of intolerance to the pyrimethamine-sulfadiazine association. Thus, clindamycin [28], azithromycin [29, 30] or clarithromycin [31] may be used in association with pyrimethamine for the treatment of severe toxoplasmosis, including cerebral toxoplasmosis.

5 Clinical Therapeutic Failures

In clinical practice, therapeutic failures and relapses have been observed. The understanding of the failure mechanisms against the main active drugs on *Toxoplasma gondii* is essential because there are currently few effective and validated therapeutic alternatives.

Congenital toxoplasmosis reveals, in its natural evolution, the emergence of chorioretinitis without having clearly established the physiological mechanisms or predictive factors [25, 32–34]. The treatment appears to be active for recent injuries and can decrease the after-effects. Chorioretinitis can appear during treatment in spite of a good observance and correct serum concentrations [21, 24, 35]. However, therapeutic failures are described with the main drugs used (pyrimethamine-sulfadiazine association) in the treatment of severe toxoplasmosis in immunocompromised patients [9, 36].

Several pharmacological parameters can contribute to these failures: individual factors of absorption, drug intolerance or poor tissue distribution. Considering a drug's action mechanism, and in analogy to the other *Apicomplexa*, the existence of a sensitivity change for drugs and/or the development of drug resistance could be feared.

6 Mechanisms of Drug Resistance in *Toxoplasma gondii*

In 1973, the World Health Organization (WHO) defined, in the case of malaria, drug resistance as 'the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given doses equal to or higher than those usually recommended but within the limits of tolerance of the subject'. Since then, this definition has extended to other species of parasites. Over the past 20 years, the number of studies on drug resistance in protozoa has continually increased, reflecting the importance of this problem for public health. Among the identified mechanisms of resistance, those based on the mutation of target genes, the overexpression of genes, and the efflux mechanisms of drugs seem to be mainly involved.

6.1 Clindamycin and Atovaquone: Mechanisms of Resistance

The mechanisms of resistance to clindamycin and atovaquone in *Toxoplasma gondii* involve experimental strains, which present a stable resistance to these drugs [37–39].

Clindamycin acts by disrupting protein synthesis at the level of apicoplasts. A point mutation of the large subunit of the rRNA in *Toxoplasma gondii* at position 1857 was identified in two mutant resistant strains for clindamycin. This mutation, corresponding to the mutation 2061 in *Escherichia coli* [40] and to the mutations 2058 and 2059 in antibiotic resistant bacteria [41], identifies apicoplasts as the clindamycin target [39].

Protozoan mitochondria were identified as therapeutic targets sensitive to ubiquinone analogues, such as the atovaquone [42, 43]. McFadden et al. [38] showed that the molecular target of the atovaquone in *Toxoplasma gondii* was the Q(o) domain of the cytochrome b gene, suggesting that mutations in this region could lead to a resistance to this drug. Two mutations obtained by mutagenicity, M129L and I254L, were identified in four strains resistant to atovaquone. These mutations take place in the regions forming the Q(o) domain, in the tertiary structure of the cytochrome b [38, 44]. Studies in *Plasmodium* and *Pneumocystis* have also shown that mutations in the Q(o) domain of cytochrome b lead to atovaquone resistance [45–47].

6.2 Inhibitors of Folic Acid Synthesis: Mechanisms of Resistance

Among the protozoa of clinical importance, *Toxoplasma gondii* and *Plasmodium* present a common evolution. They are members of the *Apicomplexa* phylum and possess vulnerability in their folic acid metabolism, which forms the base of currently used treatments [48].

Folic acid consists of three different parts: a pterin nucleus, a para-aminobenzoic acid (pABA) and a glutamate chain.

Most bacteria, yeasts and some parasites can synthesise de novo folic acid [12]. These micro-organisms must conjugate the pterin with pABA to form dihydropteroate.

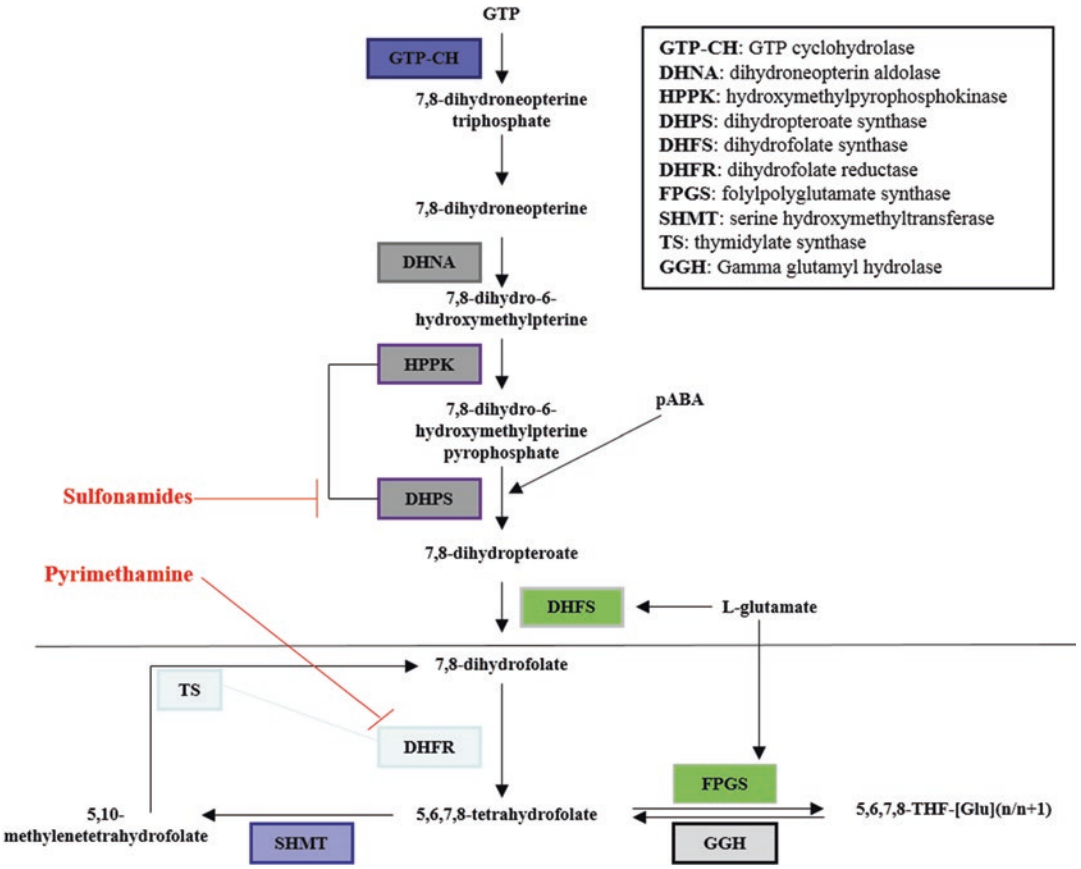


Fig. 44.3 Folic acid synthesis in *Toxoplasma gondii*

This reaction is catalysed by DHPS, which is the specific therapeutic target of sulfonamides. Most micro-organisms can synthesise folates from GTP, pABA and L-glutamate.

Dihydrofolate synthase (DHFS) catalyses the addition of glutamic acid to 7.8-dihydropteroate, for the synthesis of 7.8-dihydrofolate. DHFR reduces dihydrofolate (DHF) in tetrahydrofolate (THF). It is changed into 5.10-methylenetetrahydrofolate to serve as carbon donor for the synthesis of thymidylate, a reaction that is catalysed by thymidylate synthase (TS) (Fig. 44.3).

At the cellular level, the active forms of folic acid are the products derived from DHF and THF. The intracellular folates of micro-organisms and mammal cells are mainly in the form of polyglutamates, comprising in their structure four to six glutamate residues [49]. This polyglutamylation is realised by DHFS and folylpolyglutamate synthase (FPGS). Polyglutamates are better retained than glutamates in the cell. Furthermore, they are better substrates and co-factors of enzymes involved in folic acid metabolism.

The inhibitors of folic acid synthesis include DHFR inhibitors and DHPS inhibitors. These two anti-*Toxoplasma* drugs are used in combination because of a synergistic effect on two key enzymes of folic acid metabolism, DHPS and DHFR [10, 11]. The mechanisms of resistance to these drugs in *Toxoplasma gondii* are still little described in the literature.

6.2.1 Sulfonamides: Mechanisms of Resistance

1–dhps Gene Mutations

Aspinall et al. [50] highlighted six mutations in the *dhps* gene, after having sequenced 32 strains from human toxoplasmosis. A mutation affecting the residue 407 of the protein coded by the *dhps* gene was found in a clinical strain, Swa-20, isolated from untreated congenital toxoplasmosis. This mutation implies a natural resistance of the strain to sulfonamides. The same mutation had also been highlighted for an experimental sulfonamide resistant strain, R-SulR-5, obtained in vitro by directed mutagenesis [51], underlining the possible implication of this mutation in sulfonamide resistance. However, a study of 200 *Toxoplasma gondii* strains preserved in the Biological Resource Centre *Toxoplasma* (France) did not find this mutation (Villena, personal data).

Due to the lack of reported data on the variations of a 50 % inhibitory concentration in *Toxoplasma gondii*, the sensitivity levels to various anti-*Toxoplasma* drugs (sulfadiazine, pyrimethamine and atovaquone) were studied in 17 *Toxoplasma gondii* strains chosen according to a criterion of genomic diversity or a clinical criterion [52]. This study identified three in vitro resistant strains to sulfadiazine, in MRC-5 cells. These same resistant strains were found on

Vero cells [53]. Furthermore, among these three strains, two were responsible for congenital toxoplasmosis with the occurrence of chorioretinitis under treatment, in spite of effective serum rates. These two strains, TgH 32006 (genotype II) and TgH 32045 (atypical genotype), were isolated from human placentas during congenital toxoplasmosis, and the third strain, TgA 103001 (genotype I), was isolated from a bovine placenta. It is important to note that only the TgH 32006 strain was exposed for 12 weeks to the pyrimethamine-sulfadoxine association during pregnancy. Moreover, these data show the non-correlation between genotype and resistance to sulfadiazine in *Toxoplasma gondii*.

As part of the analysis of these 17 *Toxoplasma gondii* strains by Meneceur et al. [52], three new mutations were identified for the *dhps* gene: two silent mutations at position 370 and 654, and one causing an amino acid change (Alanine to Valine) at position 587, which was only found for the TgH 32006 strain. This mutation was not found for the two other resistant strains (TgH 32045 and TgA 103001), preventing the establishment of a formal link between this mutation and resistance. Moreover, the mutation described by Aspinall et al. [50] at position 407 was not found in this study.

2–Induction of Sulfadiazine Resistance in Vitro by Drug Pressure

In order to better understand the mechanisms of resistance to sulfadiazine in *Toxoplasma gondii*, Doliwa et al. [53] have developed, in vitro, two sulfadiazine resistant strains, RH-R^{SDZ} and ME-49-R^{SDZ}, by drug pressure. These strains will be able to be used as experimental model to characterise the mechanisms of resistance to sulfadiazine in *Toxoplasma gondii*.

3–ATP-Binding Cassette (ABC) Transporters

Sauvage et al. [54] have demonstrated that accumulation and efflux of xenobiotics from parasites are modulated by P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP) inhibitors, indicating their presence and activity in *Toxoplasma gondii*. Pgp and MRP proteins belong to the ATP-binding cassette (ABC) superfamily of transporters. So far, Sauvage et al. [55] have identified in the *Toxoplasma gondii* genome 24 genes related to the ABC whose expression was detected both in tachyzoite and bradyzoite infectious stages for the three genotypes. Among these 24 genes, two encode for whole Pgps, *TgABC.B1* (1345 amino acids) and *TgABC.B2* (1407 amino acids), and one encodes for a MRP, *TgABC.C1* (1883 amino acids). Pgp and MRP are widely reported to export xenobiotics and cause drug resistance in tumour cells [56] and protozoan parasites [57] and lead to drug resistance by increasing drug efflux from the cell, thus lowering the effective intracellular drug concentration. The increased activities of the ABC transporters could be due to an increased amount of proteins due to gene amplification or overexpression associated or not associated with point mutations in the genomic sequence. In *Plasmodium falciparum*, antimalarial resistance involves mutations and/or amplification of one Pgp and MRP genes, *PfABC.B1* (alias *PfMDR1*) and *PfABC.C1* (alias *PfMRP*), respectively. Mutations in *PfABC.B1* are identified in clinical isolates from different geographical areas. Polymorphisms are observed at five positions—codons 86, 184, 1034, 1042, and 1246. *PfABC.B1* overexpression is the only mechanism suggested to date involved in mefloquine-resistant parasites [58]. Concerning *PfABC.C1*, mutations at positions 191His and 437Ser are found to be linked 100 % to decrease quinolone resistance in south eastern Iranian isolates [59].

To identify the genotypic and/or phenotypic markers of resistance in *Toxoplasma gondii*, and in analogy with *Plasmodium* [58–60], a study was carried out that sequenced and analysed the expression levels of therapeutic targets (*dhps* and *dhfr*) and three ABC transporter genes (*TgABC.B1*, *TgABC.B2* and *TgABC.C1*). It analysed sensitive strains compared to sulfadiazine resistant strains (natural resistance or induced resistance), but neither polymorphism nor overexpression was identified [61].

4–Sulfonamides: Conclusion

The mechanisms of resistance to sulfonamides in *Toxoplasma gondii* are still not described, although ongoing studies suggest the participation of other enzymes implicated in folic acid synthesis and the participation of other transporters of xenobiotics.

6.2.2 Pyrimethamine: Mechanisms of Resistance

The 17 *Toxoplasma gondii* strains tested in vitro by Meneceur et al. [52] did not show resistance to pyrimethamine and, like Aspinall et al. [50], no polymorphism resulting in an amino acid change in the coding region of the *dhfr* gene was found. According to these results, clinical resistance to pyrimethamine has rarely been described [62]. Furthermore, the conception of a resistant strain to pyrimethamine by drug pressure in vitro is very fastidious (currently no pyrimethamine resistant strain has been described according to this method).

However, pyrimethamine resistant tachyzoites have been isolated in vitro by random mutagenesis of the *Toxoplasma gondii dhfr-ts* gene, followed by transfection and pyrimethamine selection in the parasite [63]. By modelling *Plasmodium falciparum* resistance mutations at codons equivalent to 59 and 108 [64], a high level of pyrimethamine resistance was obtained in *Toxoplasma gondii* DHFR-TS [65, 66]. Plasmids conferring a high level of pyrimethamine resistance in *Toxoplasma gondii* have been useful in the study of the resistance to pyrimethamine in various *Apicomplexa* [67]. Furthermore, these resistant mutants in *Toxoplasma gondii* are characterised by a reduction of their

fitness in vitro and in vivo (mouse model), relative to wild-type strains [68]. Consideration of this data therefore seems essential to any mechanistic analysis of these strains. However, this model is primarily used for the study of the other *Apicomplexa*, as currently the natural resistance to pyrimethamine in *Toxoplasma gondii* is still hypothetical.

References

1. Howe DK, Sibley LD. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis. 1995;172:1561–6.
2. Saeij JP, Boyle JP, Grigg ME, Arrizabalaga G, Boothroyd JC. Bioluminescence imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between strains. Infect Immun. 2005;73:695–702.
3. Su C, Khan A, Zhou P, Majumdar D, Ajzenberg D, Dardé ML, Zhu XQ, Ajioka JW, Rosenthal BM, Dubey JP, Sibley LD. Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. Proc Natl Acad Sci. 2012;109:5844–9.
4. Dubey JP. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. J Parasitol. 1995;81:410–5.
5. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet. 2004;363:1965–79.
6. Corvi MM, Berthiaume LG, De Napoli MG. Protein palmitoylation in protozoan parasites. Front Biosci (Schol Ed). 2011;3:1067–79.
7. Tenter AM. *Toxoplasma gondii* in animals used for human consumption. Mem Inst Oswaldo Cruz. 2009;104:364–9.
8. Weiss LM, Dubey JP. Toxoplasmosis: a history of clinical observations. Int J Parasitol. 2009;39:895–901.
9. Derouin F. Anti-toxoplasmosis drugs. Curr Opin Investig Drugs. 2001;2:1368–74.
10. Sheffield HG, Melton ML. Effect of pyrimethamine and sulfadiazine on the fine structure and multiplication of *Toxoplasma gondii* in cell cultures. J Parasitol. 1975;61:704–12.
11. Derouin F, Chastang C. *In vitro* effects of folate inhibitors on *Toxoplasma gondii*. Antimicrob Agents Chemother. 1989;33:1753–9.
12. Massimine KM, Doan LT, Atreya CA, Stedman TT, Anderson KS, Joiner KA, Coppens I. *Toxoplasma gondii* is capable of exogenous folate transport. A likely expansion of the BT1 family of transmembrane proteins. Mol Biochem Parasitol. 2005;144:44–54.
13. Salcedo-Sora JE, Ochong E, Beveridge S, Johnson D, Nzila A, Biagini GA, Stocks PA, O'Neill PM, Krishna S, Bray PG, Ward SA. The molecular basis of folate salvage in *Plasmodium falciparum*: characterization of two folate transporters. J Biol Chem. 2011;286:44659–68.
14. Forestier F, Daffos F, Rainaut M, Desnottes JF, Gaschard JC. Fetomaternal therapeutic follow-up of spiramycin during pregnancy. Arch Fr Pediatr. 1987;44:539–44.
15. Couvreur J, Desmonts G, Thulliez P. Prophylaxis of congenital toxoplasmosis. Effects of spiramycin on placental infection. J Antimicrob Chemother. 1988;22:193–200.
16. Stray-Pedersen B. Treatment of toxoplasmosis in the pregnant mother and newborn child. Scand J Infect Dis Suppl. 1992;84:23–31.
17. Blais J, Tardif C, Chamberland S. Effect of clindamycin on intracellular replication, protein synthesis, and infectivity of *Toxoplasma gondii*. Antimicrob Agents Chemother. 1993;37:2571–7.
18. Beckers CJ, Roos DS, Donald RG, Luft BJ, Schwab JC, Cao Y, Joiner KA. Inhibition of cytoplasmic and organellar protein synthesis in *Toxoplasma gondii*. Implications for the target of macrolide antibiotics. J Clin Invest. 1995;95:367–76.
19. Gilbert RE, Gras L, Wallon M, Peyron F, Ades AE, Dunn DT. Effect of prenatal treatment on mother to child transmission of *Toxoplasma gondii*: retrospective cohort study of 554 mother-child pairs in Lyon, France. Int J Epidemiol. 2001;30:1303–8.
20. Thiébaut R, Leproust S, Chêne G, Gilbert R. Effectiveness of prenatal treatment for congenital toxoplasmosis: a meta-analysis of individual patients' data. Lancet. 2007;369:115–22.
21. Villena I, Aubert D, Leroux B, Dupouy D, Talmud M, Chemla C, Trenque T, Schmit G, Quereux C, Guenounou M, Pluot M, Bonhomme A, Pinon JM. Pyrimethamine-sulfadoxine treatment of congenital toxoplasmosis: follow-up of 78 cases between 1980 and 1997. Reims Toxoplasmosis Group. Scand J Infect Dis. 1998;30:295–300.
22. Foulon W, Villena I, Stray-Pedersen B, Decoster A, Lappalainen M, Pinon JM, Jenum PA, Hedman K, Naessens A. Treatment of toxoplasmosis during pregnancy: a multicenter study of impact on fetal transmission and children's sequelae at age 1 year. Am J Obstet Gynecol. 1999;180:410–5.
23. Gras L, Gilbert RE, Ades AE, Dunn DT. Effect of prenatal treatment on the risk of intracranial and ocular lesions in children with congenital toxoplasmosis. Int J Epidemiol. 2001;30:1309–13.
24. Villena I, Quereux C, Pinon JM. Congenital toxoplasmosis: value of prenatal treatment with pyrimethamine-sulfadoxine combination. Prenat Diagn. 1998;18:754–6.
25. Wallon M, Kodjikian L, Binquet C, Garweg J, Fleury J, Quantin C, Peyron F. Long-term ocular prognosis in 327 children with congenital toxoplasmosis. Pediatrics. 2004;113:1567–72.
26. Wallon M, Kieffer F, Binquet C, Thulliez P, Garcia-Méric P, Dureau P, Franck J, Peyron F, Bonnin A, Villena I, Bonithon-Kopp C, Gouyon JB, Masson S, Félin A, Cornu C. Congenital toxoplasmosis: randomised comparison of strategies for retinochoroiditis prevention. Therapie. 2011;66:473–80.
27. Luft BJ, Hafner R, Korzun AH, Leport C, Antoniskis D, Bosler EM, Bourland DD, Uttamchandani R, Fuhrer J, Jacobson J. Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome. Members of the ACTG 077p/ANRS 009 Study Team. N Engl J Med. 1993;329:995–1000.
28. Dannemann B, McCutchan JA, Israelski D, Antoniskis D, Leport C, Luft B, Nussbaum J, Clumeck N, Morlat P, Chiu J, Vilde JL, Orellana M, Feigal D, Bartok A, Heseltine P, Leedom J, Remington J. Treatment of toxoplasmic encephalitis in patients with AIDS. A randomized trial comparing pyrimethamine plus clindamycin to pyrimethamine plus sulfadiazine. Ann Intern Med. 1992;116:33–43.
29. Saba J, Morlat P, Raffi F, Hazebroucq V, Joly V, Leport C, Vildé JL. Pyrimethamine plus azithromycin for treatment of acute toxoplasmic encephalitis in patients with AIDS. Eur J Clin Microbiol Infect Dis. 1993;12:853–6.
30. Jacobson JM, Hafner R, Remington J, Farthing C, Holden-Wiltse J, Bosler EM, Harris C, Jayaweera DT, Roque C, Luft BJ. Dose-escalation, phase I/II study of azithromycin and pyrimethamine for the treatment of toxoplasmic encephalitis in AIDS. AIDS. 2001;15:583–9.
31. Fernandez-Martin J, Leport C, Morlat P, Meyohas MC, Chauvin JP, Vilde JL. Pyrimethamine-clarithromycin combination for therapy of acute *Toxoplasma* encephalitis in patients with AIDS. Antimicrob Agents Chemother. 1991;35:2049–52.
32. Berrébi A, Assouline C, Bessières MH, Lathière M, Cassaing S, Minville V, Ayoubi JM. Long-term outcome of children with congenital toxoplasmosis. Am J Obstet Gynecol. 2010;203:552.e1–6.
33. Faucher B, Garcia-Meric P, Franck J, Minodier P, Francois P, Gonnet S, L'ollivier C, Piarroux R. Long-term ocular outcome in congenital toxoplasmosis: a prospective cohort of treated children. J Infect. 2012;64:104–9.
34. Wallon M, Garweg JG, Abrahamowicz M, Cornu C, Vinault S, Quantin C, Bonithon-Kopp C, Picot S, Peyron F, Binquet C. Ophthalmic outcomes of congenital toxoplasmosis followed until adolescence. Pediatrics. 2014;133:e601–8.

35. Trenque T, Simon N, Villena I, Chemla C, Quereux C, Leroux B, Jaussaud R, Rémy G, Dupouy D, Millart H, Pinon J-M, Urien S. Population pharmacokinetics of pyrimethamine and sulfadoxine in children with congenital toxoplasmosis. Br J Clin Pharmacol. 2004;57:735–41.
36. Faucher B, Moreau J, Zaegel O, Franck J, Piarroux R. Failure of conventional treatment with pyrimethamine and sulfadiazine for secondary prophylaxis of cerebral toxoplasmosis in a patient with AIDS. J Antimicrob Chemother. 2011;66:1654–6.
37. Pfefferkorn ER, Borotz SE, Nothnagel RF. Mutants of *Toxoplasma gondii* resistant to atovaquone (566C80) or decoquinate. J Parasitol. 1993;79:559–64.
38. McFadden DC, Tomavo S, Berry EA, Boothroyd JC. Characterization of cytochrome b from *Toxoplasma gondii* and Q(o) domain mutations as a mechanism of atovaquone-resistance. Mol Biochem Parasitol. 2000;108:1–12.
39. Camps M, Arrizabalaga G, Boothroyd J. An rRNA mutation identifies the apicoplast as the target for clindamycin in *Toxoplasma gondii*. Mol Microbiol. 2002;43:1309–18.
40. Douthwaite S. Interaction of the antibiotics clindamycin and lincomycin with *Escherichia coli* 23S ribosomal RNA. Nucleic Acids Res. 1992;20:4717–20.
41. Sigmund CD, Ettayebi M, Borden A, Morgan EA. Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. Methods Enzymol. 1988;164:673–90.
42. Romand S, Pudney M, Derouin F. *In vitro* and *in vivo* activities of the hydroxynaphthoquinone atovaquone alone or combined with pyrimethamine, sulfadiazine, clarithromycin, or minocycline against *Toxoplasma gondii*. Antimicrob Agents Chemother. 1993;37:2371–8.
43. Ellis JE. Coenzyme Q homologs in parasitic protozoa as targets for chemotherapeutic attack. Parasitol Today (Regul Ed). 1994;10: 296–301.
44. Kessl JJ, Meshnick SR, Trumpower BL. Modeling the molecular basis of atovaquone resistance in parasites and pathogenic fungi. Trends Parasitol. 2007;23:494–501.
45. Walker DJ, Wakefield AE, Dohn MN, Miller RF, Baughman RP, Hossler PA, Bartlett MS, Smith JW, Kazanjian P, Meshnick SR. Sequence polymorphisms in the *Pneumocystis carinii* cytochrome b gene and their association with atovaquone prophylaxis failure. J Infect Dis. 1998;178:1767–75.
46. Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB. Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. Mol Microbiol. 1999;33:704–11.
47. Kazanjian P, Armstrong W, Hossler PA, Lee CH, Huang L, Beard CB, Carter J, Crane L, Duchin J, Burman W, Richardson J, Meshnick SR. *Pneumocystis carinii* cytochrome b mutations are associated with atovaquone exposure in patients with AIDS. J Infect Dis. 2001;183:819–22.
48. Ho-Yen DO, Joss AW, Chatterton JM. Congenital toxoplasmosis. BMJ. 1992;305:651–2.
49. Moran RG. Roles of folylpoly-gamma-glutamate synthetase in therapeutics with tetrahydrofolate antimetabolites: an overview. Semin Oncol. 1999;26:24–32.
50. Aspinall TV, Joynson DH, Guy E, Hyde JE, Sims PF. The molecular basis of sulfonamide resistance in *Toxoplasma gondii* and implications for the clinical management of toxoplasmosis. J Infect Dis. 2002;185:1637–43.
51. Pfefferkorn ER, Borotz SE, Nothnagel RF. *Toxoplasma gondii*: characterization of a mutant resistant to sulfonamides. Exp Parasitol. 1992;74:261–70.
52. Meneceur P, Bouldouyre M-A, Aubert D, Villena I, Menotti J, Sauvage V, Garin J-F, Derouin F. In vitro susceptibility of various genotypic strains of *Toxoplasma gondii* to pyrimethamine, sulfadiazine, and atovaquone. Antimicrob Agents Chemother. 2008;52:1269–77.
53. Doliwa C, Escotte-Binet S, Aubert D, Velard F, Schmid A, Geers R, Villena I. Induction of sulfadiazine resistance *in vitro* in *Toxoplasma gondii*. Exp Parasitol. 2013;133:131–6.
54. Sauvage V, Aubert D, Bonhomme A, Pinon JM, Millot JM. P-glycoprotein inhibitors modulate accumulation and efflux of xenobiotics in extra and intracellular *Toxoplasma gondii*. Mol Biochem Parasitol. 2004;134:89–95.
55. Sauvage V, Millot JM, Aubert D, Visneux V, Marle-Plistat M, Pinon JM, Villena I. Identification and expression analysis of ABC protein-encoding genes in *Toxoplasma gondii. Toxoplasma gondii* ATP-binding cassette superfamily. Mol Biochem Parasitol. 2006;147:177–92.
56. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol. 1999;39:361–98.
57. Sauvage V, Aubert D, Escotte-Binet S, Villena I. The role of ATP-binding cassette (ABC) proteins in protozoan parasites. Mol Biochem Parasitol. 2009;167:81–94.
58. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet. 2004;364:438–47.
59. Ursing J, Zakeri S, Gil JP, Björkman A. Quinoline resistance associated polymorphisms in the *pfcrt*, *pfmdr1* and *pfmrp* genes of *Plasmodium falciparum* in Iran. Acta Trop. 2006;97:352–6.
60. Sibley CH, Hyde JE, Sims PF, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM, Nzila AM. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol. 2001;17:582–8.
61. Doliwa C, Escotte-Binet S, Aubert D, Sauvage V, Velard F, Schmid A, Villena I. Sulfadiazine resistance in *Toxoplasma gondii*: no involvement of overexpression or polymorphisms in genes of therapeutic targets and ABC transporters. Parasite. 2013;20:19.
62. Caramello P, Brancale T, Forno B, Lucchini A, Pollono AM, Ullio A, Gioannini P, Viano I, Tonso E. Relapse of *Toxoplasma* encephalitis and susceptibility to pyrimethamine: lack of evidence of treatment-induced resistance. Antimicrob Agents Chemother. 1995;39:2371–2.
63. Reynolds MG, Oh J, Roos DS. In vitro generation of novel pyrimethamine resistance mutations in the *Toxoplasma gondii* dihydrofolate reductase. Antimicrob Agents Chemother. 2001;45:1271–7.
64. Bzik DJ, Li WB, Horii T, Inselburg J. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc Natl Acad Sci U S A. 1987;84:8360–4.
65. Donald RG, Roos DS. Stable molecular transformation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc Natl Acad Sci U S A. 1993;90:11703–7.
66. Roos DS. Primary structure of the dihydrofolate reductase-thymidylate synthase gene from *Toxoplasma gondii*. J Biol Chem. 1993;268:6269–80.
67. Reynolds MG, Roos DS. A biochemical and genetic model for parasite resistance to antifolates. *Toxoplasma gondii* provides insights into pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. J Biol Chem. 1998;273:3461–9.
68. Fohl LM, Roos DS. Fitness effects of DHFR-TS mutations associated with pyrimethamine resistance in apicomplexan parasites. Mol Microbiol. 2003;50:1319–27.

Eimeria and *Cryptosporidium*: Recent Advances in the Therapeutic Field

45

Dominique Aubert and Loïc Favennec

1 Introduction

Coccidiosis is a disease caused by parasites of the genus *Eimeria* and *Isospora* belonging to the phylum Apicomplexa with a complex life cycle, affecting mainly the intestinal tract of many species of mammals and birds. It is of great economic significance in farm animals, especially chickens. Poultry coccidiosis is caused by protozoan parasites belonging to the genus *Eimeria* and is associated with global economic losses in excess of $3 billion annually [1]. In chickens, seven widely recognised species of *Eimeria* have been well characterised and are commonly observed within the domestic fowl: *E. acervulina*, *E. mitis*, *E. maxima*, *E. brunetti*, *E. necatrix*, *E. praecox*, and *E. tenella. Eimeria spp*. are ubiquitous in poultry and are environmentally resistant. Coccidiosis is transmitted between hosts by the ingestion of feed, water, and litter contaminated with thick-walled oocysts that are shed in the faeces of infected animals and spread by fomites or personnel moving between houses.

2 Anticoccidial Products

The protective effects of sulfonamides have been evaluated extensively and used as the first effective anticoccidial agents. In 1939, Levine [2] first reported the use of sulfanilamide against coccidiosis, and it was hypothesised that these compounds helped the bird acquire immunity against coccidiosis. Several studies carried out with *E. tenella*, a principal pathogen that was of concern, have confirmed that these compounds are effective in controlling infection, in addition to boosting immunity. Similar results were documented in studies with other species such as *E. necatrix* or *E. acervulina* in chickens and *E. meleagrimitis* in turkeys.

The use of ionophores as effective coccidiostats is well documented and has been used for decades. Ionophores act on the sporozoite/merozoite stages of the parasite life cycle, binding to cations and interfering with osmotic potential, thereby disrupting membrane integrity [3].

Anticoccidial products can be classified into three categories according to their origin [3, 4]:

1. Synthetic compounds. These compounds are produced by chemical synthesis and often referred to as ‘chemicals’. Synthetic drugs have a specific mode of action against parasite metabolism. For example, amprolium competes for the absorption of thiamine (vitamin B1) by the parasite.
2. Polyether antibiotics or ionophores. These products are produced by the fermentation of *Streptomyces spp.* or *Actinomadura spp.* and destroy coccidia by interfering with the balance of important ions like sodium and potassium. The following groups of ionophores exist:
   - Monovalent ionophores (monensin, narasin and salinomycin).
   - Monovalent glycosidic ionophores (maduramicin and semduramicin).
   - Divalent ionophores (lasalocid).
3. Mixed products. A few drug mixtures, consisting of either a synthetic compound and ionophore (nicarbazin/narasin (Maxiban®)) or two synthetic compounds (meticlorpindol/methylbenzoquate), are also used against coccidiosis.

D. Aubert, Ph.D. (✉)
Laboratory Parasitology-Mycology, Hospital Maison Blanche and EA 3800, University of Reims Champagne-Ardenne, Reims, France
e-mail: daubert@chu-reims.fr

L. Favennec, M.D., Ph.D.
Laboratory Parasitology-Mycology, Hospital Charles Nicolle and EA 3800, University of Rouen, Rouen, France

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_45

3 Drug Resistance

Drug resistance is defined in antimalarial chemotherapy as 'the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject' [5].

Generally, drug resistance in coccidia can be complete, in which case increasing doses up the maximum tolerated by the host is ineffective (i.e. diclazuril and nicarbazin). In contrast, relative resistance to anticoccidial drugs is characterised by the fact that increasing doses tolerated by the host still will show efficacy (i.e. ionophores).

In some cases, resistance is induced very quickly, as in the case of quinolones and pyridinols, which led to a decline in their use, while in other instances it may take several years as in the case of ionophores. The speed of emergence of resistant strains of coccidia in the field is given by Reid [6] as follows: (i) Glycomide—very rapid, (ii) Quinolones—rapid, (iii) Clopidol—less rapid, (iv) Sulphonamides, nitrofurans, robenidine—moderate, (v) Amprolium—slow, (vi) Nicarbazin—very slow and (vii) Monensin—absent or very slow. Resistance is more likely to develop in birds reared under intensive conditions than in farm animals.

Monensin, the first polyether ionophore, was introduced in the United States in 1971, and the first evidence of a monensin-resistant *Eimeria* isolate was seen as early as 10 years later. Research shows that monensin-resistant strains of *Eimeria* display altered characteristics, specifically, increased esterase activity [7]. However, little is understood regarding resistance associated with other ionophores.

In the early 1970s, several synthetic drugs were commercially introduced. Amprolium, nicarbazin, diclazuril, and toltrazuril have been successfully used in the control of coccidiosis for many years. The mode of action of these chemicals is similar, and most are known to inhibit sporozoite/merozoite development.

As for most antimicrobial chemicals, resistance to these drugs has become a problem. According to Chapman [3], diclazuril-resistant field isolates could not be found, even though it was possible to confer resistance to the drug in experimental conditions. Kawazoe and Fabio [8] observed variability in field isolates with respect to resistance to diclazuril, including a number of isolates that were completely resistant. Recently, it has been observed that a very high percentage of field isolates of *E. acervulina* and *E. maxima* and a significantly high percentage of *E. tenella* obtained locally from 26 broiler farms in 12 states of the USA showed either complete or partial resistance to the combination mixture of nicarbazin and narasin that has been used for a long time in the poultry industry. Most drugs are no longer as effective as when they were first introduced due to the development of drug resistance. For example, one recent report indicated that 68 and 53 % of field isolates of *E. acervulina* from chicken flocks in the EU were resistant to the synthetic drug diclazuril and the ionophore monensin, respectively [9]. Similar reports of resistance have been reported worldwide. In the turkey, drug resistance has also been shown to be widespread [10]. Details of the emergence of resistance in the 1970s to decoquinate have been provided retrospectively [11]. Although many surveys have been published indicating the extent of resistance, little research has been conducted on the mechanisms involved.

4 Mechanism of Resistance

Resistance may involve modification of the target with a decrease in sensitivity (amprolium), use of an alternative biochemical pathway (quinolones, clopidol) and altered permeability (ionophores) [12].

Amprolium competitively inhibits the uptake of thiamine by second generation schizonts of *E. tenella* [13]. An amprolium resistant line showed a decreased sensitivity to the inhibitory effect of the drug, probably reflecting change at the molecular level in the unknown target receptor [12].

Diclazuril and decoquinate are synthetic drugs to which resistance can also develop. Diclazuril has recently been shown to induce ultrastructural changes in merozoites and cause disruption of transmembrane potential in the mitochondrion [14]. It is not clear if this reflects a true mode of action or is just a consequence of cell death.

Quinolones inhibit respiration of *E. tenella* by blocking electron transport in the parasite mitochondrion [15]. Clopidol also affects electron transport in coccidian but has a different mode of action from quinolone, strains resistant to the latter are sensitive to clopidol and vice versa. Clopidol probably inhibits the electron transport chain at a different point from that of the quinolone. Hydroxynaphthoquinones (not used for the control of coccidiosis but employed for the treatment of theileriosis) are potent and selective inhibitors of electron transport in *E. tenella* [16]. The primary site of inhibition has been localised to the ubiquinone-cytochrome c reductase part of the respiratory chain. Interestingly, lines of *E. tenella* resistant to decoquinate or clopidol show no cross-resistance to the drugs, suggesting a different mode of action.

Augustine et al. [17] showed that the uptake of monensin by sporozoites of *E. tenella* resistant to the drug was significantly less than that by sensitive sporozoites. The amount of drug required to inhibit development was 20–40 times higher for resistant parasites than for sensitive parasites. It was concluded that differences in ionophore accumulation may reflect the degree of resistance. It has also been suggested, albeit with no evidence, that resistance to ionophores may also involve changes in membrane permeability of *Eimeria* [12]. Recent evidence to support this was obtained by Wang et al. [18], who found that membrane fluidity of monensin-resistant

lines of *E. tenella* was lower than that of a sensitive line. Analysis of differentially expressed genes by a monensin-resistant line of *E. tenella*, using cDNA array, indicated a six-fold upregulation of genes mainly involved in cytoskeletal rearrangement and energy metabolism compared with a sensitive parental line [19]. Like *Eimeria spp*., *Toxoplasma gondii* is highly susceptible to monensin and monensin has been used to treat toxoplasmosis in several animal species [20]. Lavine and Arrizabalaga [21] have used *T. gondii* as a model to study monensin's mode of action and possible mechanisms of resistance in apicomplexa. They suggest a novel mechanism for the mode of action of monensin (and salinomycin) on coccidial parasites, in which the drug activates an MSH-1-dependent cell cycle checkpoint by an unknown mechanism, ultimately leading to the death of the parasite.

5 Management of Resistance

To minimise the occurrence of resistance, rotation (a given anticoccidial product is used during a maximum of 2 months or two fattening periods) of various anticoccidial drugs or shuttle programmes (two or more anticoccidial drugs are used within a fattening period) is used. Due to the occurrence of cross-resistance between anticoccidial drugs, anticoccidial drugs with distinct mode of action should be used within rotation and shuttle programmes.

5.1 Cryptosporidium

The World Health Organization has established that diarrhoea accounts for 10.5 % of the nearly eight million yearly deaths of children under 5 years of age in the world, and a large clinical and epidemiological study involving 22,500 children from Africa and Asia has recently identified cryptosporidiosis as the second major cause of diarrhoeal disease and death in infants [22]. In spite of the epidemiological importance of this intestinal infection, there is no fully effective drug treatment or vaccine, and the basic research tools and infrastructure needed to discover, evaluate and develop such interventions are mostly lacking. In spite of the many drug assays aimed at the treatment of cryptosporidiosis, few consistently effective agents are presently available. Current investigations are limited by the absence of reliable long-term (more than a few days) *Cryptosporidium spp.* culture procedures, and the inadequacy of standard rodent (mouse and rat) models to support infection by *C. hominis*, the major species which infects humans and develops only in Mongolian gerbils [23]. Factors contributing to low anticryptosporidial drug efficacy may include improper targeting of parasitic molecular structures and/or biochemical pathway. Drugs may also have difficulties in reaching appropriate targets due to significant barriers established by the parasite's unique location in the host cell (intracellular but extracytoplasmic) as well described in the review by Striepen [24]. Since 2009, only few significant results have been obtained, mainly in the field of transport proteins or efflux pumps that transport drugs out of the parasite and into the host cell and intestinal lumen.

In several protozoan parasites, gene sequences which may be responsible for the expression of membrane pumps that produce multiple drug resistance (MDR) by lowering intracellular drug concentration have been detected [25]. Such members of the ATP binding cassette (ABC) transporter protein superfamily have been shown to translocate a wide range of substrates across a variety of cellular membranes [26, 27]. Recent reports suggest that *C. parvum* may possess upwards of 33 ABC transporter genes. Widmer et al. [28] found that three share a high level of homology with the NBDs of CpABC1, CpABC3, *Leishmania tropica* MDR1, and human MDR. It is therefore likely that *C. parvum* ABC transporters play a significant role in transport pathways of the parasite and may be involved in intrinsic resistance to drugs. By comparing mRNAs of three ABC transporters in *C. parvum* infected cell cultures, it was showed that the CpABC1 transporter was transcribed at a considerably higher level than other CpABC transporters [29]. Benitez et al. [30] partially characterised and analysed the expression of three *C. parvum* ABC transporters: cgd1_1350, cgd7_4510, and cgd7_4520. Li and Mun [31] described two ABC transporter proteins in *C. parvum*, one of which Cpnbd2 could be a novel member of the ABC protein superfamily.

At least one of the four ABC transporter proteins which have been detected in *C. parvum* was found at the host-parasite boundary of mature meronts, suggesting a role in crucial host-parasite transports [32]. These results were confirmed by immunofluorescence studies of Zapata et al. [33] that showed that CpABC1 was broadly distributed throughout the sporozoite and at the boundary between host cell and extracytoplasmic meront. In addition, a cyclosporin analogue that modulates the efflux of the mammalian ABC transporter MDR1 has been shown to inhibit the growth of *C. parvum* in vitro [34]. Such inhibitors may act via different mechanisms, i.e. directly by binding to the transporter and presumably blocking cytotoxic drug binding and/or indirectly by altering membrane functions or modulating gene expression, the latter having been demonstrated for the MDR1 inhibitors verapamil, doxorubicin, and mitomycin C [35, 36].

The concentration of the fluorescent dye calcein found to be higher in parasites than in host cells may reflect the important physiological function of parasite efflux pumps in physiological host-cell interactions [29]. This was recently supported by Widmer et al. [28] who, by comparing the genomes of a zoonotic and an anthroponotic *C. parvum* isolate, showed that amongst the 22 high-SNP genes, three (14 %) were annotated as ABC transporters. This proportion is much higher than the ratio (0.4 %) of the 16 ABC transporter genes *C. parvum* genome-wide (p=0.0004). The significant overrepresentation of transporters

among highly diverged genes suggests that the ability to establish an infection in a particular host species may depend in part on transporters controlling the exchange of metabolites between the host cell and intracellular developmental stages of the parasite.

References

1. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev Vaccines. 2006;5:143–63.
2. Levine PP. The effect of sulfanilamide on the course of experimental avian coccidiosis. Cornell Vet. 1939;29:309–20.
3. Chapman HD. Anticoccidial drugs and their effects upon the development of immunity to *Eimeria* infections in poultry. Avian Pathol. 1999;28:521–35.
4. Allen PC, Fetterer RH. Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidian parasites in poultry. Clin Microbiol Rev. 2002;15:58–65.
5. 5. World Health Organization. Resistance of malaria parasites to drugs. WHO Technical Report. Geneva, Switzerland: World Health Organization; 1965. Vol. 296, p. 1–65.
6. Reid WM. Progress in the control of coccidiosis with anticoccidials and planned immunization. Am J Vet Res. 1975;36:593–6.
7. McDougald LR, Seibert BP. Residual activity of anticoccidial drugs in chickens after withdrawal of medicated feeds. Vet Parasitol. 1998;74:91–9.
8. Kawazoe U, Fabio JD. Resistance to diclazuril in field isolates of *Eimeria* species obtained from commercial broiler flocks in Brazil. Avian Pathol. 1994;23:305–11.
9. Peek HW, Landman WJ. Higher incidence of *Eimeria* spp. field isolates sensitive for diclazuril and monensin associated with the use of live coccidiosis vaccination with paracox-5 in broiler farms. Avian Dis. 2006;50:434–9.
10. Rathinam T, Chapman HD. Sensitivity of isolates of *Eimeria* from turkey flocks to the anticoccidial drugs amprolium, clopidol, diclazuril, and monensin. Avian Dis. 2009;53:405–8.
11. Williams RB. Tracing the emergence of drug-resistance in coccidia (*Eimeria spp.*) of commercial broiler flocks medicated with decoquinate for the first time in the United Kingdom. Vet Parasitol. 2006;135:1–14.
12. Chapman HD. Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. Avian Pathol. 1997;26:221–44.
13. James S. Thiamine uptake in isolated schizonts of *Eimeria tenella* and the inhibitory effects of amprolium. Parasitology. 1980;80:313–22.
14. Zou J, Liu X, Shi T, Huang X, Wang H, Hao L, Yin G, Suo X. Transfection of *Eimeria* and *Toxoplasma* using heterologous regulatory sequences. Int J Parasitol. 2009;39:1189–93.
15. Wang CC. Inhibition of the respiration of *Eimeria tenella* by quinolone coccidiostats. Biochem Pharmacol. 1976;25:343–9.
16. Fry M, Hudson AT, Randall AW, Williams RB. Potent and selective hydroxynaphthoquinone inhibitors of mitochondrial electron transport in *Eimeria tenella* (Apicomplexa: Coccidia). Biochem Pharmacol. 1984;33:2115–22.
17. Augustine PC, Smith 2nd CK, Danforth HD, Ruff MD. Effect of ionophorous anticoccidials on invasion and development of *Eimeria*: Comparison of sensitive and resistant isolates and correlation with drug uptake. Poult Sci. 1987;66:960–5.
18. Wang Z, Shen J, Suo X, Zhao S, Cao X. Experimentally induced monensin-resistant *Eimeria tenella* and membrane fluidity of sporozoites. Vet Parasitol. 2006;138:186–93.
19. Chen T, Zhang W, Wang J, Dong H, Wang M. *Eimeria tenella*: analysis of differentially expressed genes in the monensin- and maduramicin-resistant lines using cDNA array. Exp Parasitol. 2008;119:264–71.
20. Lawrence K. Use of monensin sodium against *Toxoplasma*. Vet Rec. 1988;123:323.
21. Lavine MD, Arrizabalaga G. Analysis of monensin sensitivity in *Toxoplasma gondii* reveals autophagy as a mechanism for drug induced death. PLoS One. 2012;7:107.
22. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acácio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet. 2013;382:209–22.
23. Baishanbo A, Gargala G, Delaunay A, François A, Ballet JJ, Favennec L. Infectivity of *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates in immunosuppressed mongolian gerbils. Infect Immun. 2005;73:5252–5.
24. Striepen B. Drug resistance and emerging targets in the opportunistic pathogens *Toxoplasma gondii* and *Cryptosporidium parvum*. In: Mayers DL, editor. Antimicrobial drug resistance. New York: Humana Press; 2009.
25. Upcroft P. Multiple drug resistance in pathogenic protozoa. Acta Trop. 1994;56:195–212.
26. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol. 1999;39:361–98.
27. Bray PG, Ward SA. A comparison of the phenomenology and genetics of multidrug resistance in cancer cells and quinoline resistance in *Plasmodium falciparum*. Pharmacol Ther. 1998;77:1–28.
28. Widmer G, Lee Y, Hunt P, Martinelli A, Tolkoff M, Bodi K. Comparative genome analysis of two *Cryptosporidium parvum* isolates with different host range. Infect Genet Evol. 2012;12:1213–21.
29. Bonafonte MT, Romagnoli PA, McNair N, Shaw AP, Scanlon M, Leitch GJ, Mead JR. *Cryptosporidium parvum:* effect of multi-drug reversing agents on the expression and function of ATP-binding cassette transporters. Exp Parasitol. 2004;106:126–34.
30. Benitez AJ, Arrowood MJ, Mead JR. Functional characterization of the nucleotide binding domain of the *Cryptosporidium parvum* CpABC4 transporter: an iron-sulfur cluster transporter homolog. Mol Biochem Parasitol. 2009;165:103–10.
31. Li LC, Mun YF. Partial characterization of genes encoding the ATP-binding cassette proteins of *Cryptosporidium parvum*. Trop Biomed. 2005;22:115–22.
32. Perkins ME, Riojas YA, Wu TW, Le Blancq SM. CpABC, a *Cryptosporidium parvum* ATP-binding cassette protein at the host-parasite boundary in intracellular stages. Proc Natl Acad Sci U S A. 1999;96:5734–9.
33. Zapata F, Perkins ME, Riojas YA, Wu TW, Le Blancq SM. The *Cryptosporidium parvum* ABC protein family. Mol Biochem Parasitol. 2002;120:157–61.
34. Perkins ME, Wu TW, Le Blancq SM. Cyclosporin analogs inhibit in vitro growth of *Cryptosporidium parvum*. Antimicrob Agents Chemother. 1998;42:843–8.
35. Kaur J, Dey CS. Putative P-glycoprotein expression in arsenite-resistant *Leishmania donovani* down-regulated by verapamil. Biochem Biophys Res Commun. 2000;271:615–9.
36. Maitra R, Halpin PA, Karlson KH, Page RL, Paik DY, Leavitt MO, Moyer BD, Stanton BA, Hamilton JW. Differential effects of mitomycin C and doxorubicin on P-glycoprotein expression. Biochem J. 2001;355:617–24.

Drug Resistance in Nematodes

46

Roger K. Prichard

1 Introduction

Resistance to drugs used against nematode infections has become a serious problem in livestock as well as horses and is now present in species such as *Dirofilaria immitis*, which causes heartworm disease in dogs. Furthermore, there is evidence of resistance to ivermectin in *Onchocerca volvulus* in humans, and the results of efficacy evaluations coupled with detection of resistance mutations in soil transmitted helminths raise the possibility that it may be developing in gastrointestinal nematodes of humans. The main anthelmintic classes, based on drug chemistry and drug receptors/modes of action, are the benzimidazoles (BZs), the macrocyclic lactones (MLs), and a variety of drugs that act on acetylcholine receptors (AChRs), such as levamisole and monepantel. Resistance has arisen in several nematode species to all of these drug classes. Our understanding of the mechanism(s) and genetics of resistance is most advanced with BZ resistance and involves single nucleotide variants (SNVs) in *β-tubulin* genes. These changes can be detected to monitor for BZ resistance. Resistance to MLs appears more complicated. However, recently SNVs in *dyf-7* genes associated with resistance were found and a mechanism of resistance involving these genes and ABC transporters is hypothesized below. These advances may allow molecular monitoring for ML resistances in the near future. While genetic and protein changes that cause levamisole or monepantel resistance have been found, the situation appears quite complex with anthelmintics which act on AChR in nematodes and a simple panel of molecular markers for AChR anthelmintic resistances is still not available.

R.K. Prichard, Ph.D. (✉)
Institute of Parasitology, McGill University,
St. Anne-de-Bellevue, Quebec, Canada
e-mail: roger.prichord@mcgill.ca

2 Parasitic Nematodes and Resistance

Parasitic nematodes cause significant disease in animals and humans. In humans, the most important nematode-induced morbidities are due to soil-transmitted helminths, which establish, as adults, in the gastrointestinal tract, and tissue dwelling filarial nematodes which cause diseases such as onchocerciasis (River Blindness) and lymphatic filariasis (Elephantiasis). The soil transmitted helminths (STH) include the Hookworms, such as *Necator americanus* and *Ancylostoma duodenale*, the Whipworm (*Trichuris trichiura*), and the large roundworm *(Ascaris lumbricoides*). Collectively these STHs are estimated to infect over one quarter of all humans and to cause up to 39 million DALYs/year (disability adjusted life years lost each year). The major filaria parasites of humans are *Wuchereria bancrofti* and *Brugia malayi*/*B. timori* which cause lymphatic filariasis, and *O. volvulus* responsible for blindness and severe skin disease. Together these filarial infections have been estimated to cause 7.3 million DALYs/year. Other important human nematode infections include *Strongyloides stercoralis* which causes Strongyloidiasis, including the condition of disseminated Strongyloidiasis which is often fatal, and *Enterobius vermicularis* (pinworm).

In recent years, major control and elimination programs have been launched against many of these human nematode parasites worldwide, fueled by massive donations of anthelmintics, such as ivermectin (Mectizan™) by Merck Inc., albendazole by GlaxoSmithKline, and mebendazole by Johnson & Johnson. These anthelmintics are employed in mass drug administration programs in endemic areas. Over 1 billion doses of Mectizan have been administered and over 400 million doses of albendazole are being administered each year, and the deployment of anthelmintics against nematode infections form the largest chemotherapy programs for humans in the world. Currently used anthelmintics, dose rates, formulations, and treatment regimens very often do not achieve the high level of efficacy against nematodes in

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_46

humans [1–3], expected against veterinary parasites, and as a result it is much more difficult to notice an anthelmintic failure for which resistance could be suspected. In most cases, the chemotherapy has to be repeated over many years, if not for decades, and the risk for anthelmintic resistance developing in these human parasites is high. Should resistance become widespread, the control and elimination programs would be severely compromised as few alternative approaches for control are available and any such alternatives would be far less cost effective compared with anthelmintic treatment. It is important to recognize that most of the anthelmintics that are used to control nematode infections in people were originally discovered and developed as veterinary pharmaceutics [4, 5]. This is important in considering the concept of "One Health."

Nematodes, if unchecked, are a major cause of morbidity, death, and economic loss in both livestock and companion animals. In veterinary medicine, the standards of anthelmintic efficiency usually demand that ≥95 % of the parasitic nematodes be removed with a single drug treatment and efficacy below this, and certainly below 90 %, is accepted as evidence of anthelmintic resistance provided the anthelmintic has been administered at the appropriate dose rate and other conditions, such as formulation conditions, are satisfied [6]. Serious and often dramatic levels of anthelmintic resistance are being recorded, mainly in ruminant and horse gastrointestinal nematodes [7–9] and more recently in *D. immitis*, the filarial heartworm of dogs and cats [10–13]. It is therefore one of the key parasitological research issues to understand the mechanisms and genetics of anthelmintic resistance. This understanding should prove helpful in developing new anthelmintics, reducing the selection for resistance and maintaining the efficacy of anthelmintics, for monitoring the extent and spread of anthelmintic resistance using sensitive and specific markers, and in some cases for overcoming the resistance or exploiting the genetic changes involved in resistance to specifically target drug-resistant parasites.

As anthelmintic resistance has a genetic basis, it can be detected as changes in DNA sequences that affect the functioning or expression of gene products. Resistance is commonly due to either mechanisms (1) which change the effective concentration of the drug that reaches the effector site on a receptor, such as (1a) increased efflux of the drug from cells containing the receptors, (1b) reduced uptake, (1c) increased drug metabolism and inactivation, or (1d) reduced activation in the case of pro-drugs; non-receptor mechanisms of resistance, or (2) receptor-based mechanisms of resistance. The latter can be due to (2a) single nucleotide polymorphisms (SNPs) or other genetic changes (e.g., indels, deletions) which alter the amino acid sequence of the receptor (from single amino acid changes to premature stop codons or intron deletions/insertions which effectively produce a null receptor) and the affinity of the receptor for the drug, (2b) changes in ancillary proteins or other substances which affect the functioning of the receptor, (2c) alteration in regulatory components which affect the level of expression of a receptor or receptor ancillary protein, (2d) changes in the importance of the receptor function to the viability of the organism, or in (2e) remediation or compensatory mechanisms. Resistance can be due to a single gene change, or be multigenic in which more than one of these mechanisms and/or more than one gene for a given type of mechanisms can be involved. Commonly drug resistance involves more than a single genetic change and very often non-receptor-based mechanisms contribute to the resistance. To some extent, a too simplistic view of anthelmintic resistance has been assumed, based solely on the mode of action receptor.

3 Mechanisms of Action of Major Anthelmintic Classes

3.1 Benzimidazoles

Benzimidazoles (BZs) bind to nematode tubulin with high affinity and inhibit the formation and stability of microtubules [14]. Microtubules play essential roles in eukaryotic cells such as intracellular trafficking, cellular absorption and secretion, mitosis and meiosis, cellular architecture, e.g., the elongation of axons, and the migration of cells via cilia and cell pseudopods, and anchoring of membrane receptors at specific locations, such as at synapses in nerve cells [15]. β-tubulin and microtubules, which are formed by polymerization of α-β-tubulin dimers, are the targets of several pharmaceuticals, including colchicine, vinca alkaloids, BZs and others that cause microtubule instability, and other drugs such as taxol [16] and ivermectin [17, 18] which cause excessive stability of microtubules. Microtubules are dynamic polymers with a growing end where additional α- β-tubulin dimers can be added and a loss end where α- β-tubulin dimers disassociate from the polymer. It is believed that BZ anthelmintics (Fig. 46.1), which include thiabendazole, mebendazole, albendazole, oxibendazole, fenbendazole, oxfendazole, and flubendazole, bind to either the α- β-tubulin dimer or to the growing end of microtubules, "capping" the microtubule so that additional dimers cannot be added at the growing end of the polymer (Fig. 46.2). At the same time, α- β-tubulin dimers continue to be lost at the loss end of the microtubule, resulting in the disappearance of microtubules. This may disrupt the many functions performed by microtubules and lead to cell death. The exact dimensions of the BZ binding site have not been unequivocally determined [19–21]. However, allelic changes in nematode β-tubulin at either codon 200 (phenylalanine to tyrosine), codon 167 (also phenylalanine to tyrosine), or codon 198 (glutamate to alanine) result in BZ resistance [20, 22–27] and a loss of high affinity BZ binding [20, 28].

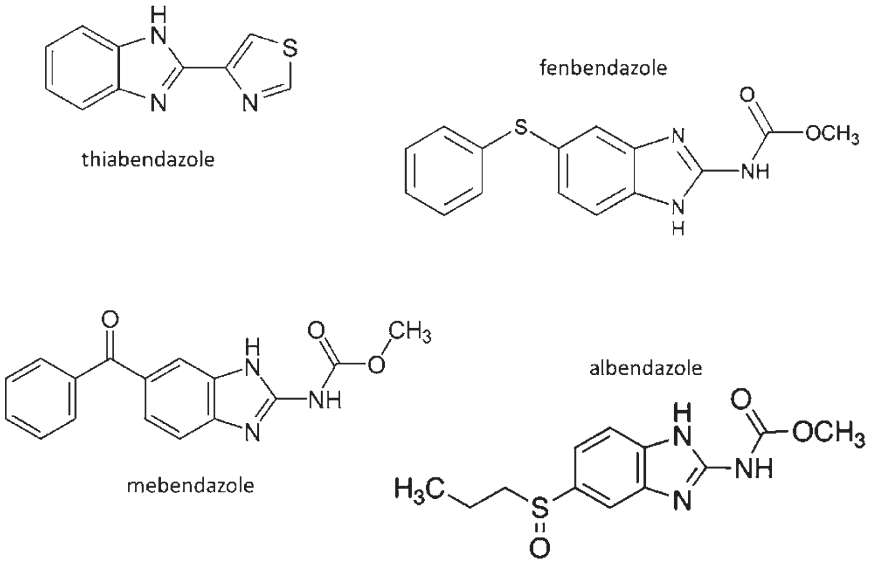


Fig. 46.1 Structures of some benzimidazole (BZ) anthelmintics. The first BZ discovered was thiabendazole which is a benzodiazol thiazole. Other BZs are benzoimidazol methylcarbamates or are pro-drugs for methylcarbamates. The methylcarbamate BZs tend to be more potent than thiabendazole, but all bind selectively to nematode tubulin

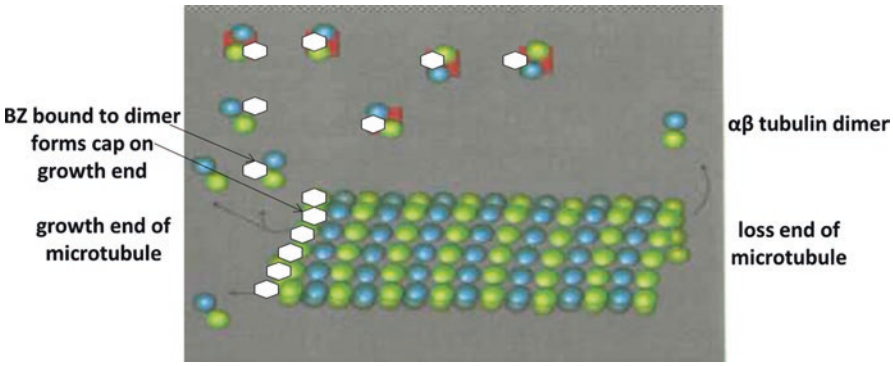


Fig. 46.2 Benzimidazole (BZ) anthelmintics bind to tubulin dimers and are added to the growth end of microtubules. This causes the growth end to become capped so that additional dimers cannot be added. The loss end of the microtubule can still depolymerize, so that the net effect of BZs binding selectively to nematode tubulin is to cause the depolymerization of microtubules and the loss or impairment of many cellular functions

3.2 Anthelmintics Acting on Ligand-Gated Cation Channels

Ligand-gated cation channels that mediate fast neurotransmission have been successfully exploited by the animal health industry in the development of a number of paralyzing anthelmintics directed at nematodes. Nicotinic acetylcholine receptors (nAChRs) are widely expressed in the nematode nervous system, both at the neuromuscular junction (on the muscle cells) and on the neurons themselves [29, 30]. The pharmacology of the nAChRs is very varied in animals because the receptors are frequently heteropentamers with different combinations of subunits markedly modulating receptor properties [31] and because they possess a plethora of modulating binding sites. They are targeted by several anthelmintics, including levamisole [32], pyrantel [33], oxantel [34], morantel, paraherquamide and derquantel [35], monepantel [36], and tribendimidine [37] (Fig. 46.3). Most of these compounds are agonists at the neuromuscular receptor, causing a spastic paralysis of the worm, though paraherquamide and its analog derquantel are antagonists [38]. It is clear that not all of these compounds act on the same receptor and it is also clear that nematodes possess multiple forms of nAChR. Martin and colleagues have identified at least three pharmacological and physiological subtypes at the body-wall neuromuscular junction and several others are likely to exist in the pharynx, head muscle, and central ganglia [38, 39]. The amino-acetonitrile derivatives (AADs), such as monepantel, agonize at a distinct group of nicotinic receptors in *C. elegans* and the sheep gastrointestinal nematode *H. contortus* [36]. These receptors contain subunits such as DES-2 and ACR-23 that are expressed on pharyngeal muscle, ventral cord interneurons, and sensory neurons [40]. These AAD sensitive nAChR subunits seem to be unique to nematodes, and this may explain the lack of AAD toxicity to mammals, insects, and other invertebrates. Resistance to monepantel was selected under laboratory conditions [41] and recently there have

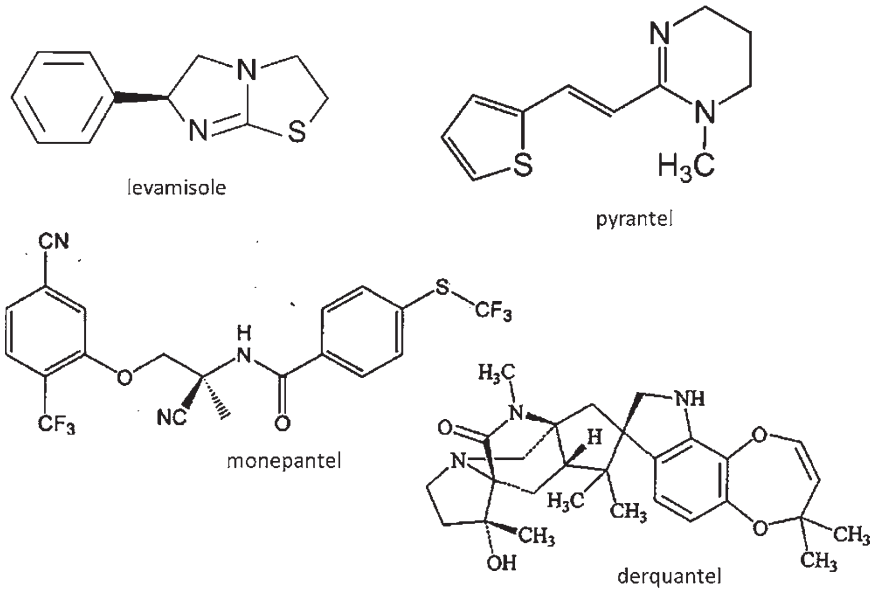


Fig. 46.3 Anthelmintic drugs which act on n-acetylcholine receptors (nAChRs) in nematodes, resulting in opening of cationic pores and leading to muscle paralysis. Most anthelmintics which act on nematode AChRs are agonists. However, derquantel is believed to be an antagonist. Nematodes have a variety of nAChRs, so that different nAChR anthelmintics may affect different NAChRs, composed of different subunits

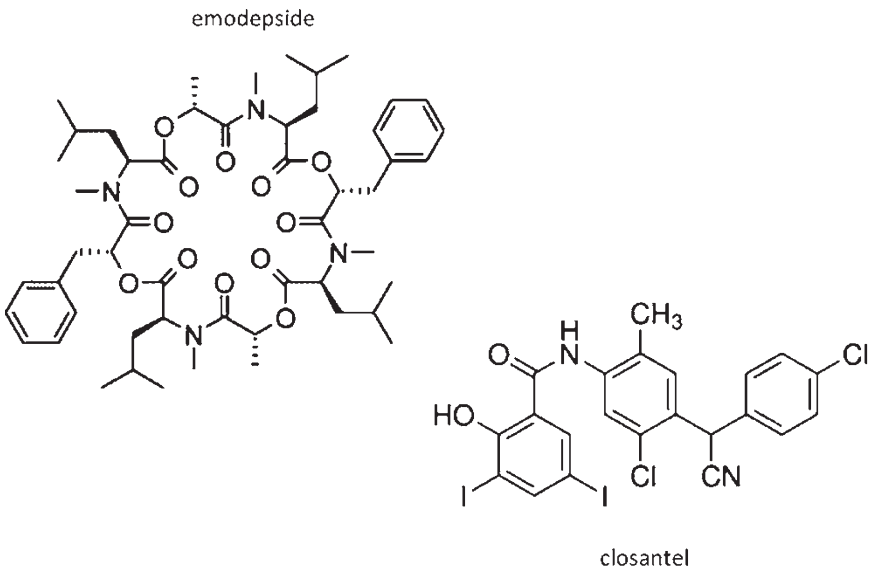


Fig. 46.4 Emodepside is a cyclooctadepsipeptide and acts on a Ca^{2+}-activated voltage-gated K^+ channel, named SLO-1. Closantel is a chlorinated salicylanilide which along with other salicylanilides and nitrophenols acts as a hydrogen ionophore

been reports of monepantel resistance arising in the field [42–45]. The current understanding about possible mechanisms of monepantel resistance will be discussed below.

3.3 Anthelmintics Acting on Ca^{2+}-Activated K^+ Channels

Full efficacy of emodepside (Fig. 46.4), a cyclooctadepsipeptide, observed against BZ, imidazothiazole, and ivermectin-resistant populations of nematodes from sheep and cattle suggested that this drug class acts by a new mode of action [46]. In vitro mutagenesis in *C. elegans*, leading to complete emodepside resistance, revealed that a large-conductance Ca^{2+}-activated voltage-gated K^+ channel, named SLO-1, is involved in the mode of action [47]. SLO-1 mutants showed resistance in the pharyngeal pumping as well as in the locomotion assays applied to investigate the emodepside effects. In contrast, only the pharyngeal pumping activity was observed to be resistant to emodepside in LAT-1 mutants, while body movement was still normally inhibited. This indicates that more than one endogenous cyclooctadepsipeptide receptor exists. The fact that, by the forward genetics approach performed by Guest et al. [47], only SLO-1 mutants were generated argues for a signaling of emodepside directly through a SLO-1 pathway, presumably

at the postsynaptic membranes at neuromuscular junctions in the body-wall musculature and at presynaptic sites in the pharynx [48]. Recent studies show that emodepside selectively activates the nematode isoform of the evolutionary conserved SLO-1 [49]. The nematode SLO-1 receptor was significantly more sensitive to emodepside than the human SLO-1 receptor, providing for the selective anthelmintic action of this drug. For commercial reasons, emodepside has been developed for use in cats and dogs only, and to date, no reports of resistance have been made.

3.4 Anthelmintics Acting on Ligand-Gated Anion Channels

Nematodes possess a greater abundance and variety of ligand-gated anion channels than are found in vertebrates. GABA (γ-amino butyric acid) is common to both phyla, but fulfills quite different roles: in nematodes it acts as an inhibitory transmitter at the neuromuscular junction and is also present in some other neurons [50, 51]. Its receptors are found on muscle cells and the anthelmintic piperazine is a GABA receptor agonist that causes a flaccid paralysis [52]. By far the most important group of compounds that act at ligand-gated chloride channels are the macrocyclic lactones (Fig. 46.5), which include the avermectin subclass (ivermectin, abamectin, eprinomectin, doramectin, and selamectin), and the milbemycin subclass (moxidectin and milbemycin oxime). All of the macrocyclic lactones are very hydrophobic and have a unique mode of action. They do not bind to the normal agonist site, but bind allosterically to irreversibly open the channel, leading to a permanent hyperpolarization of the cells and flaccid paralysis [53]. They bind to, and activate, a wide range of ligand-gated anion channels (glutamate-, GABA- and perhaps dopamine-, serotonin-, and tyramine-gated chloride channels), but in nematodes their most important targets are believed to be the glutamate-gated chloride channels (GluCls), which are expressed widely in the nervous system and on pharyngeal muscle [54–58]. There is some evidence that the avermectins, such as ivermectin, and the milbemycin, moxidectin, may not target the same ligand-gated anion channels in nematodes with similar affinities and effects. For example, ivermectin is approximately 64-fold more potent than moxidectin at inhibiting pharyngeal pumping in *C. elegans* and at nM concentrations ivermectin stimulates nematode mobility, whereas moxidectin inhibits mobility at similar concentrations [59]. In gene knock-out studies these same authors found that deletion of the glutamate channel gene *glc-3* resulted in *C. elegans* larval development becoming insensitive to moxidectin even at μM concentrations whereas nematodes with the same gene deletion were still sensitive to ivermectin at less than 20 nM. These and other studies (see below) indicate that while avermectins and moxidectin may target GluCls, diversity in these channels and in transport proteins that modulate the concentrations of macrocyclic lactones may produce marked differences between avermectins and moxidectin in effects on nematodes and on the selection and expression of resistance to the avermectins and moxidectin. Resistance has become a serious problem within the macrocyclic lactone class of anthelmintics and knowledge about the mechanisms of resistance to these anthelmintics will be discussed below.

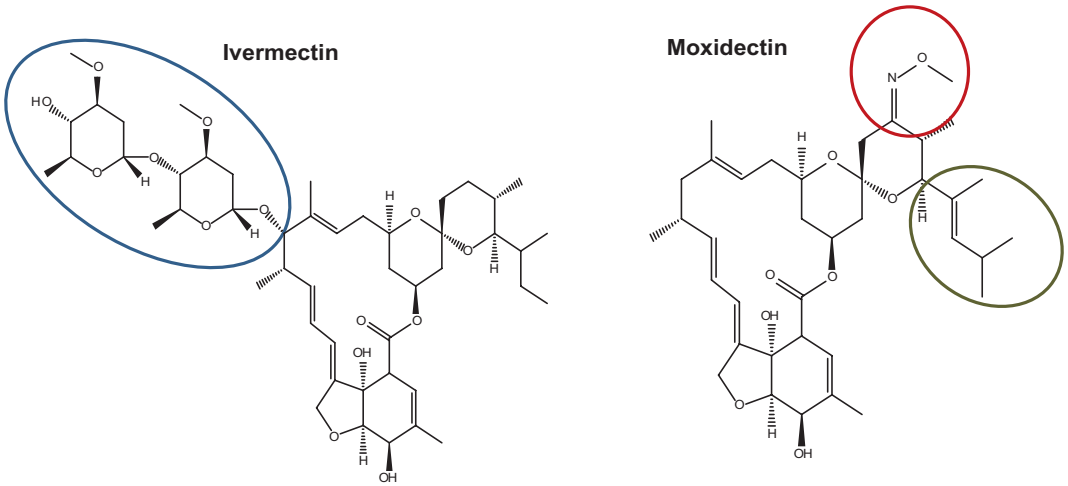


Fig. 46.5 The macrocyclic lactone anthelmintics are composed of two subclasses, the avermectins, such as ivermectin, and milbemycins, such as moxidectin. They share a common macrocycle. But have different substituents. The avermectins typically have sugar groups at C13 of the macrocyclic lactone ring, whereas the milbemycins are protonated at C13 (difference is highlighted). Moxidectin also has some unique substituents, notably a methoxime group at C23 and a substituted olefinic side chain at C25. The chemical variation between moxidectin and the avermectins lead to marked differences in lipophilicity, and some differences in interactions with glutamate-gated chloride channel receptors and with ABC transporters, which may affect potency and resistance development. Moxidectin has a longer half-life compared with ivermectin

3.5 Anthelmintics Acting as Hydrogen Ionophores

Salicylanilide and substituted nitrophenol anthelmintics, such as closantel (Fig. 46.4), rafoxanide, and disophenol, are believed to act as hydrogen ionophores, uncoupling oxidative phosphorylation [60, 61] in blood ingesting nematodes and trematodes, and have been used in some countries for the control of *H. contortus*. Specificity for blood ingesting helminths is believed to be due to their high affinity for plasma albumin. Resistance to closantel has arisen in *H. contortus* [62, 63]. However, the mechanisms involved in this resistance are not well understood. Rothwell and Sangster [64] examined the uptake and metabolism of closantel in a closantel-resistant and a -susceptible isolate of *H. contortus*. They found that neither isolate showed an ability to metabolize the drug in vitro or in vivo. They did, however, report that radiolabeled closantel accumulated at lower levels in resistant compared to susceptible *H. contortus* following administration to sheep. The mechanism responsible remained unknown; however, the authors speculated that it may be due to reduced feeding by resistant worms, a reduced level of dissociation of the drug-albumin complex in the worm gut, or increased efflux of the drug from resistant worms. Several later studies failed to detect polymorphisms in *H. contortus* P-gp genes that may have explained the reduced accumulation of drug in resistant isolates [65, 66]. However, these studies were very limited in only examining a small subset of the P-gp genes in this species, and hence the role of P-gps in closantel resistance remains unknown.

4 Mechanism and Genetics of Benzimidazole Resistance

As noted above, BZ anthelmintics work by binding to nematode tubulin and this results in depolymerization of microtubules. The evidence is overwhelming that BZ resistance is caused by the selection of single nucleotide polymorphisms at either codon 200 (TTC to TAC; phenylalanine to tyrosine), codon 167 (TTC to TAC; phenylalanine to tyrosine), or codon 198 (GAA to GCA; glutamate to alanine) of β-tubulin [22, 24–26, 67, 68]. There is evidence that these changes in the β-tubulin protein result in loss of the high affinity binding site for benzimidazole anthelmintics [14]. It is interesting that the resistance-associated changes at codons 200, 167, or 198 do not seem to occur together in the same allele [22, 26, 69], presumably because such a combination is lethal. The codon 200 mutation seems to be the most common cause of benzimidazole resistance, although the relative importance of the difference resistance causing SNPs may vary between different nematode species and isolates [9, 26, 27, 68, 70]. In trichostrongylid nematodes, selection for one of these mutations seems to occur primarily on isotype 1 β-tubulin, which corresponds to the *ben1* gene in *C. elegans*, although isotype 2 β-tubulin may also be under selection by BZ anthelmintics, resulting in certain alleles being selected [71] or in selection for a null isotype 2 β-tubulin [72]. In *H. contortus*, BZ resistance appears to be inherited as an incompletely recessive phenotype [73], so the frequency of resistance alleles may be quite high before phenotypic evidence of resistance to BZs, based on fecal egg count reduction or worm counts, becomes apparent. BZ resistance-associated mutations in β-tubulin have been found in a number of veterinary nematode parasites, including *H. contortus*, *T. colubriformis*, *T. circumcincta* in sheep, *Cooperia oncophora* in cattle, and Cyathostomes in horses [9, 27] and recently in *T. trichiura* and *N. americanus* from humans [74, 75]. There is evidence that macrocyclic lactones, such as ivermectin and moxidectin, may select on β-tubulin in nematodes such as *H. contortus* and *O. volvulus* and in the case of *H. contortus* the BZ resistance-associated SNPs at codons 200 or 167 (TAC) may be selected [26, 76, 77], so that repeated use of ML anthelmintics may predispose some nematodes to BZ resistance. Recently, it was shown that ivermectin and moxidectin bind to the taxol pocket of tubulin [17, 18]. Besides the resistance causing SNPs in β-tubulin, other mechanisms such as overexpression of some ABC transporters, as discussed below, may modulate BZ resistance. However, this resistance appears to be primarily due to selection for resistance causing mutations in β-tubulin.

Knowledge of the principal mechanisms and underlying genetics of BZ resistance has led to the development of molecular assays to detect BZ resistance. These developments have been most advanced for detection of BZ resistance in *H. contortus*, the parasite in which BZ resistance has been most problematic. Coles et al. [6] described an allele specific PCR for detection of the BZ resistance-associated F200Y SNP in *H. contortus* using DNA extracted from L3 stage larvae. Other molecular methods have also been described for detection and/or quantification of this SNP, as well as the other two associated with BZ resistance (F167Y, E198A), including restriction fragment length polymorphism-PCR (RFLP-PCR) [24, 78], real time PCR [79–81], and pyrosequencing [80]. This latter study described pyrosequencing assays for *H. contortus* codons 167, 198, and 200 of beta-tubulin isotypes 1 and 2. The method proved able to assess the BZ resistance status of a number of *H. contortus* isolates, indicating that it may be suitable for routine diagnosis of resistance in this species. A comparison of molecular data with egg hatch data showed that the molecular test was not always correlated with the degree of resistance. However, it was able to discriminate well between resistant and susceptible isolates. The inability to directly correlate with resistance levels suggests that other resistance mechanisms beside beta-tubulin mutations may also be contributing to

resistance in some isolates. The lack of a strong correlation between the molecular tests and tests such as the fecal egg count reduction test (FECRT) may also reflect on the insensitivity of the biological test used in the comparison. The ability to detect the presence of resistance in a population by a molecular test means that the test could be utilized as a diagnostic tool for detection of resistance. Two recent studies have evaluated the use of the pyrosequencing technique for diagnosis of BZ resistance in *H. contortus* under field conditions in Canada [22, 69]. Both studies showed that the molecular tests were in agreement with more laborious and expensive FECRTs in diagnosing resistance, but as the level of BZ resistance was high, it was not possible to calculate a correlation with the FECRT results.

Pyrosequencing methods have also been developed for detecting resistance-associated SNPs in β-tubulin in the human soil transmitted helminths, *T. trichiura*, *A. lumbricoides*, and *N. americanus* [74, 75, 82], and the F200Y SNP was detected in field samples of the whipworm *T. trichiura* and the hookworm *N. americanus*. In the case of the whipworm the presence of the resistance-associated SNP seemed to be in agreement with low BZ efficacies.

5 Mechanisms of Resistance to Anthelmintics Acting on Ligand-Gated Cation Channels

5.1 Mechanisms of Levamisole Resistance

An early study on levamisole resistance found that resistant *H. contortus* were considerably less sensitive to the effects of added acetylcholine [83], thereby highlighting changes to the nature of the cholinergic receptor(s) as a possible mechanism of resistance. Work by the same group demonstrated that the binding of radiolabeled levamisole to its nicotinic acetylcholine receptor (nAChR) target within the worm involved two sites, and that levamisole-resistant worms bound the drug less tightly at the low affinity site than susceptible worms [84, 85]. This indicated that the basis for the resistance was alterations at the drug target site. A study on the mode of inheritance of levamisole resistance indicated that the resistance was polygenic [86].

More recent molecular studies with *H. contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis* have provided insights into mechanisms of this altered drug/receptor affinity [31]. A number of molecular changes are seen in resistant worms:

1. *Truncated nAChR subunit genes*: truncated forms of two nAChR subunit genes, *Hco-acr-8* and *Hco-unc-63* (truncated forms denoted as *Hco-acr8b* and *Hco-unc63b*, respectively), have been shown to be present in resistant isolates of *H. contortus*, and absent in susceptible isolates in a number of studies [31, 87–89]. Boulin et al. [90] subsequently used a *Xenopus* oocyte heterologous expression system to examine the impact of the truncated *H. contortus* UNC-63b protein on the functioning of levamisole sensitive nAChRs. It was found that the truncated version hampered the normal function of the receptors when both forms of the protein (full length and truncated) were co-expressed, thereby mimicking a levamisole resistance phenotype that may occur when both forms are expressed together in nAChRs in resistant worms. The same group [87] found a truncated form of ACR8 (known as ACR8b or HAX) in some isolates of levamisole-resistant *H. contortus*. Barrère et al. [91] further examined the occurrence of the truncated *Hco-acr-8b* in resistant isolates from different geographical locations and identified an indel region of genomic DNA that was responsible for the generation of the truncated splice variant, with the presence of the 63 bp indel leading to transcription of the full-length ACR8 protein and the deletion of the 63 bp indel leading to the truncated ACR8b protein being formed and associated loss of levamisole sensitivity.
2. *Reduced transcription of nAChR subunit genes*: A significant decrease in transcription levels of *Hco-unc-29.3 and Hco-unc-63* in a resistant isolate of *H. contortus* was observed by Williamson et al. [89], and Sarai et al. [92] noted that Hco-UNC63 was downregulated in resistant isolates. In addition, they reported that all four paralogs of Hco-UNC29 were significantly downregulated in adults of one resistant isolate. Subsequently, Sarai et al. [93] subdivided this isolate into subpopulations showing different levels of levamisole resistance in vitro. A comparison of the nAChR gene expression patterns in the subpopulations showed significant downregulation of several receptor subunit genes in the most-resistant population (*Hco-unc-63a*, *-63b*, *-29.2, -29.4, -26*, and *-acr-8a*).
3. *Reduced transcription of ancillary protein genes*: Several proteins are involved as either receptor subunits or ancillary proteins in the construction of a functional nAChR that is susceptible to levamisole or other imidazothiazole anthelmintics [90]. Sarai et al. [93] found that a number of genes associated with the assembly of nAChRs were significantly downregulated in most levamisole-resistant populations of *H. contortus* (*Hco-unc-74*, *-unc-50*, *-ric-3.1*, and *-ric-3.2*), including in different life cycle stages of resistant isolates [92].

Each of these molecular changes (truncation and reduced transcription of nAChRs, reduced transcription of ancillary protein genes) could result in a reduction in the number of functional levamisole receptors in resistant worms, and hence lead to reduced drug binding in resistant worms as noted earlier [84, 85]. However, the specific changes involved

in levamisole, and other imidazothiazole anthelmintics which act on nAChRs, may vary from one nematode species and isolate to another. For example, in terms of the truncated subunit genes:

- Neveu et al. [31] detected the truncated Hco-unc-63 transcript in one resistant *H. contortus* isolate; however it was absent from a second resistant isolate.
- Williamson et al. [89] did not detect the truncated *Hco-unc-63* transcript in their resistant isolate.
- Sarai et al. [92] found that truncated *Hco-unc-63* was readily detectable in both a susceptible and three resistant isolates.
- Barrère et al. [91] and Fauvin et al. [87] found the truncated *Hco-acr-8b* was associated with levamisole resistance in a number of isolates of *H. contortus*, while Sarai et al. [92] found that truncated *Hco-acr-8b* could be detectable in larvae of both susceptible and resistant isolates, and was absent from adults of one resistant isolate.

Further inconsistencies in terms of gene expression patterns (not involving truncated forms) can be seen in the results of Sarai et al. [92] who found that the *Hco-unc-29* paralogs showed unchanged expression in levamisole-resistant isolates relative to a susceptible isolate, or significant downregulation in some levamisole-resistant isolates. Hence, it seems likely that parasitic nematodes may use a variety of variations in receptor/ancillary subunits to render nAChRs less sensitive to nicotinic agonist drugs. This diversity in the genetic changes that can lead to resistance makes a comprehensive system of molecular markers for levamisole resistance challenging.

5.2 Mechanisms of Monepantel Resistance

Prior to the commercial release of monepantel, Kaminsky et al. [36] and Rufener et al. [41] described experiments in which *H. contortus* larvae were selected with monepantel over several generations to produce mutant lines in which the adult stage showed resistance to a recommended dose of the drug administered to sheep. The resistant lines showed mutations in two nAChR subunit genes: *Hcdes-2H* and *Hcacr-23H* (subsequently renamed *Hco-mptl-1*). A panel of loss-of-function mutations were identified in the *Hco-mptl-1* gene in the mutant lines. These various mutations included deletions leading to mis-splicing, and insertions and point mutations leading to premature termination of translation of the protein. However, so far there are no publications as to whether the field isolates of *H. contortus*, *T. circumcincta*, or *T. colubriformis* that are mentioned above as having developed resistance in farmed sheep or goats have similar genetic changes as those reported after the experimental selection on the larval stages.

6 Mechanisms of Resistance to Macrocyclic Lactone Anthelmintics

Macrocyclic lactone (ML) resistance has arisen in a number of trichostrongylid parasitic nematodes of sheep, cattle, and horses, and in filarial nematode parasites of dogs and humans. It has also been experimentally selected, with ivermectin exposure in the model nematode *C. elegans*. However, the mechanisms and genetics have most often been studied in *H. contortus* and usually with the ML drug ivermectin. Rohrer et al. [94] found no differences in ivermectin binding to membrane preparations from single resistant and susceptible isolates of *H. contortus*, suggesting that the resistance shown by this single resistant isolate was not due to alterations in affinity for the target site. Nevertheless, a number of researchers have studied GluCl receptor subunits in order to determine whether there are any changes in GluCls that could be a mechanism of ML resistance. Blackhall et al. [95] reported an increased frequency for an allele of an α-subunit GluCl gene in *H. contortus* isolates laboratory-selected with ivermectin or moxidectin (in which both the selected strains became ivermectin resistant, but were still susceptible to the recommended dose of moxidectin), compared with the parental unselected strain, suggesting that a mutation in this gene could be associated with ivermectin resistance. Subsequently, Njue et al. [96] found a L256F mutation in GluClα3 (a homolog of Cel-AVR-14) in one ivermectin-resistant field isolate of *Cooperia oncophora*. When the 256 F allele was expressed in *Xenopus* oocytes as a homomeric channel, it showed a 2.5-fold reduction in sensitivity to both ivermectin and moxidectin, compared with the wild-type (L256) protein. Using experimental mutagenesis, McCavera et al. [97] showed that substitution of phenylalanine for the L256 residue in *H. contortus GluClα3B* transfected into COS-7 cells caused a reduction in the binding of ivermectin to membrane preparations of these transfected cells, thereby highlighting that this mutation produced a similar effect as Njue et al. [96] observed with the field isolate of *C. oncophora*. However, despite the fact that this change in a GluCl subunit moderately reduced sensitivity to MLs in one field resistant isolate, and another experimentally produced GluCl receptor, extensive searches have failed to find this receptor mutation as a cause of ML resistance in any other field isolates, and so it has been concluded that GluCl receptor modification is not a major mechanism of ML resistance in nematodes.

Recent studies have described the details of the interaction of ivermectin with GluCl receptors in nematodes [98–100]. *H. contortus* was found to be among a group of ivermectin-sensitive species (also including *C. elegans, C. oncophora*, and *D. immitis*) that showed the presence of a glycine residue in the third transmembrane domain of these receptors, in contrast to larger residues at the same position in the receptors from ivermectin-insensitive trematodes such

as *Schistosoma mansoni*, *S. japonica*, and *Clonorchis sinensis* [100]. In addition, mutation of this glycine residue in the *H. contortus* GluClα3B protein expressed on HEK293 cells resulted in the loss of ivermectin sensitivity [99]. However despite these insights into the important determinants of ML sensitivity, there is no evidence that changes in this amino acid residue are responsible for field resistance in *H. contortus* or other worm species. A recent study by Williamson et al. [89] failed to find a link between mutations in a number of ligand-gated chloride ion channels, including both GluCl and GABA channels (*avr-14B*, *glc-5*, *lgc-37*, and *glc-6*), and ML resistance in field-derived resistant nematodes. Hibbs and Gouaux [98] obtained a crystal structure of the *C. elegans* GluClα protein with bound ivermectin and were able to predict from that the residues responsible for ivermectin binding. When the structure of moxidectin, a milbemycin with a methoxime side chain, was superimposed over ivermectin, a number of the interaction sites relevant for ivermectin binding to the GluCl are no longer present and other possible interactions may occur [8]. These differences may contribute to the greater potency of moxidectin on most nematodes and to differences in the development of resistance between moxidectin and ivermectin. Experience has shown that when resistance to recommended dose rates of ivermectin arises in the field, moxidectin is still fully effective at its normal dose rate. Nevertheless, with a change from the use of an avermectin (e.g., ivermectin) to moxidectin, repeated use of moxidectin can lead to moxidectin resistance also being eventually selected.

Resistance to MLs in nematodes has also been examined with respect to the possible role of changes in transcription levels of genes coding for ivermectin receptors. Williamson et al. [89] found that transcription of two GluCls (*glc-3* and *glc-5*) was slightly reduced in a resistant *H. contortus* isolate. However, the changes were modest and were not considered to be significant enough to explain the observed levels of resistance.

A number of studies have indicated a role for ATP binding cassette (ABC) transporters, such as P-glycoproteins (P-gp) drug efflux pumps, in ML resistance in nematodes (reviewed by [101]). Two lines of evidence exist:

1. *Molecular*: A number of molecular analyses detected polymorphisms in nematode P-gp genes that may have been associated with resistance to MLs (e.g., [10, 11, 66, 102–104]). In addition, a number of studies have shown that expression of P-gps is increased in resistant isolates [101–107], while exposure of worm larvae and adults to ML drugs has resulted in the overexpression of a number of ABC genes in vitro [108, 109], and in vivo [109, 110].
2. *ABC transport inhibitors on ML sensitivity*: a number of multidrug resistance (mdr) inhibitors have been shown to reverse ML resistance in nematodes, both in vitro and in vivo (reviewed by [101]). In vitro studies include those by Bartley et al. [111], Heckler et al. [112], and Raza et al. [113]. In vivo, mdr inhibitors have been used to increase the efficacy of ivermectin and moxidectin against resistant *H. contortus* in jirds [104, 114] and ivermectin in sheep [101, 115]. Thus drug efflux pathways have been shown to play roles in resistance mechanisms in many nematode species and ML-resistant isolates. However, the data suggests that, on balance, efflux mechanisms can contribute part of the mechanism, but probably not all of the mechanism of ML resistance in nematodes.

A recent study by Urdaneta-Marquez et al. [116] has provided compelling evidence to indicate that another mechanism may be responsible for ML resistance in *H. contortus* and *C. elegans*, namely, changes to the anatomy and/or function of amphid sensory organs in resistant worms. This study examined the *H. contortus* homologue of a *C. elegans* gene involved in the amphid dye-filling defects observed in ML-resistant *C. elegans* worms (*Cel_dyf-7,* and its homologue *Hco_dyf-7*). Polymorphisms in the *H. contortus* gene allowed for the identification of a resistant haplotype, *Hco-dyf-7(r)*, which was consistently present in resistant *H. contortus* isolates from a range of geographical sources. This finding has significant implications for the development of resistance-monitoring diagnostics. If the changes to the amphidial neurons, caused by the *dyf-7* mutations, affect the access of MLs to GluCls expressed in the neurons innervating pharyngeal and body muscle (and perhaps other sites where GluCls can be expressed (e.g., the excretory cell, uterus, and male reproductive organs)), it can be envisioned that the DYF-7 changes may act in concert with changes in the activity of ABC efflux transporters to cause ML resistance by collectively restricting the concentration of ML which reaches the GluCl receptors (Fig. 46.6). Furthermore, the differences that have been noted in the interaction of moxidectin compared with the avermectins with ABC transporters [117, 118] may explain differences that are seen in the expression and development of resistance to moxidectin compared with the avermectins [8].

7 Is There a Fitness Costs to Drug Resistance in Nematodes?

Genetic changes that cause drug resistance in nematodes are probably present at low frequencies prior to anthelmintic selection [116], and populations of nematodes are initially susceptible, because the mutations which can cause resistance cause the nematode to lose some aspect of general fitness compared to the wild-type genomes. However, anthelmintic drugs are such powerful selective agents that the repeated use of anti-nematode drugs results in an increase in the frequency of the nematodes with resistant genomes and eventually we observe a population of worms

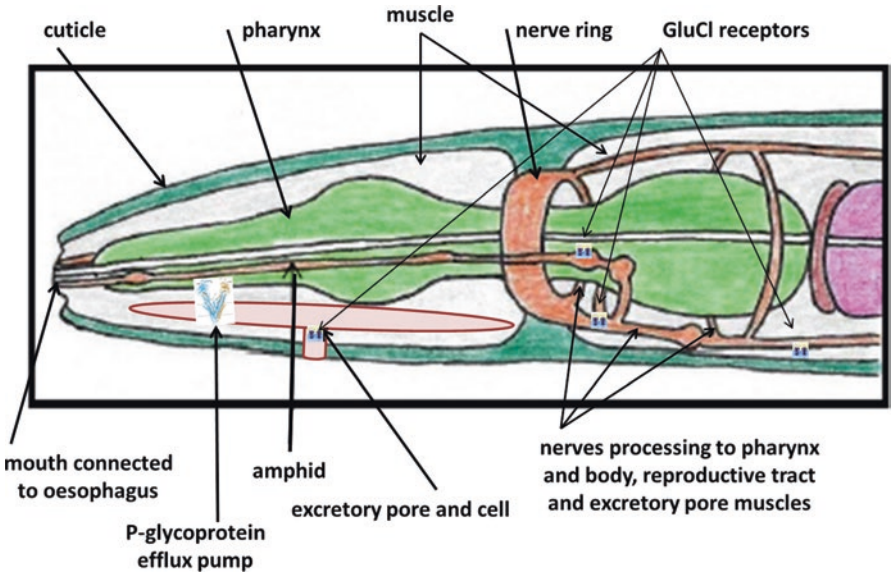


Fig. 46.6 Schematic of the anterior end of a nematode and possible factors which affect the activity of, and resistance to macrocyclic lactone (ML) anthelmintics. The amphids extend from the nerve junctions adjacent to the nerve ring to the amphidial pores each side of the mouth. The amphids are highly innervated and serve as the sensory organs of nematodes. It is hypothesized that MLs may enter the amphids via the amphidial pores and transit via the amphidial dendrites to reach the glutamate-gated chloride channels (GluCls) that are highly sensitive to MLs and are expressed in the neurons innervating the pharynx, the body muscle, reproductive tissue, and the excretory pore. MLs can cause paralysis of these muscles. In ML-resistant nematodes, changes in *dyf* genes which code for proteins that cause elongation of the dendrites in the amphids can result in shortening of these dendrites so that they no longer reach the amphidial pore. This morphological and functional change, coupled with overexpression of ABC transporters, may reduce the concentration of MLs reaching the GluCl receptors and reduce susceptibility to ML anthelmintics

that is phenotypically drug resistant. However, it is difficult to assess any fitness cost that may be associated with anthelmintic resistance and which could lead to some level of reversion to susceptibility, in the absence of further selection pressure from a specific class of anthelmintic. Quantifying any fitness cost of drug resistance is an important question for how we manage anthelmintic resistance in the long term. Because studies to attempt to assess any reversion to susceptibility of nematode populations containing a proportion of resistant worms might take several years under field conditions, data on this aspect are scarce. Nevertheless, there are some interesting findings. Because the mechanisms of resistance to different drug classes (and even to different anthelmintic molecules within a drug class) are likely to vary, the measurement of reversion, as a marker for the fitness cost of resistance, cannot be extrapolated from one drug class to another or necessarily from one nematode species to another. Given these limitations, what can we hypothesize about the possible fitness cost to different classes of anthelmintics?

Some work has been done on BZ resistance in nematode species. In a field study over a 5-year period, involving sheep with a BZ resistant strain of *H. contortus*, Waller et al. [119] found an increase in the efficacy of thiabendazole (44 mg/kg) from 72 to 91 % efficacy when the BZ resistant population was left untreated for 5 years.

In the same study, it was noted that leaving the animals untreated for 5 years resulted in the efficacy of levamisole (7.5 mg/kg) against levamisole-resistant *T. colubriformis* increasing from 14 to 60 %, suggesting a strong rate of reversion and a likely significant fitness cost for this type of resistance. Furthermore, the use of thiabendazole (44 mg/kg), eight times per year × 5 years, further increased levamisole efficacy to 84 % against this formerly very resistant strain of *T. colubriformis*. These limited data suggest that there may be some fitness cost to anthelmintic resistance in some cases. However, usually when resistance to an anthelmintic is detected, treatment is switched to another class of anthelmintic or switched to combination treatment often with the combination still containing the drug to which some of the worms have become resistant (this is common with parasite control because there are few distinct drug classes available). In this latter scenario, there would be little chance for possible reversion. In the case where a different class of anthelmintic is used once resistance is established to the first anthelmintic class, it may also be difficult to see reversion towards susceptibility because of interacting mechanisms. For example, it has been observed that MLs can select for some of the genetic changes in β-tubulin (F200Y or F167Y) which cause BZ resistance in nematodes [26, 77]. In this context it is interesting that ivermectin binds to *H. contortus* β-tubulin [17] and so there could be an effect of repeated ML treatment

limiting possible reversion to BZ susceptibility. This is of particular interest as MLs have been very commonly used alone or in combination with BZs. Considering the evidence, discussed above, it would seem quite likely that there could be a fitness cost associated with levamisole resistance (defective n-acetylcholine receptors) and with ML resistance (changes in the *dyf-7* gene which causes impaired development of dendrites in the sensory amphids). However, definitive evidence of these likely fitness costs requires further investigation.

8 Detecting Anthelmintic Resistance in Nematodes

Classically detection of anthelmintic resistance tests has been conducted using in vivo or in vitro biological tests. For gastrointestinal nematodes, the most common method is to count nematode eggs in fecal/stool samples before anthelmintic treatment and then approximately 7–14 days after anthelmintic treatment (depending on the anthelmintic used and its residence time in the host). In animals this type of test is known as the fecal egg count reduction test (FECRT) [6]. For humans, similar tests can be performed, although the Kato-Katz test [120] has been the most commonly used traditional means of assessing egg counts of soil transmitted helminthes in humans. Some in vitro tests for resistance such as the larval development assay and larval migration assays have also been described [6, 121]. All of these biological tests are quite laborious, time-consuming, and require expertise in identifying specific nematode eggs or larvae. As a result they are expensive. They also suffer from a lack of sensitivity [121] and often two assays are required to not only count eggs but also develop and differentiate larval stages in order to assess which species of nematode is responsible for the anthelmintic resistance.

Advances in our understanding of the molecular basis of anthelmintic resistances have led to the recent development of molecular-based tests for some types of drug resistance in nematodes [122, 123]. The opportunities for developing molecular tests for the different chemical classes vary, due to the varying levels of understanding of the molecular nature of the various resistances. For BZs, the work, to date, to develop molecular tests has been most successful, as this is the best understood of the resistances at the molecular level. For some other drug groups, the uncertainty about the specific nature of resistance mechanisms in field isolates, and the fact that molecular changes reported for some resistant isolates may not occur in other resistant isolates, has delayed the development of molecular diagnostics.

Coles et al. [6] described an allele-specific PCR for detection of BZ resistance-associated F200Y SNP in *H. contortus* using DNA extracted from L3 stage larvae. Other molecular methods have also been described for the detection and/or quantification of this SNP, as well as the other two associated with BZ resistance (F167Y, E198A), including restriction fragment length polymorphism-PCR (RFLP-PCR) [24, 78], real time PCR [79–81], and pyrosequencing [80]. This latter study described pyrosequencing assays for *H. contortus* codons 167, 198, and 200 of beta-tubulin isotypes 1 and 2. The method proved able to assess the BZ resistance status of a number of *H. contortus* isolates, indicating that it may be suitable for routine diagnosis of resistance in this species. A comparison of molecular data with egg hatch data showed that the molecular test was not always correlated with the degree of resistance; however, it was able to discriminate well between resistant and susceptible isolates. The inability to directly correlate with resistance levels suggests that other resistance mechanisms besides beta-tubulin mutations may also be contributing to resistance in some isolates. The lack of a strong correlation between the molecular tests and tests, such as the FECRT, may also reflect on the insensitivity of the biological test used in the comparison. The ability to detect the presence of resistance in a population by a molecular test means that the test could be utilized as a diagnostic tool for detection of resistance, even if not able to produce a quantification of the level of resistance comparable to FECRT. Two recent studies [22, 69] have evaluated the use of the pyrosequencing technique for diagnosis of BZ resistance in *H. contortus* under field conditions in Canada. Both studies showed that the molecular tests were in agreement with more laborious and expensive FECRTs in diagnosing resistance, but as the level of BZ resistance was high, it was not possible to calculate a correlation with FECRT results.

Until recently, molecular tests for the other drug classes remain to be fully evaluated in field settings. The identification of a genetic marker for levamisole resistance in *H. contortus* [91], based on the presence or absence of an indel in the *Hco-acr-8* gene, may mean that levamisole resistance caused by this genetic change could be easily detected. However, the complexity of possible changes to the levamisole receptor, which can result in loss of levamisole sensitivity (see discussion above), may make development of a simple and universal molecular assay for the detection of levamisole resistance difficult.

The recent work linking *dyf-7* gene to ML resistance in *C. elegans* and *H. contortus* [116] raises the possibility of developing molecular-based tests for detection of ML resistance in the field. The *dyf-7* gene markers have recently been used to assess ivermectin resistance in *H. contortus* on numerous sheep farms in Canada and Sweden, and initial evidence suggests a significant correlation with FECRT data (Prichard, Urdaneta, Barrere, Höglund, unpublished). These markers may prove to be useful for field-based diagnostic tests. However, the effectiveness of the *dyf-7* markers across *H. contortus* populations from different regions requires fur-

ther evaluation, as does the evaluation of similar genetic changes in other parasitic nematode species.

Resistance to ML anthelmintics has recently been reported in the filarial nematodes *D. immitis* in dogs and *O. volvulus* in people [12, 124, 125]. These are tissue dwelling nematodes which produce microfilariae in blood or skin, rather than eggs in feces. The traditional means of assessing ML activity against these filarial nematodes has been to assess microfilarial counts in blood or skin, respectively, or to use immunological tests for the adult stages. There are some disadvantages with these biological or immunological assays, such as the need to take small skin biopsy samples to assess Onchocerca microfilaraemia and possible lack of specificity with some immunological assays. Efforts to develop molecular markers are currently underway and some progress has recently been reported [10–12, 103]. The recent ease of undertaking whole genome comparisons between populations of nematodes that are drug resistant in comparison with drug susceptible populations as well as advances in understanding the mechanisms of anthelmintic resistance are greatly facilitating these efforts.

9 Conclusions

Drug resistance in parasitic nematodes of animals and humans is becoming an increasing problem, made more urgent as the rate of discovery of completely new classes of anthelmintics has been disappointingly slow. Furthermore, donations of anthelmintics such as ivermectin, albendazole, and mebendazole for the control of human onchocerciasis, lymphatic filariasis, and soil transmitted helminths have allowed huge mass drug administration (MDA) programs involving annual or more often treatment of hundreds of millions of people for nematode infections each year. While these MDA programs are significantly improving human health, inevitably selection pressure for resistance development is being dramatically increased. Similarly, in production and companion animals, the economic and welfare benefits of controlling nematode parasites have led to heavy use of anthelmintics and the current situation of, in many cases, severe problems of anthelmintic resistance. To mitigate against this drug resistance becoming dramatically worse, it is imperative that monitoring for the development of resistance be increased, so that best management and use of alternative control practices, to heavy use of anthelmintic drugs, can be employed to maintain adequate control of parasitic nematodes. Advances in understanding the mechanisms of anthelmintic resistance, with, in some cases, new molecular tools for monitoring for resistance, may facilitate improvements in following resistance development, spur new investments in drug discovery, and improve our understanding of nematode biology and novel drug target identification. Continued research to better understand the physiological and genetic bases for drug resistance in nematodes is important for success in controlling and possibly eventually eradicating major nematode infections in humans and animals.

References

1. Geary TG, Woo K, McCarthy JS, Mackenzie CD, Horton J, Prichard RK, de Silva NR, Olliaro PL, Lazdins-Helds JK, Engels DA, Bundy DA. Unresolved issues in anthelmintic pharmacology for helminthiases of humans. Int J Parasitol. 2010;40:1–13.
2. Keiser J, Utzinger J. Efficacy of current drugs against soil-transmitted helminth infections: systemic review and meta-analysis. J Am Med Assoc. 2008;299:1937–48.
3. Stepek G, Buttle DJ, Duce IR, Behnke JM. Human gastrointestinal nematode infections: are new control methods required? Int J Exp Pathol. 2006;87:325–41.
4. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L. Control of neglected tropical diseases. N Engl J Med. 2007;357:1018–27.
5. WHO. The Global Programme to Eliminate Lymphatic Filariasis (GPELF). 2015. http://www.who.int/lymphatic_filariasis/disease/en/.
6. Coles GC, Jackson F, Pomroy WE, Prichard RK, von Samson-Himmelstjerna G, Silvestre A, Taylor MA, Vercruysse J. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol. 2006;136:167–85.
7. Kaplan RM, Vidyashankar AN. An inconvenient truth: global worming and anthelmintic resistance. Vet Parasitol. 2012;186(1–2):70–8.
8. Prichard R, Ménez C, Lespine A. Moxidectin and the avermectins: consanguinity but not identity. Int J Parasitol Drugs Drug Resist. 2012;2:134–53.
9. Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC. Drug resistance in veterinary helminths. Trends Parasitol. 2004;20:469–76.
10. Bourguinat C, Keller K, Bhan A, Peregrine AS, Geary TG, Prichard RK. Macrocyclic lactone resistance in *Dirofilaria immitis*. Vet Parasitol. 2011;181:388–92.
11. Bourguinat C, Keller K, Blagburn B, Schenker R, Geary TG, Prichard RK. Correlation between loss of efficacy of macrocyclic lactone heartworm preventatives and P-glycoprotein genotype. Vet Parasitol. 2011;176:374–81.
12. Bourguinat C, Lee ACY, Lizundia R, Blagburn BL, Liotta JL, Kraus MC, Keller K, Epe C, Letourneau L, Kleinman CL, Paterson T, Carreton Gomez E, Montoya-Alonso JA, Smith H, Bhan A, Peregrine AS, Carmichael J, Drake J, Schenker R, Kaminsky R, Bowman DD, Geary TG, Prichard RK. Macrocyclic lactone resistance in *Dirofilaria immitis*: failure of heartworm preventives and investigation of genetic markers for resistance. Vet Parasitol. 2015;210:167–78.
13. Pulaski CN, Malone JB, Bourguinat C, Prichard R, Geary T, Ward D, Klei T, Guidry T, Smith G, Delcambre B, Bova J, Pepping J, Carmichael J, Schenker R, Pariaut R. Establishment of macrocyclic lactone resistant *Dirofilaria immitis* isolates in experimentally infected laboratory dogs. Parasit Vectors. 2014;7:494.
14. Lubega GW, Prichard RK. Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. Mol Biochem Parasitol. 1990;38:221–32.
15. Caviston JP, Holzbaur EL. Microtubule motors at the intersection of trafficking and transport. Trends Cell Biol. 2006;16:530–7.
16. Lacey E. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. Int J Parasitol. 1988;18:885–936.

17. Ashraf S, Beech RN, Hancock MA, Prichard RK. Ivermectin binds to *Haemonchus contortus* tubulins and promotes stability of microtubules. Int J Parasitol. 2015;45:647–54.
18. Ashraf S, Mani T, Beech R, Prichard R. Macrocyclic lactones and their relationship to the SNPs related to benzimidazole resistance. Mol Biochem Parasitol. 2015;201:128–34.
19. Nare B, Lubega G, Prichard RK, Georges E. P-azidosalicyl-5-amino-6-phenoxy-benzimidazole photolabels the N-terminal 63-103 amino acids of *Haemonchus contortus* β-tubulin 1. J Biol Chem. 1996;271:8575–81.
20. Prichard RK. Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol. 2001;17:445–53.
21. Robinson MW, McFerran N, Trudgett A, Hoey L, Fairweather I. A possible model of benzimidazole binding to beta-tubulin BZ-resistance SNPs 1085 disclosed by invoking an interdomain movement. J Mol Graph Model. 2004;23:275–84.
22. Barrère V, Keller K, von Samson-Himmelstjerna G, Prichard RK. Efficiency of a genetic test to detect benzimidazole resistant *Haemonchus contortus* nematodes in sheep farms in Quebec, Canada. Parasitol Int. 2013;62:464–70.
23. Drogemuller M, Schnieder T, von Samson-Himmelstjerna G. Beta-tubulin cDNA sequence variations observed between cyathostomins from benzimidazole-susceptible and -resistant populations. Int J Parasitol. 2004;90:868–70.
24. Ghisi M, Kaminsky R, Mäser P. Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Vet Parasitol. 2007;144:313–20.
25. Kwa MSG, Veenstra JG, Roos MH. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in [beta]-tubulin isotype-1. Mol Biochem Parasitol. 1994;63:299–303.
26. Mottier ML, Prichard RK. Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on *Haemonchus contortus*. Pharmacogenet Genomics. 2008;18:129–40.
27. von Samson-Himmelstjerna G, Blackhall WJ, McCarthy JS, Skuce PJ. Single nucleotide polymorphism (SNP) markers for benzimidazole resistance in veterinary nematodes. Parasitology. 2007;134:1077–86.
28. Lubega GW, Prichard RK. Interaction of benzimidazole anthelmintics with Haemonchus contortus tubulin binding-affinity and anthelmintic efficacy. Exp Parasitol. 1991;73:203–13.
29. Brown LA, Jones AK, Buckingham SD, Mee CJ, Sattelle DE. Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. Int J Parasitol. 2006;36:617–24.
30. Martin RJ, Verma S, Levandoski M, Clark CL, Qian H, Stewart M, Robertson AP. Drug resistance and neurotransmitter receptors of nematodes: recent studies on the mode of action of levamisole. Parasitology. 2005;131:S71–84.
31. Neveu C, Charvet C, Fauvin A, Cortet J, Beech R, Cabaret J. Genetic diversity of levamisole receptor subunits in parasitic nematode species and abbreviated transcripts associated with resistance. Pharmacogenet Genomics. 2010;20:414–25.
32. Fleming JT, Squire MD, Barnes TM, Tornoe C, Matsuda K, Ahnn J, Fire A, Sulston JE, Barnard EA, Sattelle DB, Lewis JA. Caenorhabditis elegans levamisole resistance genes lev-I, unc-29 and unc-38 encode functional nicotinic acetylcholine receptor subunits. J Neurosci. 1997;17:5843–57.
33. Robertson AP, Bjorn HE, Martin RJ. Pyrantel resistance alters nematode nicotinic acetylcholine receptor single-channel properties. Eur J Pharmacol. 2000;394:1–8.
34. Martin RJ, Clark CL, Trailovic SM, Robertson AP. Oxantel is an N-type (methyridine and nicotine) agonist not an L-type (levamisole and pyrantel) agonist: classification of cholinergic anthelmintics in *Ascaris*. Int J Parasitol. 2004;34:1083–90.
35. Robertson AP, Clark CL, Burns TA, Thompson DP, Geary TG, Trailovic SM, Martin RJ. Paraherquamide and 2-deoxy-paraherquamide distinguish cholinergic receptor subtypes in *Ascaris* muscle. J Pharmacol Exp Ther. 2002;302:853–60.
36. Kaminsky R, Ducray P, Jung M, Clover R, Rufener L, Bouvier J, Weber SS, Wenger A, Wieland-Berghausen S, Goebel T, Gauvry N, Pautrat F, Skripsky T, Froelich O, Komoin-Oka C, Westlund B, Sluder A, Mäser P. A new class of anthelmintics effective against drug-resistant nematodes. Nature. 2008;452:176–80.
37. Hu Y, Xiao SH, Aroian RV. The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis. 2009;3:e499.
38. Qian H, Martin RJ, Robertson AP. Pharmacology of N-, L-, and B-subtypes of nematode nAChR resolved at the single-channel level in Ascaris suum. FASEB J. 2006;20:2606–8.
39. Jones AK, Sattelle DB. The cys-loop ligand-gated ion channel gene superfamily of the nematode, *Caenorhabditis elegans*. Invert Neurosci. 2008;8:41–7.
40. Treinin M, Gillo B, Liebman L, Chalfie M. Two functionally dependent acetylcholine subunits are encoded in a single *Caenorhabditis elegans* operon. Proc Natl Acad Sci U S A. 1998;95:15492–5.
41. Rufener L, Mäser P, Roditi I, Kaminsky R. *Haemonchus contortus* acetylcholine receptors of the DEG-3 subfamily and their role in sensitivity to monepantel. PLoS Pathog. 2009;5(4):e1000380.
42. Bartley DJ, Devin L, Nath M, Morrison AA. Selection and characterisation of monepantel resistance in *Teladorsagia circumcincta* isolates. Int J Parasitol Drugs Drug Resist. 2015;5(2):69–76.
43. Mederos AE, Ramos Z, Banchero GE. First report of monepantel *Haemonchus contortus* resistance on sheep farms in Uruguay. Parasit Vectors. 2014;7:598.
44. Scott I, Pomroy WE, Kenyon PR, Smith G, Adlington B, Moss A. Lack of efficacy of monepantel against *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Vet Parasitol. 2013;198(1–2):166–71.
45. Van den Brom R, Moll L, Kappert C, Vellema P. *Haemonchus contortus* resistance to monepantel in sheep. Vet Parasitol. 2015;209(3–4):278–80.
46. von Samson-Himmelstjerna G, Harder A, Sangster NC, Coles GC. Efficacy of the two cyclooctadepsipeptides PF1022A and emodepside against anthelmintic resistant nematodes in sheep and cattle. Parasitology. 2005;130:343–7.
47. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, Harder A, Holden-Dye L, Hopper NA. The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclooctadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. Int J Parasitol. 2007;37:1577–88.
48. Holden-Dye L, O'Connor V, Hopper NA, Walker RJ, Harder A, Bull K, Guest M. SLO, SLO, quick, quick, slow: calcium-activated channels as regulators of *Caenorhabditis elegans* behaviour and targets for anthelmintics. Invert Neurosci. 2007;7:199–208.
49. Crisford A, Ebbinghaus-Kintscher U, Schoenhense E, Harder A, Raming K, O'Kelly I, Ndukwe K, O'Connor V, Walker RJ, Holden-Dye L. The cyclooctadepsipeptide anthelmintic emodepside differentially modulates nematode, insect and human calcium-activated potassium (SLO) channel alpha subunits. PLoS Negl Trop Dis. 2015;9(10):e0004062.
50. McIntire SL, Jorgensen EM, Kaplan J, Horvitz HR. The GABAergic nervous system of *Caenorhabditis elegans*. Nature. 1993;364:337–41.
51. Richmond JE, Jorgensen EM. One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. Nat Neurosci. 1999;2:791–7.
52. Martin RJ. Gamma-aminobutyric acid- and piperazine-activated single channel currents from *Ascaris suum* body muscle. Br J Pharmacol. 1985;84:445–61.

53. Wolstenholme AJ, Rogers AT. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology. 2005;131:S85–96.
54. Cully DF, Vassilatis DK, Liu KK, Paress P, Van der Ploeg LHT, Schaeffer JM, Arena JP. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature. 1994;371:707–11.
55. Dent JA, Davis MW, Avery L. *avr-15* encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. EMBO J. 1997;16:5867–79.
56. Liu J, Dent JA, Beech RN, Prichard RK. Genomic organization of an avermectin receptor subunit from *Haemonchus contortus* and expression of its putative promoter region in *Caenorhabditis elegans*. Mol Biochem Parasitol. 2004;134:267–74.
57. Portillo V, Jagannathan S, Wolstenholme AJ. Distribution of glutamate-gated chloride channel subunits in the parasitic nematode *Haemonchus contortus*. J Comp Neurol. 2003;462:213–22.
58. Vassilatis DK, Arena JP, Plasterk RHA, Wilkinson HA, Schaeffer JM, Cully DF, Van der Ploeg LHT. Genetic and biochemical evidence for a novel avermectin sensitive chloride channel in *C. elegans* isolation and characterisation. J Biol Chem. 1997;272:33167–74.
59. Ardelli BF, Stitt LE, Tompkins JB, Prichard RK. A comparison of the effects of ivermectin and moxidectin on the nematode *Caenorhabditis elegans*. Vet Parasitol. 2009;165:96–108.
60. Prichard RK. The pharmacology of anthelmintics in livestock. Int J Parasitol. 1987;17:473–82.
61. Van Den Bossche H, Verhoeven H, Vanparijs O, Lawers H, Thienpont H. Closantel a new antiparasitic hydrogen ionophore. Arch Int Physiol Biochim. 1979;87:851–2.
62. Rolfe PF, Boray JC, Fitzgibbon C, Parsons G, Kemsley P, Sangster N. Closantel resistance in *Haemonchus contortus* from sheep. Aust Vet J. 1990;67:29–31.
63. Van Wyk JA, Gerber HM, Alves RM. Slight resistance to the residual effect of closantel in a field strain of *Haemonchus contortus* which showed an increased resistance after one selection in the laboratory. Onderstepoort J Vet Res. 1982;49:257–61.
64. Rothwell J, Sangster NC. *Haemonchus contortus*: the uptake and metabolism of closantel. Int J Parasitol. 1997;27:313–9.
65. Kwa MS, Okoli MN, Schulz-Key H, Okongkwo PO, Roos MH. Use of P-glycoprotein gene probes to investigate anthelmintic resistance in Haemonchus contortus and comparison with Onchocerca volvulus. Int J Parasitol. 1998;28:1235–40.
66. Sangster NC, Bannan SC, Weiss AS, Nulf SC, Klein RD, Geary TG. *Haemonchus contortus*: sequence heterogeneity of internucleotide binding domains from P-glycoproteins. Exp Parasitol. 1999;91:250–7.
67. Kwa MS, Veenstra JG, Van Dijk M, Roos MH. Beta-tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. J Mol Biol. 1995;246:500–10.
68. Silvestre A, Cabaret J. Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? Mol Biochem Parasitol. 2002;120:297–300.
69. Barrère V, Falzon LC, Shakya KP, Menzies PI, Peregrine AS, Prichard RK. Assessment of benzimidazole resistance in *Haemonchus contortus* in sheep flocks in Ontario, Canada: comparison of detection methods for drug resistance. Vet Parasitol. 2013;198:159–65.
70. Chaudhry U, Redman EM, Raman M, Gilleard JS. Genetic evidence for the spread of a benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode *Haemonchus contortus*. Int J Parasitol. 2015;45(11):721–8.
71. Beech RN, Prichard RK, Scott ME. Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and -resistant strains of *Haemonchus contortus*. Genetics. 1994;138:103–10.
72. Kwa MSG, Kooyman FN, Boersema JH, Roos MH. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on beta-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun. 1993;191:413–9.
73. Le Jambre LF, Royal WM, Martin PJ. The inheritance of thiabendazole resistance in *Haemonchus contortus*. Parasitology. 1979;78:107–19.
74. Diawara A, Drake LJ, Suswillo RR, Kihara J, Bundy DAP, Scott ME, Halpenny C, Stothard JR, Prichard RK. Assays to detect β-tubulin codon 200 polymorphism in *Trichuris trichiura* and *Ascaris lumbricoides*. PLoS Negl Trop Dis. 2009;3(3):e397.
75. Diawara A, Schwenkenbecher JM, Kaplan RM, Prichard RK. Molecular and biological diagnostic tests for monitoring benzimidazole resistance in human soil-transmitted helminths. Am J Trop Med Hyg. 2013;88(6):1052–61.
76. Bourguinat C, Pion SDS, Kamgno J, Gardon J, Duke BOL, Boussinesq M, Prichard RK. Genetic selection of low fertile *Onchocerca volvulus* by ivermectin treatment. PLoS Negl Trop Dis. 2007;1(1):e72, 12–22.
77. Eng JKL, Blackhall WJ, Osei-Atweneboana MY, Bourguinat C, Galazzo D, Beech RN, Unnasch TR, Awadzi K, Lubega GW, Prichard RK. Ivermectin selection on β-tubulin: evidence in *Onchocerca volvulus* and *Haemonchus contortus*. Mol Biochem Parasitol. 2006;150:229–35.
78. Tiwari J, Kumar S, Kolte AP, Swarnkar CP, Singh D, Pathak KM. Detection of benzimidazole resistance in *Haemonchus contortus* using RFLP-PCR technique. Vet Parasitol. 2006;138:301–7.
79. Alvarez-Sánchez MA, Pérez-García J, Cruz-Rojo MA, Rojo-Vázquez FA. Real time PCR for the diagnosis of benzimidazole resistance in trichostrongylids of sheep. Vet Parasitol. 2005;129:291–8.
80. von Samson-Himmelstjerna G, Walsh TK, Donnan AA, Carriere S, Jackson F, Skuce PJ, Rohn K, Wolstenholme AJ. Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. Parasitology. 2009;136:349–58.
81. Walsh TK, Donnan AA, Jackson F, Skuce P, Wolstenholme AJ. Detection and measurement of benzimidazole resistance alleles in Haemonchus contortus using real-time PCR with locked nucleic acid Taqman probes. Vet Parasitol. 2007;144:304–12.
82. Diawara A, Halpenny CM, Churcher TS, Mwandawiro C, Kihara J, Kaplan RM, Streit TG, Idaghdour Y, Scott ME, Basáñez MG, Prichard RK. Association between response to albendazole treatment and β-tubulin genotype frequencies in soil-transmitted helminths. PLoS Negl Trop Dis. 2013a;7(5):e2247.
83. Sangster NC, Davis CW, Collins GH. Effects of cholinergic drugs on longitudinal contraction in levamisole-susceptible and -resistant Haemonchus contortus. Int J Parasitol. 1991;21:689–95.
84. Sangster NC, Gill J. Pharmacology of anthelmintic resistance. Parasitol Today. 1999;15:141–6.
85. Sangster NC, Riley FL, Wiley LJ. Binding of [3H]m-aminolevamisole to receptors in levamisole-susceptible and -resistant *Haemonchus contortus*. Int J Parasitol. 1998b;28:707–17.
86. Sangster NC, Redwin JM, Bjorn H. Inheritance of levamisole and benzimidazole resistance in an isolate of *Haemonchus contortus*. Int J Parasitol. 1998a;28:503–10.
87. Fauvin A, Charvet C, Issouf M, Cortet J, Cabaret J, Neveu C. cDNA-AFLP analysis in levamisole-resistant *Haemonchus contortus* reveals alternative splicing in a nicotinic acetylcholine receptor subunit. Mol Biochem Parasitol. 2010;170:105–7.

88. Neveu C, Charvet C, Fauvin A, Cortet J, Castagnone-Sereno P, Cabaret J. Identification of levamisole resistance markers in the parasitic nematode *Haemonchus contortus* using a cDNA-AFLP approach. Parasitology. 2007;134:1105–10.
89. Williamson SM, Storey B, Howell S, Harper KM, Kaplan RM, Wolstenholme AJ. Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of *Haemonchus contortus*. Mol Biochem Parasitol. 2011;180:99–105.
90. Boulin T, Fauvin A, Charvet C, Cortet J, Cabaret J, Bessereau J-L, Neveu C. Functional reconstitution of *Haemonchus contortu*s acetylcholine receptors in *Xenopus* oocytes provides mechanistic insights into levamisole resistance. Br J Pharmacol. 2011;164:1421–32.
91. Barrère V, Beech RN, Charvet CL, Prichard RK. Novel assay for the detection and monitoring of levamisole resistance in *Haemonchus contortus*. Int J Parasitol. 2014;44:235–41.
92. Sarai RS, Kopp SR, Coleman GT, Kotze AC. Acetylcholine receptor subunit and P-glycoprotein transcription patterns in levamisole-susceptible and -resistant *Haemonchus contortus*. Int J Parasitol Drugs Drug Resist. 2013;3:51–8.
93. Sarai RS, Kopp SR, Coleman GT, Kotze AC. Drug-efflux and target-site gene expression patterns in *Haemonchus contortus* larvae able to survive increasing concentrations of levamisole *in vitro*. Int J Parasitol Drugs Drug Resist. 2014;4:77–84.
94. Rohrer SP, Birzin ET, Eary CH, Schaeffer JM, Shoop WL. Ivermectin binding sites in sensitive and resistant *Haemonchus contortus*. J Parasitol. 1994;80:493–7.
95. Blackhall WJ, Pouliot JF, Prichard RK, Beech RN. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp Parasitol. 1998;90:42–8.
96. Njue AI, Hayashi J, Kinne L, Feng X-P, Prichard RK. Mutations in the extracellular domains of glutamate-gated chloride channel α3 and β subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity. J Neurochem. 2004;89:1137–47.
97. McCavera S, Rogers AT, Yates DM, Woods DJ, Wolstenholme AJ. An ivermectin-sensitive glutamate-gated chloride channel from the parasitic nematode *Haemonchus contortus*. Mol Pharmacol. 2009;75:1347–55.
98. Hibbs RE, Gouaux E. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature. 2011;474:54–60.
99. Lynagh T, Lynch JW. A glycine residue essential for high ivermectin sensitivity in Cys-loop ion channel receptors. Int J Parasitol. 2010;40:1477–81.
100. Lynagh T, Lynch JW. Ivermectin binding sites in human and invertebrate Cys-loop receptors. Trends Pharmacol Sci. 2012;33:432–41.
101. Lespine A, Ménez C, Bourguinat C, Prichard R. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: prospects for reversing transport-dependent anthelmintic resistance. Int J Parasitol Drugs Drug Resist. 2011;2:58–75.
102. Blackhall WJ, Liu HY, Xu M, Prichard RK, Beech RN. Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of *Haemonchus contortus*. Mol Biochem Parasitol. 1998;95:193–201.
103. Bourguinat C, Ardelli BF, Pion SDS, Kamgno J, Gardon J, Duke BOL, Boussinesq M, Prichard RK. P-glycoprotein-like protein, a possible genetic marker for ivermectin resistance selection in *Onchocerca volvulus*. Mol Biochem Parasitol. 2008;158:101–11.
104. Xu M, Molento M, Blackhall W, Ribeiro P, Beech R, Prichard R. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol. 1998;91:327–35.
105. Dicker AJ, Nisbet AJ, Skuce PJ. Gene expression changes in a P-glycoprotein (Tci-pgp-9) putatively associated with ivermectin resistance in *Teladorsagia circumcincta*. Int J Parasitol. 2011;41(9):935–42.
106. Janssen IJ, Krücken J, Demeler J, Basiaga M, Kornaś S, von Samson-Himmelstjerna G. Genetic variants and increased expression of *Parascaris equorum* P-glycoprotein-11 in populations with decreased ivermectin susceptibility. PLoS One. 2013;8(4):e61635.
107. Tydén E, Skarin M, Höglund J. Gene expression of ABC transporters in *Cooperia oncophora* after field and laboratory selection with macrocyclic lactones. Mol Biochem Parasitol. 2014;198(2):66–70.
108. Dicker AJ, Nath M, Yaga R, Nisbet AJ, Lainson FA, Gilleard JS, Skuce PJ. *Teladorsagia circumcincta*: the transcriptomic response of a multi-drug-resistant isolate to ivermectin exposure in vitro. Exp Parasitol. 2011;127(2):351–6.
109. Roulet A, Prichard RK. Ivermectin and moxidectin cause constitutive and induced over expression of different P-glycoproteins in resistant *Haemonchus contortus*. Am Assoc Vet Parasitol. 2006. Annual Meeting Honolulu, USA, Abstract No. 72.
110. Lloberas M, Alvarez L, Entrocasso C, Virkel G, Ballent M, Mate L, Lanusse C, Lifschitz A. Comparative tissue pharmacokinetics and efficacy of moxidectin, abamectin and ivermectin in lambs infected with resistant nematodes: impact of drug treatments on parasite P-glycoprotein expression. Int J Parasitol Drugs Drug Resist. 2013;3:20–7.
111. Bartley DJ, McAllister H, Bartley Y, Dupuy J, Menez C, Alvinerie M, Jackson F, Lespine A. P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. Parasitology. 2009;136:1081–8.
112. Heckler RP, Almeida GD, Santos LB, Borges DG, Neves JP, Onizuka MK, Borges FA. P-gp modulating drugs greatly potentiate the in vitro effect of ivermectin against resistant larvae of *Haemonchus placei*. Vet Parasitol. 2014;205:638–45.
113. Raza A, Kopp SR, Jabbar A, Kotze AC. Effects of third generation P-glycoprotein inhibitors on the sensitivity of drug-resistant and -susceptible isolates of *Haemonchus contortus* to anthelmintics *in vitro*. Vet Parasitol. 2015;211(1–2):80–8.
114. Molento MB, Prichard RK. Effects of the multidrug-resistance-reversing agents verapamil and CL 347,099 on the efficacy of ivermectin or moxidectin against unselected and drug-selected strains of *Haemonchus contortus* in jirds (*Meriones unguiculatus*). Parasitol Res. 1999;85:1007–11.
115. Lifschitz A, Entrocasso C, Alvarez L, Lloberas M, Ballent M, Manazza G, Virkel G, Borda B, Lanusse C. Interference with P-glycoprotein improves ivermectin activity against adult resistant nematodes in sheep. Vet Parasitol. 2010;172:291–8.
116. Urdaneta-Marquez L, Bae SH, Janukavicius P, Beech R, Dent J, Prichard R. A dyf-7 haplotype causes sensory neuron defects and is associated with macrocyclic lactone resistance worldwide in the nematode parasite *Haemonchus contortus*. Int J Parasitol. 2014;44:1063–71.
117. Godoy P, Lian J, Beech RN, Prichard RK. *Haemonchus contortus* P-glycoprotein-2: In situ localization and characterization of macrocyclic lactone transport. Int J Parasitol. 2015;45:85–93.
118. Lespine A, Martin S, Dupuy J, Roulet A, Pineau T, Orlowski S, Alvinerie M. Interaction of macrocyclic lactones with P-glycoprotein: structure-affinity relationship. Eur J Pharm Sci. 2007;30:84–94.
119. Waller PJ, Donald AD, Dobson RJ, Lacey E, Prichard RK. Changes in anthelmintic resistance status of *Haemonchus contortus* and *Trichostrongylus colubriformis* exposed to different anthelmintic selection pressures in grazing sheep. Int J Parasitol. 1989;19:99–110.

120. Cheesbrough M. “Parasitological Tests”. District laboratory practice in tropical countries, part 1. Cambridge: Cambridge University Press; 1998. p. 220–1.
121. Martin PJ, Anderson N, Jarrett RG. Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays. Aust Vet J. 1989;66:236–40.
122. Beech RN, Skuce P, Bartley DJ, Martin RJ, Prichard RK, Gilleard JS. Anthelmintic resistance: markers for resistance, or susceptibility? Parasitology. 2011;138(2):160–74.
123. Kotze AC, Hunt PW, Skuce P, von Samson-Himmelstjerna G, Martin RJ, Sager H, Krücken J, Hodgkinson J, Lespine A, Jex AR, Gilleard JS, Beech RN, Wolstenholme AJ, Demeler J, Robertson AP, Charvet CL, Neveu C, Kaminsky R, Rufener L, Alberich M, Menez C, Prichard RK. Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. Int J Parasitol Drugs Drug Resist. 2014b;4:164–84.
124. Osei-Atweneboana MY, Awadzi K, Attah SK, Boakye DA, Gyapong JO, Prichard RK. Phenotypic evidence of emerging ivermectin resistance in *Onchocerca volvulus*. PLoS Negl Trop Dis. 2011;5(3):e998.
125. Osei-Atweneboana MY, Eng JKL, Boakye DA, Gyapong JO, Prichard RK. Prevalence and intensity of *Onchocerca volvulus* infection and efficacy of ivermectin in endemic communities in Ghana: a two phase epidemiological study. Lancet. 2007;369:2021–9.

Chemotherapy and Drug Resistance in Schistosomiasis and Other Trematode and Cestode Infections

47

Robert M. Greenberg and Michael J. Doenhoff

1 Introduction

Trematodes (flukes) and cestodes (tapeworms) are members of the phylum Platyhelminthes. These parasites, including schistosomes, are estimated to infect over 400 million people worldwide (Table 47.1), with major impacts on human health and economic development [1, 2]. They represent a significant proportion of the neglected tropical diseases, a set of infections which have substantial, though often underestimated, effects on the health of more than a billion people [3]. It is now recognized that schistosome infections, which account for approximately half of these infections, are responsible for serious morbidity, mainly in sub-Saharan African countries. Flukes and tapeworms also infect agricultural animals, resulting in significant economic losses [4, 5]. Praziquantel is the drug of choice for the treatment of schistosomiasis and has been the cornerstone of ongoing mass drug administration programs. Praziquantel is effective against several other trematode and cestode infections as well. This chapter will focus primarily on schistosomes (blood flukes), the causative agents of schistosomiasis, and on praziquantel (PZQ), the drug of choice against these infections. Discussed are the drug's advantages and shortcomings, as well as the limited information available about its mode of action and the risk of drug resistance. Brief mention will also be made of other antischistosomal drugs, treatment of infections caused by other trematodes such as the liver flukes *Fasciola hepatica* and *Clonorchis sinensis* and the lung fluke *Paragonimus westermani*, and treatment of cestode infections.

R.M. Greenberg, Ph.D. (✉)
Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104, USA
e-mail: rgree@vet.upenn.edu

M.J. Doenhoff, Ph.D.
School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

1.1 Schistosomiasis

Schistosoma mansoni, *S. haematobium*, and *S. japonicum* are the three primary species of schistosomes that infect humans [6]. Estimates of the at-risk population range between 600 and 779 million people [7, 8]. In 2012, the World Health Organization estimated that almost 240 million people require preventive chemotherapy for schistosomiasis [9]. *S. mansoni* and *S. haematobium* alone infect approximately 200 million people [1, 10], accounting for roughly half of the principal flatworm infections of humans (Table 47.1). *S. mansoni* is found in Africa and parts of South America and the Caribbean (having arrived in the New World with the slave trade); *S. haematobium*, the cause of urinary schistosomiasis, is prevalent in Africa, as well as in the Middle East; and *S. japonicum* occurs largely in rural parts of China and in some Pacific islands. Other species that infect humans include *S. mekongi* and *S. intercalatum*. The large majority of people infected with schistosomes (estimated at 80 %) reside in sub-Saharan Africa [11, 12], though a small number of tourists and other visitors to endemic areas who do not take care to avoid infection are also of course at risk [13].

As with other digenetic trematodes, the life cycle of schistosomes is complex, requiring two hosts and alternating between asexual and sexual reproduction (Fig. 47.1). Schistosomiasis is a water-borne disease; humans are infected by coming into contact with fresh water in which free-swimming larval schistosomes (cercariae) have been shed by infected intermediate host snails. The cercariae attach to and penetrate the skin, transforming into schistosomula. These schistosomula leave the skin after 1–3 days (depending on the species), and enter the circulatory system via a capillary or lymphatic vessel [14–16]. The parasites reside in the vascular system of their hosts for the rest of their lives and after a short (1–2 week) period of migration throughout the body, they begin to develop into sexually mature adults, feeding on blood constituents and producing eggs. Schistosomes are unusual among trematodes in that

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_47

Table 47.1 Estimates of principal human trematode and cestode parasitic infections

Agents	Disease/distribution	Prevalence (millions)
Trematodes		
Schistosomes (blood flukes)	Schistosomiasis	~200
Schistosoma haematobium	Sub-Saharan Africa	114
S. mansoni	Sub-Saharan Africa, Brazil	83
S. japonicum	China, Southeast Asia	1.5
S. mekongi	Cambodia, Laos	0.9
S. intercalatum	Sub-Saharan Africa (limited)	1.7
Food-borne trematodes	Food-borne trematodiases	~40
Clonorchis sinensis (liver fluke)	China, southeast Asia	7
Fasciola hepatica, F. gigantica (liver flukes)	China, Egypt, Europe, Iran, South America	2.4
Fasciolopsis buski (intestinal fluke)	China, South Asia, Southeast Asia	0.2
Opisthorchis spp. (liver fluke)	Kazakhstan, Ukraine, Southeast Asia	10.3
Paragonimus spp. (lung flukes)	China, Southeast Asia, Ecuador, Peru	21.7
Tapeworms (cestodes)	Cysticercosis, hydatid disease	~175
Diphyllobothrium latum (fish tapeworm)	Worldwide, where fish are consumed	9
Echinococcus granulosus (hydatid tapeworm), *E. multilocularis*	Worldwide	2.85
Hymenolepis nana (dwarf tapeworm)	Americas, Australia, developing countries	75
Taenia saginata (beef tapeworm)	Worldwide, where beef is consumed	77
Taenia solium (pork tapeworm)	Worldwide, where pigs are raised	10
Total		~415

Adapted from [1, 2]

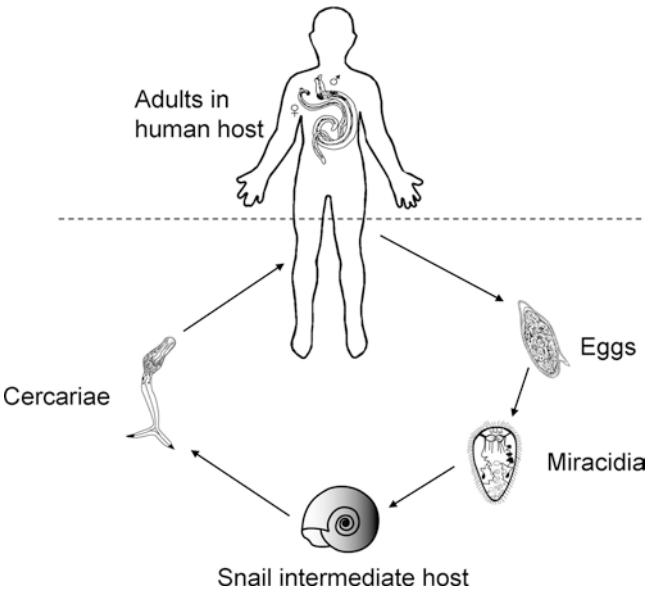


Fig. 47.1 The schistosome life cycle (simplified). Stages below the *dashed line* are aquatic

they have separate male and female sexes; as adults, males and females spend most of their time paired *in copula*, and an interaction between them is required for female reproductive development [17, 18]. *S. mansoni* and *S. japonicum* adults reside in the mesenteries, and their eggs are excreted in feces. The blood vessels of the genitourinary system are the predilection site for *S. haematobium*, and their eggs are excreted in urine. Depending on the species, an individual adult female schistosome produces between a few hundred and a few thousand eggs per day. These eggs either leave the host to continue the life cycle, or are retained within the host, where they evoke an immunopathological response. Those eggs that are excreted hatch upon contacting fresh water, releasing free-living ciliated larval miracidia. These miracidia seek and penetrate an appropriate intermediate host snail, in which they reproduce asexually via mother and daughter sporocyst stages to form many thousands of infectious cercariae. Once shed, these cercariae have approximately one day in which to find an appropriate definitive host before perishing.

Schistosomes have definitive hosts other than humans. Most notably, *S. japonicum* is zoonotic, with water buffalo and other animals, domesticated and non-domesticated, serving as major reservoirs for the parasite, complicating control strategies. Schistosomes that parasitize other mammalian or avian hosts can cause cercarial dermatitis, or swimmer's itch, a localized reaction to skin penetration by larval schistosomes that infect a variety of waterfowl (and sometimes mammals), but that cannot complete their life cycles in humans [19].

Schistosomiasis is a chronic disease. Schistosomes are estimated to live an average of 3–10 years [20, 21], though there are reports of nearly 40-year life spans within a human host [22]. The majority of morbidity and disease pathology associated with the infection is in response to the parasite eggs that remain trapped within the host, rather than to the worms themselves [6]. Eggs induce a granulomatous immune response which is usually—though not always—downregulated in chronic schistosomiasis [23]. When not modulated, the response continues, and can lead to extensive fibrosis, severe morbidity, and sometimes death. Indeed, 200,000 people are estimated to die annually in sub-Saharan Africa alone from schistosomiasis-associated complications such as kidney failure and portal hypertension [24, 25]. Schistosomiasis is also associated with other morbidities, including anemia, stunting, and reduced cognitive function [26], as well as increased susceptibility to other infections such as *Salmonella* [27] and HIV in *S. haematobium*-infected women [28].

There is at present no vaccine for schistosomiasis. Education and infrastructural improvements have proven highly effective at disease eradication, notably in Japan [29], but these efforts are expensive and difficult to implement in the developing world. Use of molluscicides to eliminate the intermediate host snails raises environmental concerns, can be expensive, and is often effective only on a limited, short-term basis [30]. Treatment and control now rely almost exclusively on chemotherapeutic intervention with a single drug, praziquantel (PZQ).

1.2 Food-Borne Trematodiases

There are several food-borne trematode infections of humans. The liver flukes *Fasciola hepatica* in temperate regions and *F. gigantica* in the tropics are important pathogens of sheep and cattle. They infect the definitive host via ingestion of metacercariae-contaminated aquatic plants such as watercress, causing reduced growth due to liver damage and killing sheep with "acute fluke disease." Human infections are associated with varying degrees of morbidity, with severity of pathogenesis related to parasite burden [31]. The intestinal fluke *Fasciolopsis buski* is also acquired via ingestion of aquatic plants.

The liver flukes *Clonorchis sinensis* and *Opisthorchis* spp. are acquired through ingestion of raw or undercooked freshwater fish containing metacercariae. Notably, in addition to their infection-associated hepatobiliary morbidity, *C. sinensis* and *O. viverrini* are strongly implicated as major etiological agents of bile duct cancer, or cholangiocarcinoma [31]. Indeed, both of these pathogens are classified as Group I carcinogens by the International Agency for Research on Cancer of the World Health Organization.

Infections with lung flukes of the genus *Paragonimus* occur via ingestion of raw or undercooked metacercariae-infested crabs or other arthropods. There are approximately 15 species known to infect humans. Pathogenesis results from damage due to migration of flukes from the gut to the lung, as well as in ectopic sites, and from parasites in the lung producing hemorrhage and inflammatory host reactions. Approximately 20 million people are thought to be infected, with an estimated 290 million at risk [32].

1.3 Cestode (Tapeworm) Infections

Adult tapeworms typically do not cause significant pathology (one important exception is the horse tapeworm *Anoplocephala perfoliata*, in which the adults can cause ulceration and inflammation). Treatment is therefore usually either for aesthetic purposes in companion animals (owners prefer not to see worm segments in their pets' feces) or to break the life cycle for those parasites in which the larval stages are pathogenic. Three cestodes in which larval stages cause considerable pathology in humans are *Echinococcus granulosus*, *E. multilocularis*, and *Taenia solium*.

Infection with tapeworm larvae, typically resulting from ingestion of oncospheres (eggs) from the definitive host, can be a serious cause of disease in humans. Humans are a definitive host for *T. solium*, the human pork tapeworm, and infection with adult *T. solium*, though unpleasant, is not typically harmful. However, if, through lapses in hygiene, humans ingest *T. solium* oncospheres found in or near feces, larval cysticerci can develop within the tissues of the human host, causing cysticercosis. Formation of cysts within the central nervous system causes neurocysticercosis, the most common parasitic disease of the nervous system, resulting in seizures, epilepsy, hydrocephalus, and dementia [33, 34]. Indeed, recent calculations indicate that nearly one-third of people with epilepsy who are living in endemic areas show lesions of neurocysticercosis in their brains [35].

Hydatid disease, or echinococcosis, occurs when humans are hosts to larval-stage cestodes of the genus *Echinococcus*. The most significant parasites are *E. granulosus* and *E. multilocularis*, which cause cystic and alveolar echinococcosis, respectively (*E. vogeli* and *E. oligarthus* cause polycystic echinococcosis). Upon ingestion of the *E. granulosus* oncosphere

by the host, the embryo is released and develops into a slowly growing, steadily enlarging hydatid cyst. The relatively long life span of humans (as compared, for instance, to sheep, another intermediate host) allows the cysts to grow quite large, interfering with the function of neighboring organs. A small percentage of cysts can also grow in the lungs or nervous system, and rupture of a cyst carries the risk of anaphylactic shock along with the possibility of spread of the infection via protoscolices, resulting in secondary echinococcosis. Traditionally, treatment has relied on surgery, though integration with chemotherapy (e.g., with albendazole) has also been used more frequently in recent years [36]. The fox tapeworm *E. multilocularis* represents one of the most dangerous zoonotic infections [37]. *E. multilocularis* produces alveolar cysts in humans, which serve as aberrant intermediate hosts. These cysts are proliferative and infiltrate surrounding tissue in a manner similar to a malignant neoplasm. Treatment is typically by surgery, which is often ineffective, and chemotherapy to suppress growth of the lesion [36]; left untreated, alveolar hydatid disease is usually fatal within 10 years.

2 Treatment of Schistosomiasis with Praziquantel

Praziquantel (PZQ) is the current drug of choice against schistosomiasis. It is effective against all species of schistosomes that infect humans [38], notably safe compared with other anthelmintics, has a relatively low cost (as low as US$ 0.07 per pill) [39], and can be administered as a single oral dose with minimal direct medical supervision [40]. Due to these advantages, other antischistosomal drugs such as oxamniquine, which for many years was a mainstay of schistosomiasis mansoni treatment in Brazil, are effectively no longer available commercially [41]. Furthermore, other than repurposed antimalarials such as artemisinins [42], no new antischistosomal drugs have entered the market since the introduction of PZQ. Thus, as of this writing, PZQ is essentially the only available therapeutic for treatment and control of a disease affecting hundreds of millions of people.

The value of PZQ has been proved repeatedly in large-scale schistosomiasis control efforts in several countries [43–47]. Indeed, based in part on this success, the Merck Group has pledged to increase its annual donation of PZQ tablets to the World Health Organization from 25 to 250 million [48]. However, the gap in PZQ availability versus need for therapy remains substantial, and is not likely to be filled by current pledges [49]. Nonetheless, these trends in targeting schistosomiasis have resulted in a massively greater rate of usage of PZQ than any other drug for treatment of trematode or cestode infections, and unfortunately this increased drug pressure may select alleles for resistance [50]. This chapter will therefore focus mainly on PZQ in the context of its activity against schistosomes. After a brief description of the drug, its mode of use, and its metabolism, the relatively limited knowledge available about PZQ resistance—both in field isolates and experimentally induced—will be discussed.

As noted above, oxamniquine is no longer in general use, in large part because it is effective against only one species, *S. mansoni*. However, oxamniquine is important because resistance to oxamniquine represents the earliest and clearest illustration of naturally selected drug resistance in a helminth parasite of humans [51] and provides an interesting "compare and contrast" exercise with PZQ. Notably, details of the mechanism of oxamniquine resistance have recently been elaborated [51], and will be discussed below.

There are several excellent reviews on antischistosomal chemotherapy and drug resistance, to which the reader is referred [40, 52–56]. Several recent reviews also discuss past, present, and plans for future control of schistosome and other helminth infections [50, 57–60].

2.1 History, Chemical Structure, and Properties of Praziquantel

In the early 1970s, pyrazino-isoquinoline derivatives, initially synthesized and assayed for tranquilizer potential, were tested for anthelmintic activity [61] under an agreement between Bayer and E. Merck, Germany. More than 400 compounds synthesized by Merck were tested by Bayer [62], and one of the most effective during in vivo screening was PZQ, then identified as EMBAY 8440 [63, 64]. Bayer initially marketed PZQ as Droncit for use as a veterinary cestocide; in 1977, PZQ was shown to be effective against infections of different schistosome species in experimental animals [63], and performed satisfactorily in toxicological and pharmacological tests.

Following successful tests on human volunteers in 1978 to assess tolerance and pharmacokinetics [65], clinical trials were performed jointly with the World Health Organization to test efficacy against *S. mansoni* [66], *S. haematobium* [67], and *S. japonicum* [68, 69]. These trials, as well as numerous subsequent ones, were very positive, and PZQ, marketed for human use as Biltricide, became, and continues to be, the clear drug of choice for treatment and control of schistosomiasis [7, 55, 70]. In 1983, the Korean company Shin Poong patented a new method for synthesizing PZQ, initiating market competition that resulted in striking price reductions. PZQ is now produced by several generic manufacturers under a variety of brand names (e.g., Distocide, Bilharzid, Epiquantel syrup, Prazitel).

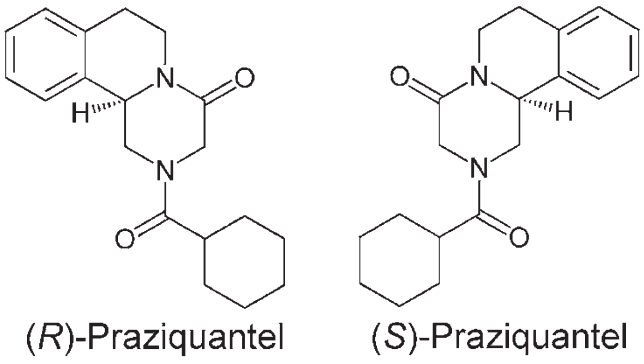


Fig. 47.2 The structure of the two enantiomers of praziquantel

PZQ is 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinoline-4-one. Its chemical structure is illustrated in Fig. 47.2. It is a white crystalline powder that is stable under normal storage conditions, with a molecular weight of 312.3. It is practically insoluble in water, but soluble in some organic solvents such as chloroform and dimethylsulfoxide. PZQ has an asymmetric center in position 11b, and the commercial preparation is a 1:1 racemate. The L-(-)-enantiomer ("levo") has the (*R*)-configuration, and the D-(+)-enantiomer ("dextro") has the (*S*)-configuration [38], and both are depicted in Fig. 47.2. Most evidence suggests that only the (*R*)-enantiomer has schistosomicidal activity, both in vivo and in vitro [38, 71–77]. The inactive (*S*)-enantiomer appears to be responsible for the majority of side effects [74], and contributes to the large tablet size and extremely bitter taste of the commercially available drug [78], factors which compromise compliance. Interestingly, however, recent data indicate that the (*S*)-enantiomer, and not the (*R*)-enantiomer, could be responsible for schistosomicidal activity against human *S. mansoni* infections [79]. More efficient recent approaches for resolving PZQ as a single enantiomer [80, 81] have the potential to be exploited for large-scale, cost-effective production of an enantiopure drug preparation, and may also allow for more definitive investigations of the roles of the different enantiomers in human schistosome infections.

Tablets of PZQ are usually oblong and contain 600 mg of the active ingredient. Epiquantel, a syrup formulation containing 600 mg PZQ/5 mL, is suitable for small children, and is produced by the Egyptian International Pharmaceutical Industries Company (EIPICO). Tested samples of PZQ tablets from different producers have generally complied well with industry standards [82, 83], though two samples from one manufacturer were counterfeit, and contained no PZQ [84]. However, this finding is now over a decade old, and it would probably be worthwhile to revisit the question in view of the enormously greater amounts of the drug currently in use. Comparisons of the efficacy, bioavailability, and metabolism of different PZQ brands in *S. mansoni*-infected mice showed that they all significantly decreased worm burden, but that some of the generic formulations had lower bioavailability that was associated with reduced efficacy [85]. As noted above, PZQ can now be purchased for as little as US$0.07–0.08 per tablet, or, for a normal treatment of 40 mg/kg body weight (2.5 tablets), US$0.20 or less per child [28, 39, 47]. Other associated expenses such as distribution, education, and administrative support essentially double the cost, to ~US$ 0.50 per person per year [47], a price still out of reach in many impoverished regions [86]. Epiquantel, the PZQ syrup formulation for children, is significantly more expensive and has major logistical challenges due to limited availability and cumbersome packaging and storage; The World Health Organization has concluded that it provides no significant improvement over administration of crushed or broken tablets [87]. Furthermore, though Epiquantel was well tolerated and effective against *S. haematobium* infections in preschool-aged children from Niger, it exhibited considerably reduced efficacy (~50 % cure rate) against *S. mansoni* infections [88]. Exhaustive evaluations of the safety and efficacy of PZQ for treating *S. mansoni* and *S. haematobium* infections have recently been published [55, 70].

2.2 Efficacy of Praziquantel

The recommended treatment dose of PZQ for *S. mansoni* and *S. haematobium* is 40 mg/kg body weight, and a dose of 60 mg/kg body weight is typically recommended for *S. japonicum* and *S. mekongi* [55, 70, 89]. Recent evidence from several regions however has shown that the higher dose of 60 mg/kg increases the risk of at least one adverse event while providing no significant efficacy advantage against intestinal schistosomiasis caused by *S. mansoni* or *S. japonicum* or urinary schistosomiasis caused by *S. haematobium* [90–92]. Indeed, even when the 40 mg/kg dose was sub-curative for *S. haematobium*, a 60 mg/kg dose did not improve cure rates [91], possibly because at the time of treatment, the host was harboring immature worms, which are insusceptible to PZQ [40]. On the other hand, recent data from Uganda suggest that 60 mg/kg improves efficacy against schistosomiasis mansoni in school-age children [79].

Use of a pole that substitutes measures of height for weight appears to be a valid approach for simplifying proper dosing adjustments in the field [93, 94], though it carries the risk of under-dosing in populations displaying a high level of obesity [95]. Splitting 40 mg/kg PZQ into two doses over 12 h appears to have few, if any, benefits over a single dose, and causes more side effects [55, 70]. However, evidence from a high-risk community suggests that repeated PZQ dosing appears to provide cost-effective incremental benefits in limiting both an individual's total years of infection and the time spent with a heavy infection [39].

The pharmacokinetic and pharmacodynamic properties of PZQ in humans have recently been reviewed and summarized [96]. Studies are few in number, particularly in the main target population of school-aged children, and only one study on the active (*R*)-PZQ enantiomer administered alone has been reported [79]. Absorption of orally administered racemic PZQ is rapid (T_{max} 2–2.6 h) and nearly complete (>80 %); it can be detected in the blood by 15 min after dosing [97]. However, systemic bioavailability of PZQ is low, with individuals showing widely varying peak plasma concentrations (200–2000 ng/mL) and kinetics, with $t_{1/2}$ ranging from 2.2 to 8.9 h for a 40 mg/kg oral dose in fasting individuals [96, 98]. Bioavailability of PZQ increases with concomitant intake of food and, in particular, a high-carbohydrate diet [98–101].

A major asset of PZQ is its activity against a broad range of platyhelminth parasites, including most of those listed in Table 47.1. Early experimental studies showed that PZQ was more or less equally effective against all schistosome species [102, 103], a result confirmed by the large data set accumulated from its use to treat endemic schistosomiasis throughout the world [43–47]. Changes in patterns of schistosome egg excretion are most often used to determine the drug's effectiveness, either in terms of a cure rate (the number of patients who are excreting no eggs after treatment as a percentage of the number found excreting eggs before treatment) or the percentage reduction in the mean number of eggs excreted by the treated group. Earliest studies showed that PZQ consistently achieved cure rates of 60 % or greater, and often 85–90 % [104]. More typically, cure rates for PZQ are in the 70–95 % range, but can be significantly lower in particular regions and age groups, with failure-to-cure rates often reaching 30–60 % [39, 105–110]. Pertinent to a consideration of PZQ resistance, a 100 % cure rate has seldom, if ever, been recorded in an endemic area. On the other hand, egg reduction rates can be as high as 95 % in patients that are not cured [39, 105–110]. As has been pointed out [105], a patient with a dramatic decrease in egg excretion would be considered a success in terms of reduction in pathology, but poses a risk by harboring egg-producing schistosomes that can survive exposure to standard PZQ dosing regimens. Further confounding the issue is the fact that the standardly used Kato-Katz technique for measuring egg counts detects only patent infections and, even within these constraints, has reliability issues and poor sensitivity and has thus been documented to underestimate levels of infection, leading to false negatives [111–113].

Also relevant to a discussion of drug resistance, and a significant shortcoming of PZQ, is its relative lack of efficacy against juvenile schistosomes in vivo and in vitro [63, 114–117]. Schistosomes have an unusual biphasic sensitivity to PZQ and some other schistosomicidal drugs [115], in which early migrating larval stages are susceptible, but susceptibility then decreases to low levels in 3–4-week-old infections and is only gradually regained as worms begin to produce eggs. Worms of experimental infections are almost fully susceptible to PZQ when they are about 6–7 weeks old. Interestingly, like PZQ-susceptible adults, PZQ-refractory juvenile schistosomes undergo a Ca^{2+}-dependent contraction and paralysis similar to that observed in adult worms in vitro (see below), but, unlike adults, recover and survive [118]. The insusceptibility of immature worms may be a cause of some of the poor cure rates and treatment failures observed in some patient groups, particularly those exposed to very high rates of transmission (see below for discussion of the reasons for poor PZQ cure rates in Senegal). A protocol involving administration of two courses of PZQ has been advocated for such situations [119] and adoption of this approach has indeed generally resulted in higher cumulative cure rates [120–122], though not in some cases for *S. mansoni* infections [107, 110, 123]. Given that this protocol recommends administration of the second treatment only a short time after the first, there is some question as to why it should necessarily be more efficacious in cases of true insusceptibility, as surviving worms may still be stunted and thus perhaps in a physiologically "immature" state; it is also not clear why the same worms that were not sensitive to the drug would become susceptible a short time later (unless, of course, it was an instance of juvenile worms maturing).

PZQ is effective against most food-borne tremadodiases, including clonorchiasis, opisthorchiasis, paragonimiasis, and intestinal fluke infections (reviewed in 124, 125). In contrast, PZQ failed to show an effect on the surface of the liver fluke *F. hepatica* [126]. This lack of activity in vitro has been reflected in several studies reporting low levels of PZQ efficacy against *F. hepatica* infections (reviewed in 127–131). Though sporadic reports of PZQ activity against *F. hepatica* have appeared [132], the consensus of opinion is that PZQ is not particularly useful against fascioliasis [124, 125]. The reasons for the variation in response and overall lack of efficacy are not understood.

PZQ is also effective against adult tapeworms and is used very widely in pets, humans, and livestock for treatment and control of cestodes (reviewed in 124). The dose used for most adult tapeworms is considerably lower (1–5 mg/kg)

than that for *Schistosoma* spp. (40 mg/kg), though higher doses are recommended for some tapeworms (e.g., *Diphyllobothrium*). A few treatment failures with PZQ have been reported, most notably in the beef tapeworm, *Taenia saginata* [133], though there is no link establishing drug resistance of the parasite as the cause. PZQ is also used for treatment of larval cestode infections, though with less effectiveness, and typically in combination with other therapies.

2.2.1 PZQ Derivatives

Since the introduction of PZQ, a small number of related compounds have been tested for antischistosomal activity [134–137]. To date, none appear to be superior to PZQ itself.

2.3 Distribution, Metabolism, Toxicity, and Side Effects of PZQ

Information on the fate of PZQ following administration has recently been reviewed extensively [96]. PZQ distributes throughout the body, concentrating especially in the liver and kidneys [38, 138]. PZQ appears to cross the blood–brain barrier [139], likely accounting for its effectiveness in neurocysticercosis. PZQ is highly protein-bound, mostly to albumin [140]. Thus, levels of free drug are likely subject to factors such as nutrition and inflammation, perhaps accounting for the variability in effectiveness between healthy volunteers and patients [96].

PZQ is subjected to pronounced metabolism on first pass through the liver, with rapid and extensive conversion into hydroxylation derivatives; it disappears relatively rapidly from the circulation with a half-life of 1–3 h. One might speculate that this rapid conversion may play a role in the insensitivity of *Fasciola* infections to PZQ (see Sect. 2.2 above), as perhaps little intact drug remains to get into the bile ducts. Elimination from the body is via urine and feces and is 80 % or more complete after 24 h [141]. Cytochrome p450 enzymes are mainly responsible for metabolism of PZQ [38, 96] and the bioavailability shows variability due to interindividual pharmacogenetic differences or liver dysfunction, as well as by concomitant exposure to agents such as grapefruit juice or cimetidine that inhibit cytochrome p450 activities [142, 143]. Subjects suffering from hepatic dysfunction, for example, because of severe schistosomal disease, metabolize PZQ more slowly [98, 144]. The main metabolite in humans is *trans*-4-hydroxypraziquantel, and a recent study using human liver microsomes identified up to nine PZQ metabolites [145]. Metabolic derivatives of PZQ have not been fully analyzed for schistosomicidal activity. Elimination of PZQ is largely renal, with the large majority of drug and metabolites removed within 24 h [38].

There is little if any information about how schistosomes themselves metabolize PZQ. However, two other platyhelminths (the trematode *Dicrocoelium dendriticum* and the cestode *Hymenolepis nana*) appear incapable of metabolizing PZQ enzymatically [146]. PZQ inhibits the mammalian ATP-binding cassette (ABC) multidrug transporter P-glycoprotein, but does not appear to be a substrate [147]. It inhibits *S. mansoni* P-glycoprotein (SMDR2) as well, but also appears to be a substrate of the parasite ABC transporter [148].

In animal tests, PZQ showed very low toxicity and minimal genotoxic risks in assays for mutagenicity [149]. A review of existing data concluded that though a few observations suggested accumulation of potentially mutagenic metabolites, they may represent anomalies amongst a massive amount of evidence indicating PZQ is a safe drug [150]. PZQ is tolerated and effective in patients of all ages and for treatment of the different forms of clinical schistosomiasis including cases of advanced hepatosplenic disease [151]. PZQ is considered safe enough that an informal consultation held by the World Health Organization recommended that it be offered to pregnant and lactating women [152]. Nonetheless, with a lack of clear evidence of PZQ safety and efficacy during pregnancy, many countries and agencies do not use PZQ during pregnancy. However, in a recent double-blind, placebo-controlled study from the Philippines, PZQ given at 12–16 weeks gestation showed no indication of safety issues for either the mother or the fetus [153].

Adverse events that are observed after treatment are generally described as relatively mild and transient, but can affect as many as 30–60 % of patients (reviewed in 55, 70). As alluded to above, patients treated with the (*R*)-enantiomer alone at half the dose of the racemate mixture had the same cure rates, but suffered fewer side effects [74], and the inactive (*S*)-enantiomer is also responsible for the bitter taste of PZQ [78]. The frequency and intensity of side effects after normal treatments is correlated with the intensity of infection as measured by the number of eggs excreted before treatment, and the most severe side effects of bloody diarrhea or edematous urticaria that are observed in areas with high intensities of infection [154] may thus be due to the release of the constituents of large numbers of dying worms or the host body's response to them.

2.4 Mechanism of Action of Praziquantel

Some of the effects of PZQ on schistosome worms have been well described, but the detailed molecular mechanisms of the drug's action continue to remain unresolved despite several decades of experiments aimed at defining the mode of action [40, 54, 155]. Recent work, however, is beginning to shed light on likely PZQ targets and downstream pathways [156, 157].

PZQ disrupts Ca^{2+} homeostasis in adult schistosomes (reviewed in 103, 158–160). Thus, an obvious and rapid response of the worms after exposure to PZQ is spastic paralysis

of the musculature, which is accompanied—and likely caused—by a rapid influx of Ca^{2+} ions [161]. Muscular contraction is not necessarily associated with worm death, however, since there are conditions of sublethal in vitro exposure [0.2–0.5 μg/mL (~500 nM–1.5 μM) for adults and 0.2–80 μg/mL (~500 nM–250 μM) for immature worms] in which even a long-lasting paralysis is reversible after drug removal, with subsequent survival of the parasites [116]. Indeed, the central role of Ca^{2+} in PZQ action has been questioned, based on pharmacological experiments on whole worms in vitro [118]. PZQ does not have the properties of an ionophore [161] and the ATPases involved in pumping Ca^{2+} out of cells are apparently unaffected by PZQ [162, 163]. PZQ appears to interfere with parasite excretory activity, perhaps by disrupting the function or expression of P-glycoprotein or other ATP-binding cassette (ABC) transporters [164, 165].

Another early effect of the drug is a morphological alteration of the worm tegument consisting of vacuolization at the base of the tegumental syncytium and blebbing at the surface [126, 166]. This effect does not occur with the inactive (*S*)-enantiomer of PZQ or in the absence of Ca^{2+} ions [167, 168], indicating that tegumental disruption is also linked to PZQ-dependent disruption of Ca^{2+} homeostasis [158, 160]. PZQ-induced tegumental damage is associated with exposure of parasite antigens on the worm surface, particularly over the tubercles [169]. These changes appear to render the parasite more susceptible to attack by antibodies and penetration by host defense cells [166]. Related to drug-induced antigen exposure, there is experimental evidence for a synergistic effect between antischistosomal drugs, including PZQ, and host antibodies in killing worms in vivo [170–174]. On the other hand, the efficacy of PZQ as measured by egg counts does not appear to be compromised in human subjects infected with HIV [175–177]. In contrast, circulating anodic antigen levels indicate that the effectiveness of PZQ is in fact reduced in HIV-infected subjects, suggesting that the drug could be affecting parasite fecundity (as indicated by egg counts) rather than survival (as indicated by circulating anodic antigen) in these immunocompromised hosts [175]. It is also possible that the HIV-infected patients had become sensitized to schistosome antigens prior to becoming infected with HIV.

Early experimental evidence showed that PZQ interacts with model membranes, indicating that the drug could be acting on schistosome surface bilayer membranes to affect fluidity, stability, or permeability [178, 179]. Subsequent follow-up experiments on adult worms in vitro showed that though both stereoisomers of PZQ decreased the average velocity of lipid molecules in the tegumental membrane, only the active enantiomer reduced the number of molecules that were able to move, perhaps indicating different insertion modalities of the stereoisomers [180].

Since the introduction of PZQ, there have been several molecular targets postulated for the drug, with varying degrees of supporting evidence (reviewed in 181). Several years ago, glutathione *S*-transferase was suggested as a PZQ receptor based on the three-dimensional structure of this protein having a "pocket" in which a molecule of the drug could fit [182], but the failure of PZQ to affect the activity of the enzyme is not consistent with this hypothesis [183]. PZQ has also been shown to stereoselectively inhibit nucleoside uptake in schistosomes, but not mammals, perhaps linked to Ca^{2+} uptake [184]. PZQ inhibits basal and stimulated phosphoinositide turnover in schistosomes, though at higher concentrations and longer timescales than required for paralysis and tegumental disruption [185]. Other proposed targets include myosin light chain [186] and actin [186, 187], two highly abundant proteins [188].

There is a strong body of evidence supporting parasite voltage-gated Ca^{2+} (Ca_v) channels as molecular targets for PZQ. Ca_v channels are the major conduit for entry of extracellular Ca^{2+}, and they couple cellular excitation to a wide array of Ca^{2+}-dependent responses, and PZQ interaction with these channels would be consistent with the Ca^{2+}-dependence of PZQ action and the drug's rapid disruption of parasite Ca^{2+} homeostasis. Schistosomes express an unusual Ca_v channel auxiliary "variant" β subunit (β_{var}) that appears to be found exclusively in platyhelminths, including free-living turbellarians, trematodes, and cestodes [189, 190]. When either the *S. mansoni* or *S. japonicum* β_{var} subunit is expressed in *Xenopus* oocytes in combination with a mammalian Ca_v channel pore-forming α_1 subunit, the schistosome β subunit confers PZQ sensitivity to this normally PZQ-insensitive mammalian channel. The capability of this subunit to confer PZQ sensitivity to the mammalian subunit can be eliminated by altering a single amino acid residue in the schistosome β subunit; changing a mammalian β subunit to resemble the schistosome subunit at that same residue creates a subunit that now behaves like the schistosome subunit and can confer PZQ sensitivity (reviewed in 155).

Recent studies on free-living planaria are consistent with a role for Ca_v channels in PZQ action [156, 191]. Planarians have the remarkable capability to regenerate their head and tail when cut at both ends. When exposed to PZQ, planaria instead regenerate two heads. However, planaria in which Ca_v channel subunit expression has been suppressed by RNA interference show normal regenerative patterning when exposed to PZQ, although the β_{var} subunit unexpectedly does not appear to be the key subunit [191]. Despite such differences in details, however, these results support a central role for platyhelminth Ca_v channels in PZQ action. Recent experiments show that PZQ-dependent effects on planarian head regeneration occur via inappropriate activation of neuronal Ca_v channels, with subsequent dysregulation of dopaminergic and serotonergic pathways. Dopaminergic and serotonergic agents that disrupt normal planarian regeneration also exhibit schistosomicidal activity, and compounds with antischistosomal activity cause

bipolar planarian regeneration. Thus, both outcomes (death or axis duplication) appear to depend on a conserved initial signal (Ca^{2+} entry via PZQ activation of Ca_v channels) with divergent downstream outputs [156].

There is a rich pharmacology for mammalian Ca_v channels, and, since PZQ appears to affect these channels in the schistosome, Ca_v channel-modulating agents have been tested for their effects on schistosomes either alone or in combination with PZQ, with variable results. Thus, one group found that dihydropyridine Ca_v channel antagonists such as nifedipine and nicardipine partially rescued worms from the schistosomicidal effects of PZQ [192], while another found that nifedipine instead enhanced the effects of PZQ against schistosomula and adults and, in fact, exhibited schistosomicidal activity on its own [193]. To make matters even more puzzling, the Ca_v channel blockers apparently did not block Ca^{2+} influx in whole worms [118], and the limited pharmacology that has been done on native currents from flatworm muscle and neuronal cells suggests that their Ca^{2+} currents are either insensitive to Ca_v channel blockers such as nifedipine or require relatively high concentrations for only partial inhibition [194–197]. Furthermore, pre-incubation of adult schistosomes with cytochalasin-D, an actin depolymerizing agent, protected schistosomes from the effects of PZQ; yet, contrary to expectation that such an effect might be reflected in reduced PZQ-dependent Ca^{2+} influx, cytochalasin-D instead appeared to increase Ca^{2+} influx into the parasite [118]. Nonetheless, based on the totality of evidence, it seems clear that schistosome Ca_v channels are almost certainly important mediators of PZQ action; development and exploitation of more precise molecular, genetic, and electrophysiological methodologies may help resolve some of the currently perplexing details.

3 Resistance to Praziquantel

3.1 Evidence for Resistance to Praziquantel in Schistosomes

Dependence on a single drug for treatment and control of a disease as prevalent as schistosomiasis is very risky; there are few if any alternatives should resistance arise. Fortunately, as of this writing, there is no indication of widespread resistance to PZQ in schistosomes, perhaps reflecting lack of systematic monitoring or difficulties in distinguishing resistance from other confounding factors (reviewed in 53, 105). Nonetheless, several reports from both the field and the laboratory indicate that schistosomes can in fact develop resistance to PZQ.

3.1.1 Field Isolates

The first report of apparent PZQ resistance in the field emanated from Senegal, where the construction of a barrage dam to control the flow of the Senegal River in the mid-1980s resulted in an expansion of endemic schistosomiasis mansoni. By the early 1990s, the rate of transmission had reached epidemic proportions in inland villages in Northern Senegal that had begun to benefit from agricultural irrigation projects along the dammed river [198, 199]. When PZQ was used in an attempt to control the disease it gave cure rates of only 18–39 % [200, 201], alarmingly low when compared with the 60–90 % cure rates normally expected. Increasing the dose of PZQ from 40 to 60 mg/kg body weight did not significantly improve cure rates [202]. A later study involving treatment in the same area of Northern Senegal again resulted in relatively low cure rates [123]. Two further observations indicated that *S. mansoni* in northern Senegal was responding aberrantly to PZQ: (a) a parasite line taken into laboratory passage from snails with patent infections collected in that area was found to have a decreased susceptibility to PZQ [203–205]; (b) when the effect of oxamniquine, a different antischistosomal, was later tested in this area, the routine dose of 20 mg/kg gave a cure rate of 79 %, compared with 36 % in a simultaneously treated control group given 40 mg/kg PZQ [206]. Similar results were found in mouse infections with these worms [203]. Since oxamniquine, like PZQ, is relatively ineffective against immature worms, these results cast some doubt on arguments suggesting that the low cure rates in this area reflect large numbers of PZQ-refractory juvenile worms due to high rates of transmission [207]. On the other hand, follow-up studies and data analysis pointed to at least some portion of this failure of PZQ being attributable to factors other than drug resistance, including high-intensity infection, rapid reinfection and transmission, presence of PZQ-refractory juvenile worms, variations in methodology for analysis of efficacy, and perhaps native tolerance of these schistosomes [207–209]. For example, when infected patients were relocated to urban areas, where there is little or no transmission, cure rates rose to near-normal levels [207]. However, even after accounting for intensity of infection and sensitivity of diagnosis, a meta-analysis suggests that Senegal remains atypical, showing cure rates significantly lower than expected and "the suspicion of tolerance or resistance to PZQ…cannot be ruled out" [209]. Fortunately, despite the reduced cure rates in the area of interest, treatment with PZQ still lowered the infection intensity significantly and reduced morbidity in treated individuals.

Another focus of potential PZQ resistance has been in Egypt. During the 1990s, PZQ was used widely in Egypt in a consolidated effort to control schistosomal disease [210], with an estimated 60 million tablets taken between 1997 and 1999 alone [82]. During this period Ismail et al. [211] treated 1607 *S. mansoni*-infected patients in the Nile delta region with 40 mg PZQ/kg body weight, and after an additional two treatments, the last at 60 mg/kg, a reported 2.4 % of the patients were still passing eggs. Eggs passed by several of these uncured patients were subsequently used to establish

laboratory life cycles and were found in mice to exhibit 2–5-fold lower sensitivity to PZQ (as measured by ED_{50}) than isolates that had been established from eggs passed before treatment by patients who were easily cured [211, 212]. Though significant, the difference in susceptibility is comparatively small, with no evidence for emergence of "super-resistant" worms [213]. The reduced PZQ susceptibility of these Egyptian isolates in vivo was consistent with responses of individual worms to PZQ in vitro, where confounding host influences are not a factor [212, 214, 215].

Approximately half of the Egyptian isolates retained their reduced susceptibility to PZQ following multiple passages through the life cycle in the absence of drug pressure, suggesting that drug pressure is not required for maintenance of the PZQ insusceptibility trait [216]. Those isolates that continued to show lower PZQ susceptibility also often exhibited evidence of compromised biological fitness such as decreased production of cercariae by infected snails [217], an observation consistent with others [218], and indicative of fitness costs associated with reduced susceptibility to PZQ. These costs may serve to constrain spread of PZQ resistance. Interestingly, 10 years following initial characterization of the Egyptian PZQ-insusceptible isolates, a visit to the same site in Egypt revealed no evidence of uncured patients despite a decade of drug pressure [219], suggesting at the least that PZQ "resistance" had not expanded. It has been postulated that PZQ-refractory immature schistosomes can act as a refugia (see Sect. 5.1, below) for PZQ-susceptible worms, and that this and other large refugia in endemic areas may act to limit the spread of resistance [220].

Evidence for PZQ insusceptibility has also been reported more recently in Kenya [221]. In this case, miracidia hatched from eggs excreted by *S. mansoni*-infected car washers on the shores of Lake Victoria were screened for susceptibility to PZQ, and it was found that different patients produced eggs that hatched into miracidia with variable sensitivity to killing by PZQ, with miracidia from previously treated patients showing significantly lower sensitivity to the drug. Adult worms derived from eggs excreted by a patient (KCW) who was never fully cured by PZQ were less sensitive to PZQ, both in vivo, in murine infections, and in vitro, as assayed by schistosome length. The reduced susceptibility of one sub-isolate of KCW was heritable and persisted through at least 6 life cycle passages in the absence of drug pressure while a second KCW sub-isolate had reverted to a PZQ-susceptible state when retested after eight generations. The sub-isolate that reverted survived, while the sub-isolate that continued to exhibit reduced PZQ susceptibility eventually perished [221]. Thus, similar to the Egyptian isolates, these Kenyan isolates also showed variable stability of PZQ tolerance and apparent biological costs associated with PZQ insusceptibility.

There is currently little evidence for emergence of PZQ resistance in other species of schistosomes that infect humans (*S. japonicum*, *S. haematobium*). Monitoring of various endemic areas of China for evidence of PZQ insusceptibility revealed little if any evidence for emerging PZQ resistance [222]. Thus, despite decades of intensive chemotherapy with PZQ, it continues to be effective in treating schistosomiasis japonica in China [223–226]. Despite isolated reports of failure of PZQ to cure *S. haematobium* infections [227–229], there is currently no evidence for emergence of heritable resistance in carefully studied populations [230].

As of this writing, PZQ resistance has not been documented in the food-borne trematodes or in the cestodes [58, 124]. An unusually low cure rate (29 %) following PZQ treatment of clonorchiasis patients in Vietnam may be attributable to use of low PZQ doses [231]. A few cases of unsuccessful PZQ treatment of beef tapeworm (*T. saginata*) infections have also been reported [133], but there is no evidence that these failures reflect parasite resistance to the drug.

3.1.2 Experimentally Induced Praziquantel Resistance

In 1994, Fallon and Doenhoff [232] reported that resistance to PZQ could be selected for in laboratory-maintained *S. mansoni*. This selection was achieved by applying drug pressure to successive mouse passages of a "hybrid" isolate that had earlier been raised from a pool of cercariae from four laboratory-maintained *S. mansoni* lines from different geographic areas. All the isolates contributing to the hybrid had been taken into laboratory passage before PZQ began to be used. By the seventh life cycle passage, PZQ drug pressure produced a population of schistosomes in which 93 % of the worms survived a PZQ dose that killed 89 % of control, unselected worms. This and subsequent reports confirmed that worms produced as a result of this selection pressure were less sensitive to PZQ than controls not exposed to PZQ [205, 232, 233]. Using a similar approach, PZQ resistance has been experimentally induced in *S. mansoni* from Brazil [234] and in *S. japonicum* [235].

In addition to its effects on adult worms, PZQ also reduces shedding of cercariae from *S. mansoni*-infected *Biomphalaria glabrata* snails [236], which opens the possibility of using drug selection on the asexual stages of the life cycle in the snail host to induce PZQ resistance. Subsequently, Couto et al. [237] exploited this effect, selecting for PZQ insusceptibility by exposing *S. mansoni*-infected *B. glabrata* to successive treatments of 100 mg/kg PZQ. Following completion of this regimen, cercariae shed by these snails were used to infect mice and developed into adult worms with significantly reduced susceptibility to PZQ. Specifically, these LE-PZQ worms were approximately five-fold less sensitive to PZQ than those from the parental LE strain. They were also less contracted by PZQ in vitro, exhibited decreased PZQ-induced tegumental damage, and unlike the parental worms, appeared to retain a functional excretory system following exposure to

PZQ [238]. This strategy of selecting for drug resistance at the snail stage is far less costly and labor intensive than earlier approaches that applied drug pressure at the intra-mammalian stage, and has the potential to generate new PZQ-insusceptible lines that could provide insight into the mechanisms underlying emergence and maintenance of PZQ resistance.

3.2 Mechanisms and Markers of Resistance to Praziquantel

In the absence of firm knowledge of the mode of action of PZQ (see Sect. 2.4 above), hypotheses about mechanisms of resistance to this drug are bound to be speculative, and no clear changes in candidate targets have been found to date [53]. It is important to note that thus far only a very limited number of putatively resistant and susceptible isolates have been compared with each other; extension of these comparative investigations is needed. The availability of isolates of *S. mansoni* from several different sources with confirmed differences in sensitivity to PZQ [233] should facilitate the search for the genetic and physiological mechanisms responsible for drug resistance. Furthermore, resistance to a drug can arise via several mechanisms other than target modification. For example, heritable changes that alter drug uptake/permeability, drug activation/metabolism, or drug efflux can all underlie resistance. Nevertheless, some genetically based differences between PZQ-resistant and sensitive isolates have been identified.

Genetic studies on worms with reduced sensitivity to PZQ suggest either dominant [239] or partially dominant [240] inheritance of the trait. An analysis using a subtractive polymerase chain reaction (PCR) indicated that adult worms of a laboratory-selected PZQ-resistant isolate were expressing subunit 1 of the mitochondrial enzyme cytochrome C-oxidase (SCOX-1) at a 5–10-fold higher rate than worms of the parental hybrid isolate from which the former was derived [241]. Unexpectedly, however, the actual activity of the enzyme was fourfold lower in the resistant worms.

Use of a random amplified polymorphic DNA (RAPD) PCR showed that there was differential amplification of at least two major DNA nucleotide sequences between an Egyptian PZQ-resistant isolate and several PZQ-sensitive isolates from the same endemic area [242]. It has not been established whether these differences might serve as markers for resistance or have any functional implications.

More recently, several groups have investigated whether genetic diversity of schistosomes is altered by drug pressure; reduction in such diversity could reflect, at least in part, selection of these parasites for decreased PZQ susceptibility [243]. Field studies on different populations have produced widely varying results, ranging from no change in genetic diversity following PZQ treatment [244–246] to significant loss of genetic diversity following a single round of preventative therapy with PZQ [243, 247]. Additionally, a group working in Brazil found that *S. mansoni* that persist following PZQ treatment have genotypes that do not differ significantly from susceptible worms, indicating that they do not represent a subpopulation selected for PZQ resistance [248]. In contrast, experimental *S. mansoni* infections selected for reduced PZQ susceptibility show decreased genetic diversity, increased endogamy, and an increased ratio of female-to-male worms, all of which correlate with the decrease in drug susceptibility [234].

More specifically, the discovery that a difference in the amino acid sequence of the β subunits of schistosome Ca_v channels of schistosomes and other platyhelminths may account for differential sensitivity to PZQ (see Sect. 2.4 above) stimulated an investigation to compare the sequence of these molecules in several PZQ-resistant and -sensitive isolates [249]. No meaningful differences were found in sequence or expression of cDNAs coding for either the conventional or variant schistosome Ca_v channel β subunit that could account for differences in PZQ sensitivity between different isolates or between PZQ-susceptible adult and PZQ-refractory juvenile worms. This negative evidence does not however disprove the hypothesis that β subunits of Ca_v channels may be involved in PZQ activity, since, as discussed above, drug insusceptibility can arise from several mechanisms other than modification of the drug's target.

Mechanisms that increase drug efflux have attracted more attention recently, with a focus on ABC multidrug transporters such as P-glycoprotein. ABC transporters are found in organisms from all living kingdoms. They bind and hydrolyze ATP and use the resultant energy to translocate a diversity of compounds, including drugs, across the membrane. Changes in expression or structure of these transporters are associated with multidrug resistance in a variety of organisms (reviewed in 250–252) and they have also been implicated in drug resistance in a variety of parasites, including helminths (reviewed in 253–259).

Fluorescent substrates of mammalian P-glycoprotein and other ABC transporters localize to the excretory system of schistosomes [260, 261], and PZQ dramatically disrupts the distribution of resorufin, a P-glycoprotein substrate, in PZQ-susceptible worms [164, 165]. As noted above (Sect. 3.1.2), the experimentally induced LE-PZQ PZQ-resistant isolate appears to be refractory to the PZQ-induced disruption of this localization [238]. Furthermore, exposure of schistosomes to sublethal concentrations of PZQ increases expression of P-glycoprotein (and another ABC transporter), and the EE2 PZQ-resistant isolate from Egypt shows dramatically increased expression of P-glycoprotein [262, 263]. Since PZQ is a likely substrate of (i.e., is transported by) schistosome P-glycoprotein [148], enhanced P-glycoprotein expression could in theory reduce effective intra-worm drug concentration. This hypothesis is supported by recent studies

showing that PZQ susceptibility of *S. mansoni* is enhanced in vitro by ABC transporter inhibitors or by knockdown of ABC transporter RNAs [264]. Interestingly, juvenile schistosomes express higher levels of ABC transporters than adults, and when exposed to PZQ in the presence of ABC transporter inhibitors, they are no longer refractory to PZQ [264]. Though these findings are consistent with a role for ABC transporters in modulating drug susceptibility, the question of their importance in emergence or maintenance of PZQ resistance awaits more definitive experiments.

Detection of PZQ resistance currently has to rely primarily on either in vivo tests on infected mice or in vitro tests on adult worms derived from infected mice. Performance of these tests is therefore constrained to laboratories with the capacity to maintain and passage life cycles and thus generally unsuitable for application in the field or clinic. More recently, however, several groups have found that the eggs and larvae (both miracidia and cercariae), as well as worms of resistant isolates, express phenotypic differences in terms of changes in survival or morphology that can be detected following exposure to PZQ in vitro, and in some cases have used these changes to assess PZQ insusceptibility [205, 221, 265, 266]. These assays could prove useful for detecting drug resistance in the field or clinic, but there is a need for markers that can be detected simply, for example, by PCR. Nonetheless, for the foreseeable future, it will likely be necessary that tests for detection of drug resistance be performed "centrally" in laboratories with appropriate facilities.

The immune-dependent action of PZQ and other schistosomicidal drugs has been noted (see Sect. 2.4 above). In the context of drug resistance mechanisms, isolates deemed to be less sensitive to PZQ have been found to suffer a lesser degree of damage to their surface membranes compared to susceptible isolates after exposure to the same dose of drug in vitro [214] and in vivo [267]. This factor may modulate the susceptibility of worms to immune attack in vivo, and thus confer a modicum of drug resistance that presently available in vivo assays for resistance would not be able to distinguish from, for example, the effects of a mutation in a specific drug receptor molecule. Indeed, PZQ-sensitive and -insensitive isolates from Egypt appear to induce different immune responses in mice [268]. Changes in antigens or antigen exposure cannot however alone account for PZQ resistance in *S. mansoni*, as worms from the laboratory-selected resistant isolates survive higher doses of PZQ in vitro than those of susceptible isolates [240].

As noted, immature schistosome worms are relatively "resistant" to schistosomicidal chemotherapy and, as discussed (see Sect. 3.1.1 above), it has been argued that the poor cure rates and treatment failures that have been observed in areas such as northern Senegal with high infection and transmission rates may reflect the presence of immature worms in the patients at the time they are treated, perhaps due to delayed maturation of these worms [207, 208]. This argument is supported by the higher cumulative cure rates that are achieved when two treatments are given a few weeks apart [120, 121]. However, a *S. mansoni* isolate collected in patent snails (not from treated patients) before much PZQ had been used in northern Senegal was found to have a decreased susceptibility to the drug [204]. Though this isolate did mature relatively slowly in mice, it nevertheless was still less susceptible to PZQ than a similarly slow-maturing isolate from Kenya [203]. Furthermore, as discussed (see Sect. 3.1.1 above), oxamniquine is a drug that, like PZQ, is relatively ineffective against immature worms in experimental infections [115], but when used at the normal dose rate in humans in Senegal it was more effective than PZQ [206].

4 Alternative Agents for Schistosomiasis

4.1 Oxamniquine

Oxamniquine provides some interesting contrasts with PZQ, particularly with respect to the factors that affected its market potential and the amount of information we have about its mechanisms of action and of schistosome resistance to it. It was synthesized by Pfizer in the late 1960s and initial laboratory studies in mice and primates indicated that it was effective against *S. mansoni* [269]. The first clinical trials were performed in Brazil, and these showed it was safe and effective against *S. mansoni* [270]. Through the late 1990s, oxamniquine was used as a first-line drug for the control of schistosomiasis mansoni in Brazil, where more than 12 million doses were administered as part of that country's "Special Program for Schistosomiasis Control" [271].

Oxamniquine is 6-hydroxymethyl-2-isopropyl aminomethyl-7-nitro-1,2,3,4,-tetrahydoquinolone. Its structure is similar to that of hycanthone, a related compound which also has activity against *S. mansoni*. Hycanthone had to be abandoned, however, because of suspected mutagenic, carcinogenic, and teratogenic activity [272]. Hycanthone has a 3-ring planar structure that is typical of DNA-intercalating agents, whereas oxamniquine has a simpler structure that has been shown to be devoid of intercalating activity [273].

In contrast to PZQ, oxamniquine is ineffective against *S. haematobium* and *S. japonicum*, the other two main schistosome species that infect humans (hycanthone is active against *S. mansoni* and *S. haematobium*, but not *S. japonicum*). Because of its limited spectrum of anti-parasitic activity, use of oxamniquine was almost entirely restricted to Brazil and other South American countries, which imposed severe restraints on its marketability. Unlike PZQ, the price of oxamniquine has therefore remained practically unchanged. Indeed, it has not been in general use since 2010, having been replaced by PZQ even in Brazil [51, 274], the

country that used oxamniquine for all its schistosome control activities throughout the 1980s and 1990s [271].

4.1.1 The Activity of Oxamniquine Against *S. mansoni*

Worms exposed to oxamniquine in vitro show no immediate adverse effects. Instead, depending on the dose, 2–7 days after an initial 30-min exposure followed by culture in normal medium, damage begins to be apparent, with death of the worms occurring some days later [52]. A similar delay in schistosomicidal effect occurs within the host [275]. However, only short times of exposure to oxamniquine are required to cause worm death: 15 min in vitro or 2 h in vivo before transfer, respectively, to drug-free medium or untreated animals [276]. Oxamniquine is more effective against male worms than against female worms by a ratio of approximately 2:1, but like PZQ and some other schistosomicides, it is largely ineffective against immature worms [115].

Nucleic acid synthesis is the first metabolic activity to be inhibited by oxamniquine; protein synthesis and all other metabolic pathways are affected only later [277]. Experiments with tritiated oxamniquine indicated that the drug formed stable covalent bonds with worm DNA. Worms from *S. mansoni* isolates that were resistant to oxamniquine failed to bind significant amounts of radioactive drug, as did *S. haematobium* and *S. japonicum* worms, species intrinsically insusceptible to oxamniquine [273].

4.1.2 Schistosome Oxamniquine Resistance and Oxamniquine Mode of Action

S. mansoni can become resistant to very high concentrations of oxamniquine (generally with concomitant cross-resistance to hycanthone (reviewed in 277)). Genetic crosses between sensitive and resistant *S. mansoni* isolates, achieved by transplanting single male and female worms of opposite genotypes to new mouse hosts and testing F1 and F2 progeny for resistance, clearly indicated that resistance to oxamniquine is an autosomal recessive character [278]. It was therefore hypothesized that oxamniquine (and hycanthone) per se is inactive as a schistosomicide, and that it requires activation by chemical transformation [279]. Oxamniquine-resistant schistosomes lack the factor necessary for activation of the drug, which was subsequently demonstrated to be a parasite sulfotransferase that biotransforms oxamniquine to its active form. Once activated, the drug acts as an alkylating agent of schistosome DNA and other macromolecules, interfering with nucleic acid synthesis [277]. This model also accounts for why the drug is not active against all schistosome species; those species that do not express this sulfotransferase activity cannot activate oxamniquine (or hycanthone) and are therefore not susceptible to it [280, 281].

Recent work has confirmed and extended this model for oxamniquine resistance and provided a detailed mechanism of action [51]. Using linkage mapping in *S. mansoni*, oxamniquine resistance mapped to a single quantitative trait locus (QTL). Subsequently, RNA interference and biochemical complementation studies identified a single sulfotransferase within that QTL (SmSULT-OR; Smp_089320) as the single causative gene. An independently derived oxamniquine-resistant field isolate also maps to this gene. The crystal structure of the *S. mansoni* sulfotransferase provides insights into the bases for oxamniquine action and resistance, and clues to differences in the drug-binding site in *S. haematobium* and *S. japonicum* that likely account for the lack of drug activity in these species. It also offers the opportunity for structure-based redesign of oxamniquine derivatives that exhibit activity against multiple schistosome species [51].

Table 47.2 summarizes the main differences between praziquantel and oxamniquine.

4.2 Artemisinin Derivatives

Artemisinin is the active ingredient of the sweet wood-worm *Artemisia annua*, a medicinal herb used in China. It is a sesquiterpene lactone which contains a peroxide bridge, from which synthetic derivatives such as artemether and artesunate have been synthesized. Artemisinins are potent antimalarial drugs and millions of doses have been administered for this purpose [282]. These drugs also have activity against other diseases, including schistosomiasis [283, 284]. Artemisinin activity against *S. japonicum* was discovered in the early 1980s [285] and effectiveness against other schistosome species confirmed subsequently [286]. These compounds are well tolerated and have only mild side effects. Their mode of action is not yet fully understood, though there is evidence for oxidative killing by glu-

Table 47.2 Comparison of praziquantel and oxamniquine

	Praziquantel	Oxamniquine
Effective against:	All schistosome species	*S. mansoni* alone
Mechanism of action:	Not known	Activation of prodrug by sulfotransferase; DNA alkylation
Potential to develop resistance:	Relatively low	Very high
Mechanism of resistance:	Not known	Lack of drug activation
Price and usage:	Inexpensive; extensive	Expensive; extremely limited

tathione depletion and lipid peroxidation [287, 288], effects on parasite glycolysis [289], and damage to the tegument and musculature of schistosomula, perhaps with schistosomicidal effects exerted through synergy with heme-containing compounds [290]. Formation of free radicals due to cleavage of an endoperoxide bridge in the structure of arteminisins is implicated in their mode of action [291], and synthetic endoperoxide-containing compounds have shown promise as drug leads that are effective in vivo against both adult and immature schistosomes [292]. Interestingly, unlike PZQ, the activity of artemisinins does not require T cell immunity and was found to be equally effective against *S. mansoni* in both athymic and immunocompetent mice [293].

In contrast to PZQ and oxamniquine, artemisinins are more active against immature than mature worms (reviewed in 56, 283, 284) and it is in this context that artemether and artesunate have been used effectively in China as "prophylactics" against *S. japonicum* infection during major floods [294]. Chemoprophylactic effectiveness has also been demonstrated against both *S. mansoni* [295] and *S. haematobium* [296]. Meta-analyses show that cure rates with artesunate alone are lower than with PZQ, but that artemisinins combined with PZQ are more effective than PZQ monotherapy [297, 298].

Although resistance to artemisinins in malaria has become a concern, particularly in Southeast Asia [299, 300], there are currently no examples of confirmed resistance to artemisinins in schistosomes. There has been one report of a decrease in *S. japonicum* sensitivity to artesunate after 10 years of use in China [301], though that conclusion has been challenged on methodological grounds [302]. Because of the poor cure rates given by PZQ in areas with high rates of infection transmission (which in turn may be due in part or wholly to the insensitivity of immature schistosomes to this drug) artemisinins may be of most use in these areas. Proposals for large-scale use of artemisinins in areas where *Plasmodium* spp. and schistosomes coexist, particularly in sub-Saharan Africa, have naturally raised concerns about creation of conditions for inducing drug resistance in the former [303].

4.3 Mefloquine

The quinoline-based drug mefloquine is another antimalarial drug that exhibits activity against schistosomes [304, 305]. It appears to be effective against all three major human schistosome species, is active against juveniles as well as adults, and shows synergistic effects when combined with PZQ or artemisinins in laboratory animals (reviewed in 42, 306), though apparently not when tested in human infections [307]. Mefloquine-related compounds have been evaluated for antischistosomal activity, with promising results [308].

4.4 Ro 15-5458

With the marketing of PZQ, an interesting schistosomicide discovered by Hoffmann La-Roche, Ro 15-5458 [10-(2-(diethylamino)ethyl)-9(10H)-acridanone(2-thiazolin-2-ylidene)hydrazone], was not further developed. Since the realization that resistance to PZQ could become a problem and discovery of novel schistosomicides is very difficult, there was some renewed interest in the development of Ro 15-5458 in the early 2000s.

Early reports of activity of Ro 15-5458 and 9-acridanone-hydrazone derivatives against *S. mansoni* were in primates [309, 310]. Ro 15-5458 killed almost all skin schistosomula in mice at 100 mg/kg and in Cebus monkeys it was fully effective at 25 mg/kg 7 days after infection [311]. Against adult worms in mice a dose of 20 mg/kg removed 95 % of *S. mansoni* and resulted in a disappearance of all immature stages in worm eggs [312]. If 4-week-old *S. haematobium* was treated in hamsters at 25 mg/kg, Ro 15-5458 was more effective than PZQ (total dose of 1000 mg/kg), but at 8 and 12 weeks cure rates were similar [313]. Combined one-third doses of Ro 15-5458 and PZQ gave a 99.4 % cure rate for *S. mansoni* in susceptible CD mice [314]. Since combination therapy is a well-known method of slowing the development of resistance, use of both products together would be beneficial, but costs and evidence for the beginning of PZQ resistance make it impractical. However, the activity of Ro 15-5458 against differing ages of schistosomes and at least two species suggests that if the toxicity is acceptable and manufacturing costs are cheap enough, it could provide a useful alternative to PZQ.

4.5 Meclonazepam (Ro 11-3128)

In the 1970s, more than 400 benzodiazepines were screened for antischistosomal activity at Hoffmann-La Roche and some members of the group turned out to be quite active [315]. Among them were the anticonvulsant clonazepam and its 3-methyl derivative designated Ro 11-3128, or meclonazepam.

Meclonazepam, given as a single oral dose of about 80 mg/kg, cured 90 % of mice or hamsters infected with *S. mansoni* or *S. haematobium*, while *S. japonicum* was completely refractory to treatment. A lower dose (25 mg/kg) was curative in monkeys. Notably, the drug was active against immature stages. Initial toxicology and mutagenicity trials proved that the drug is well tolerated in animals [315]. A clinical study in South Africa showed that a dose of 0.2–0.3 mg/kg was curative for most patients infected with either *S. mansoni* or *S. haematobium* [316]. Unfortunately, the drug causes a severe and long lasting sedation, accompanied by ataxia and muscle relaxation [317]. Due to these side effects,

further development of the drug was abandoned, but interest has been rekindled by efforts to develop derivatives that retain selective schistosomicidal activity with minimal side effects [318, 319].

The molecular target of meclonazepam remains an enigma, making efforts at elimination of these side effects through structural-functional approaches problematical. In mammals, meclonazepam is, like other benzodiazepines, an allosteric modulator of Cys-loop GABA channels [320, 321]. In contrast, the effects of meclonazepam do not appear to be mediated by *S. mansoni* benzodiazepam receptors [322]. Indeed, though schistosomes have high-affinity binding sites for benzodiazepines [323], the *S. mansoni* genome does not appear to contain any GABA channels, and the related glutamate-gated Cl^- channels that schistosomes do express are insensitive to meclonazepam [324]. Extensive early experimental work on meclonazepam [161] demonstrated that the drug has effects in vitro that are very similar to those of PZQ (spastic paralysis, influx of Ca^{2+}, tegumental vacuolization, and blebbing). Nonetheless, meclonazepam and PZQ do not appear to compete for the same binding sites in schistosomes [325].

4.6 Oxadiazoles

One of the more promising candidate targets for new antischistosomals is a unique antioxidant enzyme found in the parasite. In mammals, two enzymes, thioredoxin reductase and glutathione reductase, act to detoxify reactive oxygen species, but in schistosomes, these two functions are combined into a single enzyme, thioredoxin glutathione reductase (TGR). Oxadiazole-2 oxides inhibit TGR, and have been identified as promising lead compounds for schistosomiasis chemotherapy. They exhibit potency in vitro against *S. mansoni*, *S. japonicum*, and *S. haematobium*, and against juvenile and adult *S. mansoni* both in vitro and in experimental infections in mice [326].

4.7 Statins

Statins, blockbuster cholesterol-reducing drugs which inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR), have a negative impact on egg production and worm survival in mouse models of *S. mansoni* infection [327–329], and in vitro, on both schistosomula and adults [329–331]. The role of *S. mansoni* HMGR as a target of these drugs is supported by the lethal effects of RNAi knockdown of this enzyme in schistosomula, effects that are rescued by mevalonate in vitro, the product of HMGR [330]. Those schistosomula in which HMGR was suppressed by RNAi also showed significantly reduced survival compared to controls when transferred into mice [330]. Statins are approved, widely used compounds available off patent, and may hold particular promise for repurposing as antischistosomals.

5 Cross-Resistance and Spread of Resistance to Schistosomicides

So far, the only noteworthy instance of cross-resistance in schistosomicidal drugs is that between oxamniquine and hycanthone and it is most likely due to a structural similarity in the two drugs (see Sect. 4.1 above). However, in contrast to oxamniquine, development of hycanthone for treatment of schistosomiasis had to be abandoned because of its apparent carcinogenicity and mutagenic potential [52].

Several pieces of evidence indicate there is no cross-resistance between oxamniquine and PZQ. For example, the resistance to each of these drugs that was selectively bred into two respective lines of laboratory-maintained *S. mansoni* was drug-specific [232] and oxamniquine gave normally expected cure rates against schistosomiasis mansoni in an area in northern Senegal in which PZQ had given poor cure rates [206]. There also appears to be no cross-resistance between artemisinins and PZQ in *S. japonicum* [332, 333]. Additional evidence for an absence of cross-resistance is given by other clinical data [334, 335].

The evidence on PZQ accumulated so far only indicates that there is variation between schistosome isolates with respect to their sensitivity to the drug and that the degree of variation uncovered is small—no more than 3–5-fold differences in the ED50s of putatively resistant and sensitive control parasites. Fortunately as yet no case can be made for the occurrence of resistance against PZQ that is comparable with the levels of resistance that have developed against drugs for treatment of many bacterial or protozoan infections, or even against the antischistosomal drug oxamniquine. Indeed, as noted (see Sect. 3.1.1 above), there was no evidence for the presence, let alone expansion of Egyptian PZQ-insusceptible isolates characterized ten years previously, despite a decade of drug pressure [219]. Nonetheless, there seems little doubt that the mass drug administration programs currently in place in many parts of Africa and likely to be expanded will subject schistosomes to much higher levels of drug pressure than in the past. However, since oxamniquine has an entirely different mode of action from PZQ [51], does not show cross-resistance with PZQ in mice [203], and provides good cure rates in regions of Senegal with reduced PZQ efficacy [206], it may be worthwhile to "bring oxamniquine back" as a second-line drug for use when PZQ fails. Recent advances [51] may allow for newer, less expensive synthesis of oxamniquine, as well as derivatives that might be active against all species.

The contribution that offspring of worms that survive PZQ pressure will make to the genetic constitution of an

endemic population of schistosomes will depend on a number of factors, of which perhaps the two most important are the relative sizes of the "refugia" into which they are entering and their relative "biological fitness."

5.1 Refugia

Refugia are defined as subpopulations of parasites that are not selected by drug treatment [336], thereby providing a pool of susceptible genes that can dilute the resistant genes in the population [220, 336]. The concept of "refugia" has assumed importance in analysis of the dynamics of drug resistance in helminths of sheep and cattle [336–338], and has more recently attracted increasing attention in strategies for human mass drug administration programs [339]. Provided refugia populations remain large relative to the number of incoming offspring of drug-treated and uncured schistosomes, the impact of the latter on the genetic constitution of the population as a whole will be small. Large refugia are likely to be found in human populations living in areas of high infection intensity and prevalence, and that are subjected to chemotherapy only randomly or selectively. Similarly, infested environments in which intense transmission is occurring without interference from measures intended to control it (e.g., molluscicide) are likely to provide relatively large refugia.

Human populations subjected to mass-chemotherapy and/or endemic areas with low transmission rates will provide smaller refugia. As has been pointed out in regard to PZQ however [220], immature worms, which are refractory to the drug, can be considered a "natural" refugium, and several other factors (significant proportion of untreated individuals, short action of the drug) will tend to maintain large refugia. However, alternative scenarios that could enhance the impact of genetically drug-resistant organisms on the schistosome population as a whole can be envisaged: for example, if mass chemotherapy was performed at a time when an intermediate host snail population was reestablishing itself and was therefore largely uninfested, as might occur soon after flooding or application of molluscicide.

As with so much else with regard to our knowledge about PZQ, firm and consistent evidence about the relative biological fitness of putatively resistant and susceptible isolates is lacking. However, most evidence suggests that there is a biological cost to PZQ insusceptibility. Thus, as discussed (see Sect. 3.1.1, above) approximately half of the Egyptian isolates with reduced PZQ susceptibility that were further characterized [217] retained that trait in the absence of drug pressure, while others reverted to drug sensitivity that was no different from controls. Those isolates that retained the decreased drug sensitivity showed evidence of decreased cercarial production by infected snails, thus indicating there may be a cost of biological fitness in PZQ resistance. Similar results were reported for PZQ-insusceptible Kenyan isolates [221]. Furthermore, 10 years following recovery of the Egyptian isolates, there was no evidence for spread, or even existence, of PZQ resistance in the village in which the original Egyptian were originally found, despite a decade of continuing drug pressure [219].

On the other hand, when three *S. mansoni* lines that had been isolated from uncured Senegalese patients in the mid-1990s and subsequently passaged in laboratory mice without drug pressure for approximately 5–6 years were tested for susceptibility to PZQ they were found to be still less susceptible than several control isolates [218]. Similar to the Egyptian isolates, however, the three Senegalese isolates shed fewer cercariae per snail than other nonresistant, non-Senegalese isolates, though the snails infected with the Senegalese isolates survived longer [218].

6 Drugs for Liver Fluke Infections

As noted above (Sect. 2.2), *F. hepatica* does not appear to be susceptible to PZQ.

However, liver fluke infections can be treated with a number of fasciolicides (Table 47.3), some of which only kill adult flukes while others kill immature stages as well. An extensive review of this topic is available [340].

Given the importance of fasciolosis, it is surprising that there are no validated tests for resistance other than a controlled trial, that the molecular mechanisms of action remain unknown for all fasciolicides, and that the mechanisms of resistance, where they occur, are not completely understood. Recently, however, an egg-hatch assay for albendazole resistance has

Table 47.3 Fasciolicides licensed in the UK

Action group	Name	Effective against immatures?
Uncouplers	Closantel	Yes
	Nitroxynil	Yes
	Oxyclozanide	No
Tubulin inhibitors	Albendazole	No
	Ricobendazole	No
Unknown	Triclabendazole	Yes
Glycolysis inhibitor	Clorsulon	No

been reported [341, 342], and evidence is accruing that implicates altered P-glycoprotein expression or activity in reduced susceptibility to triclabendazole (see below).

6.1 Triclabendazole

The drug of choice against *F. hepatica* infections in both livestock and humans is triclabendazole [343–345], though it is not approved for use for ruminants or humans in the USA. A major advantage of triclabendazole is that it kills flukes at all intramammalian stages, from 1 week of age and older. Triclabendazole is structurally a benzimidazole, and there is some evidence that, like other benzimidazoles, it binds to β-tubulin and disrupts microtubule-based functions (reviewed in 343). On the other hand, triclabendazole does not act on nematodes, it is active against albendazole-resistant flukes [346], and albendazole is active against adult triclabendazole-resistant *F. hepatica* [347], indicating that the drugs have different mechanisms of action. Furthermore, since all other fasciolicides kill adult triclabendazole-resistant flukes, they must also have a different mode of action from triclabendazole [347, 348]. The exact mechanism of action of triclabendazole has not been established, but in common with some other fasciolicides one of the first events is disruption of the tegument as well as secretory activity. Thus, triclabendazole sulfoxide, the active sulfoxide metabolite of triclabendazole, disrupts the tegumental surface of adult and juvenile flukes in vitro [349], and adult [350] and juvenile [351, 352] parasites (*F. hepatica* or *F. gigantica*) recovered from triclabendazole-treated sheep and goats show surface disruption by 48 h post treatment. Triclabendazole sulfoxide also inhibits mitotic division of spermatogenic cells [353]. These effects are compatible with disruption of microtubule function that clearly does not occur at the same site as for other benzimidazoles; colchicine-binding data are contradictory [354, 355]. The presence in *F. hepatica* of multiple α- and β-tubulin isotypes, some of which diverge from other trematode and nematode tubulin sequences [356], may help account for some of these seemingly paradoxical observations. In addition, triclabendazole sulfoxide appears to inhibit protein synthesis [357], perhaps reflecting a general stress response.

Resistance to triclabendazole has been reported in livestock from several countries, including Australia, Ireland, the UK, The Netherlands, and Spain (reviewed in 343). In at least one of these isolates there is evidence that reduced triclabendazole susceptibility is associated with enhanced drug efflux via parasite P-glycoprotein [358, 359].

6.2 Uncouplers

Rafoxanide has been withdrawn from sale in many countries, but closantel, a structurally related salicylanilide, is widely used for control of both flukes and the nematode *Haemonchus contortus*. Both closantel and the halogenated phenol, nitroxynil, have activity against immature flukes. Oxyclozanide, another salicylanilide, only has activity against adult flukes at normal doses. As all are uncouplers of energy formation in mammalian mitochondria, it has been suggested that they have a similar mechanism of action in flukes. However, nitroxynil might be acting on ion permeability in muscle cells [360] rather than affecting mitochondria in what are largely anaerobic animals. Morphological and metabolic studies using the salicylanilides are compatible with effects on mitochondria [360]. There is cross-resistance between rafoxanide, closantel, and nitroxynil, but apparently not to oxyclozanide. The difference with oxyclozanide could be related to blood profiles with a very short persistence for oxyclozanide but long half-lives for closantel [340]. This is an area requiring further investigation as closantel and nitroxynil will become the major fasciolicides as resistance to triclabendazole spreads.

6.3 Benzimidazoles

Resistance has been selected in the laboratory to the experimental benzimidazole luxabendazole [340], and has recently been reported for albendazole [346]. Fluke eggs are prevented from embryonation by incubation in solutions of benzimidazoles [361] in a way similar to inhibition of embryonation in nematode eggs [362], suggesting a similar mechanism of action on β-tubulin. Albendazole-resistant isolates show increased resistance to suppression of egg hatching by albendazole [341, 342], though to date, no structural differences in *F. hepatica* tubulin genes have been reported for albendazole-resistant isolates.

6.4 Clorsulon

Present evidence suggests that clorsulon acts by inhibition of glycolysis leading to slow paralysis [340, 363, 364]. There is a report of an isolate that is resistant to clorsulon, and also albendazole [365], but little is known about possible resistance mechanisms.

7 Other Drugs for Tapeworms

7.1 Pyrantel

Pyrantel is used exclusively for the treatment of *Anoplocephala perfoliata* in horses where a double dose (38 mg/kg as pyrantel embonate) is effective [366] and is not used in other species for control of tapeworms. It is assumed that the drug affects nicotinic acetylcholine

receptors as in nematodes, but the action of levamisole and pyrantel resistance has not yet been elucidated precisely at the molecular level in parasitic nematodes [367, 368]. No resistance to pyrantel has been reported but a change in behavior is widespread, with worms attaching to the cecal wall rather than the ileocecal junction. It seems reasonable to assume that pyrantel concentrations are less in the cecum than in the lower small intestine, so this change in attachment site may represent an adaptation to under-dosing with pyrantel, which happens when the normal dose is used for treatment of nematodes in horses. In vitro tests suggest that the worms attaching to the cecal wall are not resistant [369].

7.2 Benzimidazoles

Benzimidazoles have been used successfully to treat various cestode infections. The mechanism of action is not known but they presumably act similarly as in nematodes, where the drugs bind to β-tubulin, preventing polymerization to form microtubules. Albendazole is used to treat neurocysticercosis, and may be superior to PZQ at usual doses [34]. Multiple doses of albendazole and mebendazole are used to treat hydatid cysts of *E. granulosus* and *E. multilocularis* (reviewed in 370, 371). Albendazole resistance in *E. granulosus* has been found in laboratory studies [372]. The detailed mechanism of resistance is not known, though recent evidence [373] suggests a point mutation in β-tubulin as occurs, for example, in *H. contortus* [374]. Benzimidazoles (e.g., fenbendazole, oxfendazole, and albendazole) are also used to treat *Moniezia expansa* infection in sheep [375]. Reduced drug activity has been reported in sheep in New Zealand [376, 377] and in South Africa (cited in 378) but the mechanism of resistance is again not known.

7.3 Nitroscanate

Nitroscanate is used to treat tapeworms in dogs and cats. The detailed mechanism of action is not known and no cases of resistance have been reported.

7.4 Niclosamide

Reduced activity may have developed in sheep in South Africa (cited in 378), and there is a report of insusceptibility in a human infection with the monkey tapeworm *Bertiella studeri* [379], but there are no further details of the exact mechanism of action of this uncoupler on cestodes, or of the mechanism of resistance.

7.5 Treatment and Control of Larval Tapeworms

7.5.1 *Taenia solium*

Mass treatment of people with PZQ at a dose of 10 mg/kg is recommended for reducing infection in both humans and pigs [380]. Mass chemotherapy with niclosamide (2 g per patient) has also been used to reduce prevalence [381]. Cysts in pigs are killed by treatment with a single oral dose of 30 mg/kg of oxfendazole [382], and combined mass treatment of people (5 mg/kg PZQ) and pigs (30 mg/kg oxfendazole) decreased infection pressure, but did not eliminate transmission [383]. Both albendazole (15 mg/kg/day in 2–3 divided doses for 28 days) and PZQ (50 mg/kg/day for 15 days) are used for treatment of neurocysticercosis, though albendazole is the current cysticidal drug of choice, as it appears to be more effective and better tolerated (reviewed in 384).

7.5.2 *Echinococcus granulosus*

Treatment options for cystic echinococcosis have recently been reviewed [370, 371, 385]. The cornerstone for treatment of hepatic cysts is albendazole administered in 10–15 mg/kg doses (usually 400 mg) twice daily. Mebendazole may also be used, but albendazole is generally preferred as it has greater activity in vitro and better absorption and bioavailability. There is some evidence that combination chemotherapy with albendazole plus PZQ may be more effective than monotherapy. However, as has been pointed out [371], there is a dearth of systematic controlled trials analyzing standardized benzimidazole treatment at different cyst stages or comparing this therapy with other options.

7.5.3 *Echinococcus multilocularis*

Treatment options for alveolar echinococcosis have also recently been reviewed [370, 371, 385, 386]. Treatment with long-term courses of benzimidazoles (mebendazole or albendazole) is essential. For patients with inoperable alveolar hydatid cysts, this means continuous lifetime treatment; a minimum of two years of treatment is recommended following surgical resection of cysts. Such treatment increases survival rates of patients to 80 % (vs. 6–25 % in historical controls). Benzimidazoles do not kill metacestodes, but instead inhibit proliferation. Anthelmintic treatment is not recommended before surgery [385]. A few compounds appear to have parasitostatic activity in vitro or show effectiveness in animal studies (e.g., nitazoxanide), but have either not been tested or have showed no efficacy in clinical studies (reviewed in 386). Interestingly, a recent report indicates that triclabendazole, already in clinical use for treatment of *F. hepatica* infections, shows parasitostatic, and perhaps parasitocidal activity against *E. multilocularis* metacestodes in vitro [387].

8 Concluding Remarks

For the foreseeable future, PZQ will continue to be the drug used overwhelmingly for treatment of trematode (except for fasciolosis) and cestode infections in humans, particularly schistosomiasis. The large reductions in its price that started in the 1990s have led to a much greater rate of usage under the auspices of national and multi-national control programs such as The Schistosomiasis Control Initiative. Indeed, as noted above, Merck recently pledged to provide a tenfold increase in its annual donation of PZQ tablets, to 250 million. There is currently much debate whether PZQ is destined to suffer the fate of becoming less useful because of drug resistance, as has been the case with very many other anti-infection drugs. Some of the recent discussion has been concerned with whether or not PZQ-resistant schistosomes already exist in the field. However, it is now at least clear that variation in susceptibility to PZQ does exist in *S. mansoni* [233]. Some isolates that have been exposed to PZQ either in the field or in the laboratory have a decreased drug sensitivity in comparison with isolates that have never been exposed to the drug, either because the latter were established before the advent of PZQ or because they came from patients that were later successfully treated. The ED_{50} differences are relatively small, but generally reproducible, and no "super-resistant" isolates have been encountered so far [213], even after continuous application of drug pressure in the laboratory (Doenhoff et al., unpublished results) and in the field [219].

The degree of variation in susceptibility found so far in *S. mansoni* may not pose a real problem for human chemotherapy, especially since the doses routinely employed in clinical practice—at least theoretically—eliminate the large majority of parasites. However, as the objectives of mass drug administration progress from reduction of incidence and pathology to elimination of transmission [388] or even disease eradication, these more ambitious goals may be difficult or even impossible to accomplish with PZQ alone [57, 389]. Notably, recent results suggest that, despite a clear improvement in the health of individuals, four years of mass PZQ administration of PZQ in central Kenya had no clear effect on reducing transmission or significantly impacting the schistosome population [390]. Clearly, continued monitoring seems necessary, particularly during the course of prolonged chemotherapy-based control programs, since we could be party to only the first step of an escalation to resistance. Even with a high cure rate and a 95 % reduction in egg counts in uncured patients, those remaining eggs are from worms that survived PZQ and may be carrying (and transmitting) a drug resistance trait [105]. Furthermore, much of the recent debate on resistance to PZQ has to a large extent obscured the fact that even when this drug is used according to recommended schedules, it often results in relatively low cure rates, for example, in Senegal [198, 201], Uganda [108–110], Niger [107], and Zimbabwe [106]. One obvious remedial strategy is to increase the dose; unfortunately that does not always appear to improve cure rates [90–92, 202], though it has helped in *S. mansoni* infections in young children in Uganda [79]. Adoption of protocols involving two successive closely spaced treatments with the same drug has increased efficacy in some cases [121–123], but not in others [108, 111, 124]; treatment of initial therapeutic failures with a different drug [207] may also be effective, but both of these strategies will of course be more expensive. Combination chemotherapy is currently not standardly used for schistosomiasis, but such an approach holds unrealized potential for enhancing treatment options and possibly reducing the likelihood of resistance [55, 70, 392].

Situations now unquestionably exist or will develop where schistosomiasis is not treatable very effectively with PZQ and this will most likely be in part due to the intrinsic limitations of the drug when dealing with recent infections. In spite of its enormous usefulness, PZQ is therefore not the perfect drug. Unfortunately, with the possible exception of artemisinin derivatives (yet to be developed into front-line antischistosome drugs), the last schistosomicides were introduced in the 1970s, and since then, little systematic attempt has been made to discover new drugs apart from a small investment recently made by WHO/TDR to promote compound screening [393]. Pharmaceutical companies have not been at all motivated to invest in research for antischistosomal drugs, since the prospects of economic returns are far from realistic. Nonetheless, the notion of repurposing drugs already in use, a strategy that avoids most of the bottlenecks and costs associated with new drug approval, has gained momentum recently, and several candidates—mefloquine, statins—are worthy of further investigation. Additionally, strategies that take advantage of lead compounds identified in other parasites (e.g., malaria) have provided candidates for antischistosomal therapeutics [394].

Similarly, the cost of developing new drugs for the control of fasciolosis of domestic animals is such that there is little interest in searching for new alternatives. Once resistance to both triclabendazole and closantel/nitroxynil emerges, only products for treating adult flukes will be available, which will restrict effective disease control. Before that situation develops, validated tests for resistance are urgently required so that meaningful management systems can be put in place to try to limit the development and spread of fasciolicide resistance. Similarly validated tests are required for the detection of resistance in adult tapeworms.

Due to lack of commercial interest, public institutions, international organizations, and charitable foundations will need to continue leading at least in the initial stages of drug discovery. It is also important that an effort is made to create and sustain research environments that will attract the best minds to tackle the neglected infectious diseases that affect overwhelming numbers of people and animals in resource-poor countries.

Acknowledgements R.M.G. was supported by NIH grants R21AI112713, R21AI106268, and R21AI100505, and by a Bill & Melinda Gates Foundation Grand Challenges Explorations award.

References

1. Crompton DWT. How much human helminthiasis is there in the world? J Parasitol. 1999;85:397–403.
2. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Investig. 2008;118:1311–21.
3. Fenwick A. The global burden of neglected tropical diseases. Public Health. 2012;126:233–6.
4. Charlier J, van der Voort M, Kenyon F, Skuce P, Vercruysse J. Chasing helminths and their economic impact on farmed ruminants. Trends Parasitol. 2014;30:361–7.
5. Khan MK, Sajid MS, Riaz H, Ahmad NE, He L, Shahzad M, Hussain A, Khan MN, Iqbal Z, Zhao J. The global burden of fasciolosis in domestic animals with an outlook on the contribution of new approaches for diagnosis and control. Parasitol Res. 2013;112:2421–30.
6. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. The Lancet. 2014;383:2253–64.
7. Chitsulo L, Engels D, Montresor A, Savioli L. The global status of schistosomiasis and its control. Acta Trop. 2000;77:41–51.
8. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis. 2006;6:411–25.
9. World Health Organization. Schistosomiasis: population requiring preventive chemotherapy and number of people treated in 2010. Wkly Epidemiol Rec. 2012;87:37–44.
10. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380:2163–96.
11. Southgate VR, Rollinson D, Tchuem Tchuente LA, Hagan P. Towards control of schistosomiasis in sub-Saharan Africa. J Helminthol. 2005;79:181–5.
12. Engels D, Chitsulo L, Montresor A, Savioli L. The global epidemiological situation of schistosomiasis and new approaches to control and research. Acta Trop. 2002;82:139–46.
13. Whitty CJ, Mabey DC, Armstrong M, Wright SG, Chiodini PL. Presentation and outcome of 1107 cases of schistosomiasis from Africa diagnosed in a non-endemic country. Trans R Soc Trop Med Hyg. 2000;94:531–4.
14. McKerrow JH, Salter J. Invasion of skin by *Schistosoma* cercariae. Trends Parasitol. 2002;18:193–5.
15. Curwen RS, Wilson RA. Invasion of skin by schistosome cercariae: some neglected facts. Trends Parasitol. 2002;19:63–6.
16. He YX, Salafsky B, Ramaswamy K. Comparison of skin invasion among three major species of *Schistosoma*. Trends Parasitol. 2005;21:201–3.
17. Beckmann S, Quack T, Burmeister C, Buro C, Long T, Dissous C, Grevelding CG. *Schistosoma mansoni*: signal transduction processes during the development of the reproductive organs. Parasitology. 2010;137:497–520.
18. LoVerde PT, Niles EG, Osman A, Wu W. *Schistosoma mansoni* male-female interactions. Can J Zool. 2004;82:357–74.
19. Kolorova L, Horak P, Skirnisson K, Mareckova H, Doenhoff M. Cercarial dermatitis, a neglected allergic disease. Clin Rev Allergy Immunol. 2013;45:63–74.
20. Warren KS, Mahmoud AA, Cummings P, Murphy DJ, Houser HB. *Schistosomiasis mansoni* in Yemeni in California: duration of infection, presence of disease, therapeutic management. Am J Trop Med Hyg. 1974;23:902–9.
21. Goddard MJ, Jordan P. On the longevity of *Schistosoma mansoni* in man on St. Lucia, West Indies. Trans R Soc Trop Med Hyg. 1980;74:185–91.
22. Chabasse D, Bertrand G, Leroux JP, Gauthey N, Hocquet P. Developmental bilharziasis caused by *Schistosoma mansoni* discovered 37 years after infestation. Bull Soc Pathol Exot Filiales. 1985;78:643–7.
23. Pearce EJ, Macdonald CA. The immunobiology of schistosomiasis. Nat Rev Immunol. 2002;2:499–511.
24. van der Werf MJ, de Vlas SJ, Brooker S, Looman CW, Nagelkerke NJ, Habbema JD, Engels D. Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. Acta Trop. 2003;86:125–39.
25. Savioli L, Albonico M, Engels D, Montresor A. Progress in the prevention and control of schistosomiasis and soil-transmitted helminthiasis. Parasitol Int. 2004;53:103–13.
26. King CH, Dangerfield-Cha M. The unacknowledged impact of chronic schistosomiasis. Chronic Illn. 2008;4:65–79.
27. Melhem RF, LoVerde PT. Mechanism of interaction of *Salmonella* and *Schistosoma* species. Infect Immun. 1984;44:274–81.
28. Ndeffo Mbah ML, Poolman EM, Atkins KE, Orenstein EW, Meyers LA, Townsend JP, Galvani AP. Potential cost-effectiveness of schistosomiasis treatment for reducing HIV transmission in Africa—the case of Zimbabwean women. PLoS Negl Trop Dis. 2013;7:e2346.
29. Tanaka H, Tsuji M. From discovery to eradication of schistosomiasis in Japan: 1847–1996. Int J Parasitol. 1997;27:1465–80.
30. Sturrock RF. Schistosomiasis epidemiology and control: how did we get here and where should we go? Mem Inst Oswaldo Cruz. 2001;96(Suppl):17–27.
31. Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ. Food-borne trematodiases in Southeast Asia epidemiology, pathology, clinical manifestation and control. Adv Parasitol. 2010;72:305–50.
32. Keiser J, Utzinger J. Food-borne trematodiases. Clin Microbiol Rev. 2009;22:466–83.
33. Carpio A. Neurocysticercosis: an update. Lancet Infect Dis. 2002;2:751–62.
34. Nash TE, Garcia HH. Diagnosis and treatment of neurocysticercosis. Nat Rev Neurol. 2011;7:584–94.
35. Ndimubanzi PC, Carabin H, Budke CM, Nguyen H, Qian YJ, Rainwater E, Dickey M, Reynolds S, Stoner JA. A systematic review of the frequency of neurocyticercosis with a focus on people with epilepsy. PLoS Negl Trop Dis. 2010;4:e870.
36. Brunetti E, White ACJ. Cestode infestations: hydatid disease and cysticercosis. Infect Dis Clin North Am. 2012;26:421–35.
37. Torgerson PR, Keller K, Magnotta M, Ragland N. The global burden of alveolar echinococcosis. PLoS Negl Trop Dis. 2010;4:e722.
38. Andrews P, Thomas H, Pohlke R, Seubert J. Praziquantel. Med Res Rev. 1983;3:147–200.
39. King CH, Olbrych SK, Soon M, Singer ME, Carter J, Colley DG. Utility of repeated praziquantel dosing in the treatment of schistosomiasis in high-risk communities in Africa: a systematic review. PLoS Negl Trop Dis. 2011;5:e1321.
40. Cioli D, Pica-Mattoccia L, Basso A, Guidi A. Schistosomiasis control: praziquantel forever? Mol Biochem Parasitol. 2014;195:23–9.
41. Fenwick A, Savioli L, Engels D, Robert Bergquist N, Todd MH. Drugs for the control of parasitic diseases: current status and development in schistosomiasis. Trends Parasitol. 2003;19:509–15.
42. Keiser J, Utzinger J. Antimalarials in the treatment of schistosomiasis. Curr Pharm Des. 2012;18:3531–8.
43. Toure S, Zhang Y, Bosque-Oliva E, Ky C, Ouedraogo A, Koukounari A, Gabrielli AF, Bertrand S, Webster JP, Fenwick

A. Two-year impact of single praziquantel treatment on infection in the national control programme on schistosomiasis in Burkina Faso. Bull World Health Organ. 2008;86:780–7.
44. Vennervald BJ, Booth M, Butterworth AE, Kariuki HC, Kadzo H, Ireri E, Amaganga C, Kimani G, Kenty L, Mwatha J, et al. Regression of hepatosplenomegaly in Kenyan school-aged children after praziquantel treatment and three years of greatly reduced exposure to *Schistosoma mansoni*. Trans R Soc Trop Med Hyg. 2005;99:150–60.
45. Xianyi C, Liying W, Jiming C, Xiaonong Z, Jiang Z, Jiagang G, Xiaohua W, Engels D, MInggang C. Schistosomiasis control in China: the impact of a 10-year World Bank Loan Project (1992-2001). Bull World Health Organ. 2005;83:43–8.
46. French MD, Churcher TS, Gambhir M, Fenwick A, Webster JP, Kabatereine NB, Basanez MG. Observed reductions in *Schistosoma mansoni* transmission from large-scale administration of praziquantel in Uganda: a mathematical modelling study. PLoS Negl Trop Dis. 2010;4:e897.
47. Fenwick A, Webster JP, Bosque-Oliva E, Blair L, Fleming FM, Zhang Y, Garba A, Stothard JR, Gabrielli AF, Clements AC, et al. The Schistosomiasis Control Initiative (SCI): rationale, development and implementation from 2002–2008. Parasitology. 2009;136:1719–30.
48. Barry MA, Simon GG, Mistry N, Hotez PJ. Global trends in neglected tropical disease control and elimination: impact on child health. Arch Dis Child. 2013;98:635–41.
49. World Health Organization. Schistosomiasis: number of people receiving preventive chemotherapy in 2012. Wkly Epidemiol Rec. 2014;89:21–8.
50. Geary TG. Are new anthelmintics needed to eliminate human helminthiases? Curr Opin Infect Dis. 2012;25:709–17.
51. Valentim CL, Cioli D, Chevalier FD, Cao X, Taylor AB, Holloway SP, Pica Mattoccia L, Guidi A, Basso A, Tsai IJ, et al. Genetic and molecular basis of drug resistance and species-specific drug action in schistosome parasites. Science. 2013;342:1385–9.
52. Cioli D, Pica-Mattoccia L, Archer S. Antischistosomal drugs: past, present…and future? Pharmacol Ther. 1995;68:35–85.
53. Greenberg RM. New approaches for understanding mechanisms of drug resistance in schistosomes. Parasitology. 2013;140:1534–46.
54. Doenhoff MJ, Cioli D, Utzinger J. Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Curr Opin Infect Dis. 2008;21:659–67.
55. Danso-Appiah A, Olliaro PL, Donegan S, Sinclair D, Utzinger J. Drugs for treating *Schistosoma mansoni* infection. Cochrane Database Syst Rev. 2013;2013:CD000528.
56. Thetiot-Laurent SA, Boissier J, Robert A, Meunier B. Schistosomiasis chemotherapy. Angew Chem. 2013;52:7936–56.
57. Rollinson D, Knopp S, Levitz S, Stothard JR, Tchuem Tchuente LA, Garba A, Mohammad KA, Schur N, Person B, Colley DG, et al. Time to set the agenda for schistosomiasis elimination. Acta Trop. 2013;128:423–40.
58. Keiser J, Utzinger J. The drugs we have and the drugs we need against major helminth infections. Adv Parasitol. 2010;73:197–230.
59. Webster JP, Molyneux DH, Hotez PJ, Fenwick A. The contribution of mass drug administration to global health: past, present and future. Philos Trans R Soc Lond Ser B Biol Sci. 2014;369:20130434.
60. Hotez P. Enlarging the "Audacious Goal": elimination of the world's high prevalence neglected tropical diseases. Vaccine. 2011;29:D104–10.
61. Groll E. Praziquantel. Adv Pharmacol Chemother. 1984;20:219–38.
62. Seubert J, Pohlke R, Loebich F. Synthesis and properties of praziquantel, a novel broad spectrum anthelmintic with excellent activity against schistosomes and cestodes. Experientia. 1977;33:1036–7.
63. Gonnert R, Andrews P. Praziquantel, a new broad-spectrum antischistosomal agent. Z Parasitenkd. 1977;52:129–50.
64. Thomas H, Gonnert R. The efficacy of praziquantel against cestodes in animals. Z Parasitenkd. 1977;52:117–27.
65. Leopold G, Ungethum W, Groll E, Diekmann HW, Nowak H, Wegner DH. Clinical pharmacology in normal volunteers of praziquantel, a new drug against schistosomes and cestodes. An example of a complex study covering both tolerance and pharmacokinetics. Eur J Clin Pharmacol. 1978;14:281–91.
66. Katz N, Rocha RS, Chaves A. Preliminary trials with praziquantel in human infections due to *Schistosoma mansoni*. Bull World Health Organ. 1979;57:781–5.
67. Davis A, Biles JE, Ulrich AM. Initial experiences with praziquantel in the treatment of human infections due to *Schistosoma haematobium*. Bull World Health Organ. 1979;57:773–9.
68. Ishizaki T, Kamo E, Boehme K. Double-blind studies of tolerance to praziquantel in Japanese patients with *Schistosoma japonicum* infections. Bull World Health Organ. 1979;57:787–91.
69. Santos AT, Blas BL, Nosenas JS, Portillo GP, Ortega OM, Hayashi M, Boehme K. Preliminary clinical trials with praziquantel in *Schistosoma japonicum* infections in the Philippines. Bull World Health Organ. 1979;57:793–9.
70. Kramer CV, Zhang F, Sinclair D, Olliaro PL. Drugs for treating urinary schistosomiasis. Cochrane Database Syst Rev. 2013;2013:CD000053.
71. Liu YH, Qian MX, Wang XG, Jia J, Wang QN, Jiang YF, Wang RQ, Yan SH, Chen BY, Li JS, et al. Comparative efficacy of praziquantel and its optic isomers in experimental therapy of schistosomiasis japonica in rabbits. Chinese Med J Peking. 1986;99:935–40.
72. Tanaka M, Ohmae H, Utsunomiya H, Nara T, Irie Y, Yasuraoka K. A comparison of the antischistosomal effect of levo- and dextro-praziquantel on *Schistosoma japonicum* and *S. mansoni* in mice. Am J Trop Med Hyg. 1989;41:198–203.
73. Xiao SH, Catto BA. Comparative *in vitro* and *in vivo* activity of racemic praziquantel and its levorotated isomer on *Schistosoma mansoni*. J Infect Dis. 1989;159:589–92.
74. Wu MH, Wei CC, Xu ZY, Yuan HC, Lian WN, Yang QJ, Chen M, Jiang QW, Wang CZ, Zhang SJ, et al. Comparison of the therapeutic efficacy and side effects of a single dose of levo-praziquantel with mixed isomer praziquantel in 278 cases of schistosomiasis japonica. Am J Trop Med Hyg. 1991;45:345–9.
75. Staudt U, Schmahl G, Blaschke G, Mehlhorn H. Light and scanning electron microscopy studies on the effects of the enantiomers of praziquantel and its main metabolite on *Schistosoma mansoni in vitro*. Parasitol Res. 1992;78:392–7.
76. Meister I, Ingram-Sieber K, Cowan N, Todd M, Robertson MN, Meli C, Patra M, Gasser G, Keiser J. Activity of praziquantel enantiomers and main metabolites against *Schistosoma mansoni*. Antimicrob Agents Chemother. 2014;58:5466–72.
77. Sharma LK, Cupit PM, Goronga T, Webb TR, Cunningham C. Design and synthesis of molecular probes for the determination of the target of the anthelmintic drug praziquantel. Bioorg Med Chem Lett. 2014;24:2469–72.
78. Meyer T, Sekljic H, Fuchs S, Bothe H, Schollmeyer D, Miculka C. Taste, a new incentive to switch to (R)-praziquantel in schistosomiasis treatment. PLoS Negl Trop Dis. 2009;3:e357.
79. Bustinduy AL, Waterhouse D, de Sousa-Figueiredo JC, Roberts SA, Atuhaire A, Van Dam GJ, Corstjens PL, Scott JT, Stanton MC, Kabatereine NB, Ward S, Hope WW, Stothard JR. Population pharmacokinetics and pharmacodynamics of praziquantel in Ugandan children with intestinal schistosomiasis: higher dosages are required for maximal efficacy. mBio. 2016;7:e00227–16.
80. Woelfle M, Seerden JP, de Gooijer J, Pouwer K, Olliaro P, Todd MH. Resolution of praziquantel. PLoS Negl Trop Dis. 2011;5:e1260.
81. Woelfle M, Olliaro P, Todd MH. Open science is a research accelerator. Nat Chem. 2011;3:745–8.

82. Doenhoff M, Kimani G, Cioli D. Praziquantel and the control of schistosomiasis. Parasitol Today. 2000;16:364–6.
83. Appleton CC, Mbaye A. Praziquantel—quality, dosages and markers of resistance. Trends Parasitol. 2001;17:356–7.
84. Sulaiman SM, Traore M, Engels D, Hagan P, Cioli D. Counterfeit praziquantel. Lancet. 2001;358:666–7.
85. Botros S, El-Lakkany N, Seif el-Din SH, Sabra AN, Ibrahim M. Comparative efficacy and bioavailability of different praziquantel brands. Exp Parasitol. 2011;127:515–21.
86. Hotez PJ, Engels D, Fenwick A, Savioli L. Africa is desperate for praziquantel. Lancet. 2010;376:496–8.
87. Stothard JR, Sousa-Figueiredo JC, Betson M, Bustinduy A, Reinhard-Rupp J. Schistosomiasis in African infants and preschool children: let them now be treated! Trends Parasitol. 2013;29:197–205.
88. Garba A, Lamine MS, Djibo A, Tahirou A, Aouami MA, Alfari A, Phillips AE, Fenwick A, Utzinger J. Safety and efficacy of praziquantel syrup (Epiquantel®) against Schistosoma haematobium and Schistosoma mansoni in preschool-aged children in Niger. Acta Trop. 2013;128:318–25.
89. Gilbert DN, Moellering RC, Eliopoulos GM. The Sanford guide to antimicrobial therapy 2012. 42nd ed. Sperryville: Antimicrobial Therapy; 2012.
90. Olliaro PL, Vaillant MT, Belizario VJ, Lwambo NJ, Ouldabdallahi M, Pieri OS, Amarillo ML, Kaatano GM, Mamadou D, Domingues AC, et al. A multicentre randomized controlled trial of the efficacy and safety of single-dose praziquantel at 40 mg/kg vs. 60 mg/kg for treating intestinal schistosomiasis in the Philippines, Mauritania, Tanzania and Brazil. PLoS Negl Trop Dis. 2011;5:e1165.
91. Ouldabdallahi M, Ousmane B, Ouldbezeid M, Mamadou D, Konate D, Chitsulo L. Comparison of the efficacy and safety of praziquantel administered in single dose of 40 versus 60 mg/kg for treating urinary schistosomiasis in Mauritania. Bull Soc Pathol Exot. 2013;106:167–9.
92. Belizario VY, Amarillo ML, Martinez RM, Mallari AO, Tai CMC. Efficacy and safety of 40 mg/kg and 60 mg/kg single doses of praziquantel in the treatment of schistosomiasis. J Pediatr Infect Dis. 2008;3:27–34.
93. Erfe JM, Belizario VY, Chua PL, Naig JR. Validating the WHO dose pole in the Philippines for school-based mass drug administration of praziquantel for morbidity control of schistosomiasis. Trans R Soc Trop Med Hyg. 2013;107:620–6.
94. Palha De Sousa CA, Brigham T, Chasekwa B, Mbuya MN, Tielsch JM, Humphrey JH, Prendergast AJ. Dosing of praziquantel by height in sub-Saharan African adults. Am J Trop Med Hyg. 2014;90:634–7.
95. Strandgaard H, Johansen MV, Montresor A, Ornbjerg N. Field testing of the WHO dose pole for administration of praziquantel in the treatment of opisthorchiasis in Lao PDR. Trans R Soc Trop Med Hyg. 2007;101:1120–3.
96. Olliero P, Delgado-Romero P, Keiser J. The little we know about the pharmacokinetics and pharmacodynamics of praziquantel (racemate and R-enantiomer). J Antimicrob Chemother. 2014;69:863–70.
97. Valencia CI, Catto BA, Webster LT, Barcelon E, Ofendo-Reyes R. Concentration time course of praziquantel in Filipinos with mild *Schistosoma japonicum* infection. Southeast Asian J Trop Med Public Health. 1994;25:409–14.
98. Mandour ME, el Turabi H, Homeida MM, el Sadiq T, Ali HM, Bennett JL, Leahey WJ, Harron DW. Pharmacokinetics of praziquantel in healthy volunteers and patients with schistosomiasis. Trans R Soc Trop Med Hyg. 1990;84:389–93.
99. Castro N, Medina R, Sotelo J, Jung H. Bioavailability of praziquantel increases with concomitant administration of food. Antimicrob Agents Chemother. 2000;44:2903–4.
100. Homeida M, Leahy W, Copeland S, Ali MM, Harron DW. Pharmacokinetic interaction between praziquantel and albendazole in Sudanese men. Ann Trop Med Parasitol. 1994;88:551–9.
101. Lopez-Gomez M, Castro N, Jung H, Sotelo J, Corona T. Optimization of the single-day praziquantel therapy for neurocysticercosis. Neurology. 2001;57:1929–30.
102. Webbe G, James C. A comparison of the susceptibility to praziquantel of *Schistosoma haematobium*, *S. japonicum*, *S. mansoni*, *S. intercalatum* and *S. mattheei* in hamsters. Z Parasitenkd. 1977;52:169–77.
103. Andrews P. Praziquantel: mechanisms of anti-schistosomal activity. Pharmacol Ther. 1985;29:129–56.
104. Wegner DH. The profile of the trematodicidal compound praziquantel. Arzneimittelforschung. 1984;34:1132–6.
105. Day TA, Botros S. Drug resistance in schistosomes. In: Maule A, Marks NJ, editors. Parasitic flatworms: molecular biology, biochemistry, immunology and physiology. Oxfordshire: CAB International; 2006. p. 256–68.
106. Mutapi F, Rujeni N, Bourke C, Mitchell K, Appleby L, Nausch N, Midzi N, Mduluza T. *Schistosoma haematobium* treatment in 1–5 year old children: safety and efficacy of the antihelminthic drug praziquantel. PLoS Negl Trop Dis. 2011;5:e1143.
107. Garba A, Lamine MS, Barkire N, Djibo A, Sofo B, Gouvras AN, Labbo R, Sebangou H, Webster JP, Fenwick A, et al. Efficacy and safety of two closely spaced doses of praziquantel against *Schistosoma haematobium* and *S. mansoni* and re-infection patterns in school-aged children in Niger. Acta Trop. 2013;128:334–44.
108. Sousa-Figueiredo JC, Betson M, Atuhaire A, Arinaitwe M, Navaratnam AM, Kabatereine NB, Bickle Q, Stothard JR. Performance and safety of praziquantel for treatment of intestinal schistosomiasis in infants and preschool children. PLoS Negl Trop Dis. 2012;6:e1864.
109. Tukahebwa E, Vennervald BJ, Nuwaha F, Kabatereine NB, Magnussen P. Comparative efficacy of one versus two doses of praziquantel on cure rate of *Schistosoma mansoni* infection and re-infection in Mayuge District, Uganda. Trans R Soc Trop Med Hyg. 2013;107:397–404.
110. Kabatereine NB, Kemijumbi J, Ouma JH, Sturrock RF, Butterworth AE, Madsen H, Ornbjerg N, Dunne DW, Vennnervald BJ. Efficacy and side effects of praziquantel treatment in a highly endemic *Schistosoma mansoni* focus at Lake Albert, Uganda. Trans R Soc Trop Med Hyg. 2003;97:599–603.
111. Kongs A, Marks G, Verle P, Van der Stuyft P. The unreliability of the Kato-Katz technique limits its usefulness for evaluating *S. mansoni* infections. Trop Med Int Health. 2008;6:163–9.
112. Lin DD, Liu JX, Liu YM, Hu F, Zhang YY, Xu JM, Li JY, Ji MJ, Bergquist R, Wu GL, et al. Routine Kato–Katz technique underestimates the prevalence of *Schistosoma japonicum*: a case study in an endemic area of the People's Republic of China. Parasitol Int. 2008;57:281–6.
113. de Vlas SJ, Gryseels B. Underestimation of *Schistosoma mansoni* prevalences. Parasitol Today. 1992;8:274–7.
114. Xiao SH, Catto BA, Webster Jr LT. Effects of praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo*. J Infect Dis. 1985;151:1130–7.
115. Sabah AA, Fletcher C, Webbe G, Doenhoff MJ. *Schistosoma mansoni*: chemotherapy of infections of different ages. Exp Parasitol. 1986;61:294–303.
116. Pica-Mattoccia L, Cioli D. Sex- and stage-related sensitivity of *Schistosoma mansoni* to *in vivo* and *in vitro* praziquantel treatment. Int J Parasitol. 2004;34:527–33.
117. Aragon AD, Imani RA, Blackburn VR, Cupit PM, Melman SD, Goronga T, Webb T, Loker ES, Cunningham C. Towards an understanding of the mechanism of action of praziquantel. Mol Biochem Parasitol. 2009;164:57–65.

118. Pica-Mattoccia L, Orsini T, Basso A, Festucci A, Liberti P, Guidi A, Marcatto-Maggi AL, Nobre-Santana S, Troiani AR, Cioli D, et al. *Schistosoma mansoni*: lack of correlation between praziquantel-induced intra-worm calcium influx and parasite death. Exp Parasitol. 2008;119:332–5.
119. Renganathan E, Cioli D. An international initiative on praziquantel use. Parasitol Today. 1998;14:390–1.
120. Picquet M, Vercruysse J, Shaw DJ, Diop M, Ly A. Efficacy of praziquantel against *Schistosoma mansoni* in northern Senegal. Trans R Soc Trop Med Hyg. 1998;92:90–3.
121. Utzinger J, N'Goran EK, N'Dri A, Lengeler C, Tanner M. Efficacy of praziquantel against *Schistosoma mansoni* with particular consideration for intensity of infection. Trop Med Int Health. 2000;5:771–8.
122. N'Goran EK, Gnaka HN, Tanner M, Utzinger J. Efficacy and side-effects of two praziquantel treatments against *Schistosoma haematobium* infection, among schoolchildren from Cote d'Ivoire. Ann Trop Med Parasitol. 2003;97:37–51.
123. Tchuem Tchuente LA, Southgate VR, Mbaye A, Engels D, Gryseels B. The efficacy of praziquantel against *Schistosoma mansoni* infection in Ndombo, northern Senegal. Trans R Soc Trop Med Hyg. 2001;95:65–6.
124. Chai J-Y. Praziquantel treatment in trematode and cestode infections: an update. Infect Chemother. 2013;45:32–43.
125. Keiser J, Utzinger J. Chemotherapy for major food-borne trematodes: a review. Expert Opin Pharmacother. 2004;5:1711–26.
126. Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J. Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) *in vitro*. Z Parasitenkd. 1980;63:113–28.
127. Schubert S, Phetsouvanh R. Praziquantel is mostly ineffective: treatment of *Fasciola hepatica* infection (large liver fluke). Z Arztl Fortbild. 1990;84:705–7.
128. Price TA, Tuazon CU, Simon GL. Fascioliasis: case reports and review. Clin Infect Dis. 1993;17:426–30.
129. Patrick DM, Isaac-Renton J. Praziquantel failure in the treatment of *Fasciola hepatica*. Can J Infect Dis. 1992;3:33–6.
130. Farag HF, Ragab M, Salem A, Sadek N. A short note on praziquantel in human fascioliasis. J Trop Med Hyg. 1986;89:79–80.
131. Ishii Y, Nakamura-Uchiyama F, Nawa Y. A praziquantel-ineffective fascioliasis case successfully treated with triclabendazole. Parasitol Int. 2002;51:205–9.
132. Schiappacasse RH, Mohammadi D, Christie AJ. Successful treatment of severe infection with *Fasciola hepatica* with praziquantel. J Infect Dis. 1985;152:1339–40.
133. Lateef M, Zargar SA, Khan AR, Nazir M, Shoukat A. Successful treatment of niclosamide- and praziquantel-resistant beef tapeworm infection with nitazoxanide. Int J Infect Dis. 2008;12:80–2.
134. Ronketti F, Ramana AV, Chao-Ming X, Pica-Mattoccia L, Cioli D, Todd MH. Praziquantel derivatives I: modification of the aromatic ring. Bioorg Med Chem Lett. 2007;17:4154–7.
135. Dong YX, Chollet J, Vargas M, Mansour NR, Bickle Q, Alnouti Y, Huang JG, Keiser J, Vennerstrom JL. Praziquantel analogs with activity against juvenile *Schistosoma mansoni*. Bioorg Med Chem Lett. 2010;20:2481–4.
136. Sadhu PS, Kumar SN, Chandrasekharam M, Pica-Mattoccia L, Cioli D, Rao VJ. Synthesis of new praziquantel analogues: potential candidates for the treatment of schistosomiasis. Bioorg Med Chem Lett. 2012;22:1103–6.
137. Liu H, William S, Herdtweck E, Botros S, Domling A. MCR synthesis of praziquantel derivatives. Chem Biol Drug Des. 2012;79:470–7.
138. Steiner K, Garbe A. The fate of praziquantel in the organism II. Distribution in rats. Eur J Drug Metab Pharmacokinet. 1976;1:97–106.
139. Spina-Franca A, Machado LR, Nobrega LPS, Livramento JA, Diekmann HW, Groll E, de Rezende GL. Praziquantel in the cerebrospinal fluid in neurocysticercosis. Arq Neuro-Psiquiatr. 1985;43:243–59.
140. Gonzalez-Esquivel D, Rivera J, Castro N, Yepez-Mulia L, Jung Cook H. *In vitro* characterization of some biopharmaceutical properties of praziquantel. Int J Pharm. 2005;295:93–9.
141. Steiner K, Garbe A, Diekmann HW, Nowak H. The fate of praziquantel in the organism I. Pharmacokinetics in animals. Eur J Drug Metab Pharmacokinet. 1976;1:85–95.
142. Castro N, Jung H, Medina R, Gonzalez-Esquivel D, Lopez M, Sotelo J. Interaction between grapefruit juice and praziquantel in humans. Antimicrob Agents Chemother. 2002;46:1614–6.
143. Jung H, Medina R, Castro N, Corona T, Sotelo J. Pharmacokinetic study of praziquantel administered alone and in combination with cimetidine in a single-day therapeutic regimen. Antimicrob Agents Chemother. 1997;41:1256–9.
144. el Guiniady MA, el Touny MA, Abdel-Bary MA, Abdel-Fatah SA, Metwally A. Clinical and pharmacokinetic study of praziquantel in Egyptian schistosomiasis patients with and without liver cell failure. Am J Trop Med Hyg. 1994;51:809–18.
145. Huang J, Bathena SP, Alnouti Y. Metabolite profiling of praziquantel and its analogs during the analysis of *in vitro* metabolic stability using information-dependent acquisition on a hybrid triple quadrupole linear ion trap mass spectrometer. Drug Metab Pharmacokinet. 2010;25:487–99.
146. Vokřál I, Jirásko R, Jedličková V, Bártíková H, Skálová L, Lamka J, Holčapek M, Szotáková B. The inability of tapeworm *Hymenolepis diminuta* and fluke *Dicrocoelium dendriticum* to metabolize praziquantel. Vet Parasitol. 2012;185:168–74.
147. Hayeshi R, Masimirembwa C, Mukanganyama S, Ungell AL. The potential inhibitory effect of antiparasitic drugs and natural products on P-glycoprotein mediated efflux. Eur J Pharm Sci. 2006;29:70–81.
148. Kasinathan RS, Goronga T, Messerli SM, Webb TR, Greenberg RM. Modulation of a *Schistosoma mansoni* multidrug transporter by the antischistosomal drug praziquantel. FASEB J. 2010;24:128–35.
149. Frohberg H. Results of toxicological studies on praziquantel. Arzneimittelforschung. 1984;34:1137–44.
150. Montero R, Ostrosky P. Genotoxic activity of praziquantel. Mutat Res. 1997;387:123–39.
151. Bassily S, Farid Z, Dunn M, el-Masry NA, Stek M. Praziquantel for treatment of schistosomiasis in patients with advanced hepatosplenomegaly. Ann Trop Med Parasitol. 1985;79:629–34.
152. Allen HE, Crompton DW, de Silva N, LoVerde PT, Olds GR. New policies for using anthelmintics in high risk groups. Trends Parasitol. 2002;18:381–2.
153. Friedman JF, Acosta LP, Baltazar P, Tallo V, McDonald EA, Wu H, Pond-Tor S, Watson NL, Kurtis JD, Olveda RM. The impact of praziquantel given at 12–16 weeks gestation on pregnancy outcomes: results of double blind, placebo controlled trial. Am J Trop Med Hyg. 2014;91:A56.
154. Polderman AM, Gryseels B, Gerold JL, Mpamila K, Manshande JP. Side effects of praziquantel in the treatment of *Schistosoma mansoni* in Maniema, Zaire. Trans R Soc Trop Med Hyg. 1984;78:752–4.
155. Greenberg RM. Are Ca^{2+} channels targets of praziquantel action? Int J Parasitol. 2005;35:1–9.
156. Chan JD, Agbedanu PN, Zamamian M, Gruba SM, Haynes CL, Day TA, Marchant JS. 'Death and axes': unexpected Ca^{2+} entry phenologs predict new anti-schistosomal agents. PLoS Pathog. 2014;10:e1003942.
157. Chan JD, Zarowiecki M, Marchant JS. Ca^{2+} channels and praziquantel: a view from the free world. Parasitol Int. 2013;62:619–28.

158. Day TA, Bennett JL, Pax RA. Praziquantel: the enigmatic antiparasitic. Parasitol Today. 1992;8:342–4.
159. Greenberg RM. Ca^{2+} signalling, voltage-gated Ca^{2+} channels and praziquantel in flatworm neuromusculature. Parasitology. 2005;131(Suppl):S97–108.
160. Redman CA, Robertson A, Fallon PG, Modha J, Kusel JR, Doenhoff MJ, Martin RJ. Praziquantel: an urgent and exciting challenge. Parasitol Today. 1996;12:14–20.
161. Pax R, Bennett JL, Fetterer R. A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. Naunyn Schmiedebergs Arch Pharmacol. 1978;304:309–15.
162. Cunha VM, Noel F. Praziquantel has no direct effect on $(Na^{+}+K^{+})$-ATPases and $(Ca^{2+}$-$Mg^{2+})$-ATPases of *Schistosoma mansoni*. Life Sci. 1997;60:289–94.
163. Nechay BR, Hillman GR, Dotson MJ. Properties and drug sensitivity of adenosine triphosphatases from *Schistosoma mansoni*. J Parasitol. 1980;66:596–600.
164. Kusel JR, Oliveira FA, Todd M, Ronketti F, Lima SF, Mattos AC, Reis KT, Coelho PM, Thornhill JA, Ribeiro F. The effects of drugs, ions, and poly-l-lysine on the excretory system of *Schistosoma mansoni*. Mem Inst Oswaldo Cruz. 2006;101:293–8.
165. Oliveira FA, Kusel JR, Ribeiro F, Coelho PM. Responses of the surface membrane and excretory system of *Schistosoma mansoni* to damage and to treatment with praziquantel and other biomolecules. Parasitology. 2006;132:321–30.
166. Mehlhorn H, Becker B, Andrews P, Thomas H, Frenkel JK. *In vivo* and *in vitro* experiments on the effects of praziquantel on *Schistosoma mansoni*: a light and electron microscopic study. Arzneimittelforschung. 1981;31:544–54.
167. Bricker CS, Depenbusch JW, Bennett JL, Thompson DP. The relationship between tegumental disruption and muscle contraction in *Schistosoma mansoni* exposed to various compounds. Z Parasitenkd. 1983;69:61–71.
168. Xiao SH, Friedman PA, Catto BA, Webster Jr LT. Praziquantel-induced vesicle formation in the tegument of male *Schistosoma mansoni* is calcium dependent. J Parasitol. 1984;70:177–9.
169. Harnett W, Kusel JR. Increased exposure of parasite antigens at the surface of adult male *Schistosoma mansoni* exposed to praziquantel *in vitro*. Parasitology. 1986;93:401–5.
170. Brindley PJ, Sher A. The chemotherapeutic effect of praziquantel against *Schistosoma mansoni* is dependent on host antibody response. J Immunol. 1987;139:215–20.
171. Doenhoff MJ, Sabah AA, Fletcher C, Webbe G, Bain J. Evidence for an immune-dependent action of praziquantel on *Schistosoma mansoni* in mice. Trans R Soc Trop Med Hyg. 1987;81:947–51.
172. Brindley PJ. Relationships between chemotherapy and immunity in schistosomiasis. Adv Parasitol. 1994;34:133–61.
173. Sabah AA, Fletcher C, Webbe G, Doenhoff MJ. *Schistosoma mansoni*: reduced efficacy of chemotherapy in infected T-cell-deprived mice. Exp Parasitol. 1985;60:348–54.
174. Doenhoff MJ, Modha J, Lambertucci JR. Anti-schistosome chemotherapy enhanced by antibodies specific for a parasite esterase. Immunology. 1988;65:507–10.
175. Kallestrup P, Zinyama R, Gomo E, Butterworth AE, van Dam GJ, Gerstoft J, Erikstrup C, Ullum H. Schistosomiasis and HIV in rural Zimbabwe: efficacy of treatment of schistosomiasis in individuals with HIV coinfection. Clin Infect Dis. 2006;42:1781–90.
176. Karanja DM, Boyer AE, Strand M, Colley DG, Nahlen BL, Ouma JH, Secor WE. Studies on schistosomiasis in western Kenya: II. Efficacy of praziquantel for treatment of schistosomiasis in persons coinfected with human immunodeficiency virus-1. Am J Trop Med Hyg. 1998;59:307–11.
177. Mwanakasale V, Vounatsou P, Sukwa TY, Ziba M, Ernest A, Tanner M. Interactions between *Schistosoma haematobium* and human immunodeficiency virus type 1: the effects of coinfection on treatment outcomes in rural Zambia. Am J Trop Med Hyg. 2003;69:420–8.
178. Harder A, Goossens J, Andrews P. Influence of praziquantel and Ca^{2+} on the bilayer-isotropic-hexagonal transition of model membranes. Mol Biochem Parasitol. 1988;29:55–9.
179. Schepers H, Brasseur R, Goormaghtigh E, Duquenoy P, Ruysschaert JM. Mode of insertion of praziquantel and derivatives into lipid membranes. Biochem Pharmacol. 1988;37:1615–23.
180. Lima SF, Vieira LQ, Harder A, Kusel JR. Effects of culture and praziquantel on membrane fluidity parameters of adult *Schistosoma mansoni*. Parasitology. 1994;109:57–64.
181. Angelucci F, Miele AE, Boumis G, Dimastrogiovanni D, Bellelli A. Macromolecular bases of antischistosomal therapy. Curr Top Med Chem. 2011;11:2012–28.
182. McTigue MA, Williams DR, Tainer JA. Crystal structures of a schistosomal drug and vaccine target: glutathione S-transferase from *Schistosoma japonica* and its complex with the leading antischistosomal drug praziquantel. J Mol Biol. 1995;246:21–7.
183. Milhon JL, Thiboldeaux RL, Glowak K, Tracy JW. *Schistosoma japonicum* GSH S-transferase Sj26 is not the molecular target of praziquantel action. Exp Parasitol. 1997;87:268–74.
184. Angelucci F, Basso A, Bellelli A, Brunori M, Pica Mattoccia L, Valle C. The anti-schistosomal drug praziquantel is an adenosine antagonist. Parasitology. 2007;134:1215–21.
185. Wiest PM, Li Y, Olds GR, Bowen WD. Inhibition of phosphoinositide turnover by praziquantel in *Schistosoma mansoni*. J Parasitol. 1992;78:753–5.
186. Gnanasekar M, Salunkhe AM, Mallia AK, He YX, Kalyanasundaram R. Praziquantel affects the regulatory myosin light chain of *Schistosoma mansoni*. Antimicrob Agents Chemother. 2009;53:1054–60.
187. Tallima H, El Ridi R. Praziquantel binds *Schistosoma mansoni* adult worm actin. Int J Antimicrob Agents. 2007;29:570–5.
188. Troiani AR, Pica-Mattoccia L, Valle C, Cioli D, Mignogna G, Ronketti F, Todd M. Is actin the praziquantel receptor? Int J Antimicrob Agents. 2007;30:280–1.
189. Jeziorski MC, Greenberg RM. Voltage-gated calcium channel subunits from platyhelminths: potential role in praziquantel action. Int J Parasitol. 2006;36:625–32.
190. Cho PY, Yoo WG, Kim TI, Ahn SK, Cho SH, Kim TS, Hong SJ. Molecular characterization of voltage-gated calcium channel β-subunits of *Clonorchis sinensis*. Parasitol Res. 2014;113:121–9.
191. Nogi T, Zhang D, Chan JD, Marchant JS. A novel biological activity of praziquantel requiring voltage-operated Ca^{2+} channel β subunits: subversion of flatworm regenerative polarity. PLoS Negl Trop Dis. 2009;3:e464.
192. Pica-Mattoccia L, Valle C, Basso A, Troiani AR, Vigorosi F, Liberti P, Festucci A, Cioli D. Cytochalasin D abolishes the schistosomicidal activity of praziquantel. Exp Parasitol. 2007;115:344–51.
193. Silva-Moraes V, Couto FF, Vasconcelos MM, Araujo N, Coelho PM, Katz N, Grenfell RF. Antischistosomal activity of a calcium channel antagonist on schistosomula and adult *Schistosoma mansoni* worms. Mem Inst Oswaldo Cruz. 2013;108:600–4.
194. Novozhilova E, Kimber MJ, Qian H, McVeigh P, Robertson AP, Zamanian M, Maule AG, Day TA. FMRFamide-like peptides (FLPs) enhance voltage-gated calcium currents to elicit muscle contraction in the human parasite *Schistosoma mansoni*. PLoS Negl Trop Dis. 2010;4:e790.
195. Blair KL, Anderson PAV. Physiology and pharmacology of turbellarian neuromuscular systems. Parasitology. 1996;113:S73–82.
196. Blair KL, Anderson PAV. Physiological and pharmacological properties of muscle cells isolated from the flatworm *Bdelloura candida* (Tricladia). Parasitology. 1994;109:325–35.
197. Blair KL, Anderson PAV. Properties of voltage-activated ionic currents in cells from the brains of the triclad flatworm *Bdelloura candida*. J Exp Biol. 1993;185:267–86.

198. Stelma FF, Talla I, Polman K, Niang M, Sturrock RF, Deelder AM, Gryseels B. Epidemiology of *Schistosoma mansoni* infection in a recently exposed community in northern Senegal. Am J Trop Med Hyg. 1993;49:701–6.
199. Southgate VR. Schistosomiasis in the Senegal River Basin: before and after the construction of the dams at Diama, Senegal and Manantali, Mali and future prospects. J Helminthol. 1997;71:125–32.
200. Gryseels B, Stelma FF, Talla I, van Dam GJ, Polman K, Sow S, Diaw M, Sturrock RF, Doehring-Schwerdtfeger E, Kardorff R, et al. Epidemiology, immunology and chemotherapy of *Schistosoma mansoni* infections in a recently exposed community in Senegal. Trop Geogr Med. 1994;46:209–19.
201. Stelma FF, Talla I, Sow S, Kongs A, Niang M, Polman K, Deelder AM, Gryseels B. Efficacy and side effects of praziquantel in an epidemic focus of *Schistosoma mansoni*. Am J Trop Med Hyg. 1995;53:167–70.
202. Guisse F, Polman K, Stelma FF, Mbaye A, Talla I, Niang M, Deelder AM, Ndir O, Gryseels B. Therapeutic evaluation of two different dose regimens of praziquantel in a recent *Schistosoma mansoni* focus in Northern Senegal. Am J Trop Med Hyg. 1997;56:511–4.
203. Fallon PG, Mubarak JS, Fookes RE, Niang M, Butterworth AE, Sturrock RF, Doenhoff MJ. *Schistosoma mansoni*: maturation rate and drug susceptibility of different geographic isolates. Exp Parasitol. 1997;86:29–36.
204. Fallon PG, Sturrock RF, Niang AC, Doenhoff MJ. Short report: diminished susceptibility to praziquantel in a Senegal isolate of *Schistosoma mansoni*. Am J Trop Med Hyg. 1995;53:61–2.
205. Liang YS, Coles GC, Doenhoff MJ, Southgate VR. *In vitro* responses of praziquantel-resistant and -susceptible *Schistosoma mansoni* to praziquantel. Int J Parasitol. 2001;31:1227–35.
206. Stelma FF, Sall S, Daff B, Sow S, Niang M, Gryseels B. Oxamniquine cures *Schistosoma mansoni* infection in a focus in which cure rates with praziquantel are unusually low. J Infect Dis. 1997;176:304–7.
207. Gryseels B, Mbaye A, De Vlas SJ, Stelma FF, Guisse F, Van Lieshout L, Faye D, Diop M, Ly A, Tchuem-Tchuente LA, et al. Are poor responses to praziquantel for the treatment of *Schistosoma mansoni* infections in Senegal due to resistance? An overview of the evidence. Trop Med Int Health. 2001;6:864–73.
208. Fallon PG. Schistosome resistance to praziquantel. Drug Resist Updat. 1998;1:236–41.
209. Danso-Appiah A, De Vlas SJ. Interpreting low praziquantel cure rates of *Schistosoma mansoni* infections in Senegal. Trends Parasitol. 2002;18:125–9.
210. El Khoby T, Galal N, Fenwick A. The USAID/Government of Egypt's Schistosomiasis Research Project (SRP). Parasitol Today. 1998;14:92–6.
211. Ismail M, Metwally A, Farghaly A, Bruce J, Tao LF, Bennett JL. Characterization of isolates of *Schistosoma mansoni* from Egyptian villagers that tolerate high doses of praziquantel. Am J Trop Med Hyg. 1996;55:214–8.
212. Ismail M, Botros S, Metwally A, William S, Farghally A, Tao LF, Day TA, Bennett JL. Resistance to praziquantel: direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers. Am J Trop Med Hyg. 1999;60:932–5.
213. Cioli D, Pica-Mattoccia L. Current and future antischistosomal drugs. In: Secor WE, Colley DG, editors. Schistosomiasis, World class parasites, vol. 10. New York: Springer; 2005. p. 191–206.
214. William S, Botros S, Ismail M, Farghally A, Day TA, Bennett JL. Praziquantel-induced tegumental damage in vitro is diminished in schistosomes derived from praziquantel-resistant infections. Parasitology. 2001;122(Pt 1):63–6.
215. William S, Botros S. Validation of sensitivity to praziquantel using *Schistosoma mansoni* worm muscle tension and Ca^{2+}-uptake as possible in vitro correlates to in vivo ED50 determination. Int J Parasitol. 2004;34:971–7.
216. Sabra AN, Botros SS. Response of *Schistosoma mansoni* isolates having different drug sensitivity to praziquantel over several life cycle passages with and without therapeutic pressure. J Parasitol. 2008;94:537–41.
217. William S, Sabra A, Ramzy F, Mousa M, Demerdash Z, Bennett JL, Day TA, Botros S. Stability and reproductive fitness of *Schistosoma mansoni* isolates with decreased sensitivity to praziquantel. Int J Parasitol. 2001;31:1093–100.
218. Liang YS, Coles GC, Dai JR, Zhu YC, Doenhoff MJ. Biological characteristics of praziquantel-resistant and -susceptible isolates of *Schistosoma mansoni*. Ann Trop Med Parasitol. 2001;95:715–23.
219. Botros S, Sayed H, Amer N, El-Ghannam M, Bennett JL, Day TA. Current status of sensitivity to praziquantel in a focus of potential drug resistance in Egypt. Int J Parasitol. 2005;35:787–91.
220. Webster JP, Gower CM, Norton AJ. Evolutionary concepts in predicting and evaluating the impact of mass chemotherapy schistosomiasis control programmes on parasites and their hosts. Evol Appl. 2008;1:66–83.
221. Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, Wynn NB, Mutuku MW, Karanja DM, Colley DG, Black CL, et al. Reduced susceptibility to praziquantel among naturally occurring Kenyan isolates of *Schistosoma mansoni*. PLoS Negl Trop Dis. 2009;3:e504.
222. Wang W, Wang L, Liang YS. Susceptibility or resistance of praziquantel in human schistosomiasis: a review. Parasitol Res. 2012;111:1871–7.
223. Wang W, Dai JR, Li HJ, Shen XH, Liang YS. The sensitivity of *Schistosoma japonicum* to praziquantel: a field evaluation in areas with low endemicity of China. Am J Trop Med Hyg. 2012;86:834–6.
224. Wang W, Dai JR, Li HJ, Shen XH, Liang YS. Is there reduced susceptibility to praziquantel in *Schistosoma japonicum*? Evidence from China. Parasitology. 2010;137:1905–12.
225. Yu DB, Li Y, Sleigh AC, Yu XL, Li YS, Wei WY, Liang YS, McManus DP. Efficacy of praziquantel against *Schistosoma japonicum*: field evaluation in an area with repeated chemotherapy compared with a newly identified endemic focus in Hunan, China. Trans R Soc Trop Med Hyg. 2001;95:537–41.
226. Seto EY, Wong BK, Lu D, Zhong B. Human schistosomiasis resistance to praziquantel in China: should we be worried? Am J Trop Med Hyg. 2011;85:74–82.
227. Silva IM, Thiengo R, Conceição MJ, Rey L, Lenzi HL, Perreira Filho E, Ribeiro PC. Therapeutic failure of praziquantel in the treatment of *Schistosoma haematobium* infection in Brazilians returning from Africa. Mem Inst Oswaldo Cruz. 2005;100:445–9.
228. Herwaldt BL, Tao LF, van Pelt W, Tsang VC, Bruce JI. Persistence of *Schistosoma haematobium* infection despite multiple courses of therapy with praziquantel. Clin Infect Dis. 1995;20:309–15.
229. Alonso D, Munoz J, Gascon J, Valls ME, Corachan M. Failure of standard treatment with praziquantel in two returned travelers with *Schistosoma haematobium* infection. Am J Trop Med Hyg. 2006;74:342–4.
230. Guidi A, Andolina C, Ame SM, Albonico M, Cioli D, Haji HJ. Praziquantel efficacy and long-term appraisal of schistosomiasis control in Pemba Island. Trop Med Int Health. 2010;15:614–8.
231. Tinga N, De N, Vien HV, Chau L, Toan ND, Kager PA, Vries PJ. Little effect of praziquantel or artemisinin on clonorchiasis in Northern Vietnam. A pilot study. Trop Med Int Health. 1999;4:814–8.
232. Fallon PG, Doenhoff MJ. Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific. Am J Trop Med Hyg. 1994;51:83–8.

233. Cioli D, Botros SS, Wheatcroft-Francklow K, Mbaye A, Southgate V, Tchuente LA, Pica-Mattoccia L, Troiani AR, El-Din SH, Sabra AN, et al. Determination of ED_{50} values for praziquantel in praziquantel-resistant and -susceptible *Schistosoma mansoni* isolates. Int J Parasitol. 2004;34:979–87.
234. Coeli R, Baba EH, Araujo N, Coelho PM, Oliveira G. Praziquantel treatment decreases *Schistosoma mansoni* genetic diversity in experimental infections. PLoS Negl Trop Dis. 2013;7:e2596.
235. Liang YS, Li HJ, Dai JR, Wang W, Qu GL, Tao YH, Xing YT, Li YZ, Qian K, Wei JY. Studies on resistance of *Schistosoma* to praziquantel XIII: resistance of *Schistosoma japonicum* to praziquantel is experimentally induced in laboratory. Chin J Schisto Control. 2011;23:605–10.
236. Mattos AC, Pereira GC, Jannotti-Passos LK, Kusel JR, Coelho PM. Evaluation of the effect of oxamniquine, praziquantel and a combination of both drugs on the intramolluscan phase of *Schistosoma mansoni*. Acta Trop. 2007;102:84–91.
237. Couto FF, Coelho PM, Araujo N, Kusel JR, Katz N, Jannotti-Passos LK, Mattos AC. *Schistosoma mansoni*: a method for inducing resistance to praziquantel using infected *Biomphalaria glabrata* snails. Mem Inst Oswaldo Cruz. 2011;106:153–7.
238. Couto FF, Coelho PM, Araujo N, Kusel JR, Katz N, Mattos AC. Use of fluorescent probes as a useful tool to identify resistant *Schistosoma mansoni* isolates to praziquantel. Parasitology. 2010;137:1791–7.
239. Liang YS, Dai JR, Zhu YC, Coles GC, Doenhoff MJ. Genetic analysis of praziquantel resistance in *Schistosoma mansoni*. Southeast Asian J Trop Med Public Health. 2003;34:274–80.
240. Pica-Mattoccia L, Doenhoff MJ, Valle C, Basso A, Troiani AR, Liberti P, Festucci A, Guidi A, Cioli D. Genetic analysis of decreased praziquantel sensitivity in a laboratory strain of *Schistosoma mansoni*. Acta Trop. 2009;111:82–5.
241. Pereira C, Fallon PG, Cornette J, Capron A, Doenhoff MJ, Pierce RJ. Alterations in cytochrome-c oxidase expression between praziquantel-resistant and susceptible strains of *Schistosoma mansoni*. Parasitology. 1998;117(Pt 1):63–73.
242. Tsai MH, Marx KA, Ismail MM, Tao L. Randomly amplified polymorphic DNA (RAPD) polymerase chain reaction assay for identification of *Schistosoma mansoni* strains sensitive or tolerant to anti-schistosomal drugs. J Parasitol. 2000;86:146–9.
243. French MD, Churcher TS, Basanez MG, Norton AJ, Lwambo NJ, Webster JP. Reductions in genetic diversity of *Schistosoma mansoni* populations under chemotherapeutic pressure: the effect of sampling approach and parasite population definition. Acta Trop. 2013;128:196–295.
244. Betson M, Sousa-Figueiredo JC, Kabatereine NB, Stothard JR. New insights into the molecular epidemiology and population genetics of *Schistosoma mansoni* in Ugandan pre-school children and mothers. PLoS Negl Trop Dis. 2013;7:e2561.
245. Huyse T, Van den Broeck F, Jombart T, Webster BL, Diaw O, Volckaert FA, Balloux F, Rollinson D, Polman K. Regular treatments of praziquantel do not impact on the genetic make-up of *Schistosoma mansoni* in Northern Senegal. Infect Genet Evol. 2013;18:100–5.
246. Webster BL, Culverwell CL, Khamis IS, Mohammad KA, Rollinson D, Stothard JR. DNA barcoding of *Schistosoma haematobium* on Zanzibar reveals substantial genetic diversity and two major phylogenetic groups. Acta Trop. 2013;128:206–17.
247. Norton AJ, Gower CM, Lamberton PH, Webster BL, Lwambo NJ, Blair L, Fenwick A, Webster JP. Genetic consequences of mass human chemotherapy for *Schistosoma mansoni*: population structure pre- and post-praziquantel treatment in Tanzania. Am J Trop Med Hyg. 2010;83:951–7.
248. Blanton RE, Blank WA, Costa JM, Carmo TM, Reis EA, Silva LK, Barbosa LM, Test MR, Reis MG. *Schistosoma mansoni* population structure and persistence after praziquantel treatment in two villages of Bahia, Brazil. Int J Parasitol. 2011;41:1093–9.
249. Valle C, Troiani AR, Festucci A, Pica-Mattoccia L, Liberti P, Wolstenholme A, Francklow K, Doenhoff MJ, Cioli D. Sequence and level of endogenous expression of calcium channel beta subunits in *Schistosoma mansoni* displaying different susceptibilities to praziquantel. Mol Biochem Parasitol. 2003;130:111–5.
250. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem. 2002;71:537–92.
251. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM. P-glycoprotein: from genomics to mechanism. Oncogene. 2003;22:7468–85.
252. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 2006;5:219–34.
253. Kerboeuf D, Blackhall W, Kaminsky R, von Samson-Himmelstjerna G. P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. Int J Antimicrob Agents. 2003;22:332–46.
254. Jones PM, George AM. Multidrug resistance in parasites: ABC transporters, P-glycoproteins and molecular modelling. Int J Parasitol. 2005;35:555–66.
255. James CE, Hudson AL, Davey MW. An update on P-glycoprotein and drug resistance in *Schistosoma mansoni*. Trends Parasitol. 2009;25:538–9.
256. Leprohon P, Legare D, Ouellette M. ABC transporters involved in drug resistance in human parasites. Essays Biochem. 2011;50:121–44.
257. Lespine A, Menez C, Bourguinat C, Prichard RK. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: prospects for reversing transport-dependent anthelmintic resistance. Int J Parasitol Drugs Drug Resist. 2012;2:58–75.
258. Ardelli BF. Transport proteins of the ABC systems superfamily and their role in drug action and resistance in nematodes. Parasitol Int. 2013;62:639–46.
259. Greenberg RM. ABC multidrug transporters in schistosomes and other parasitic flatworms. Parasitol Int. 2013;62:647–53.
260. Sato H, Kusel JR, Thornhill J. Excretion of fluorescent substrates of mammalian multidrug resistance-associated protein (MRP) in the *Schistosoma mansoni* excretory system. Parasitology. 2004;128:43–52.
261. Sato H, Kusel JR, Thornhill J. Functional visualization of the excretory system of adult *Schistosoma mansoni* by the fluorescent marker resorufin. Parasitology. 2002;125:527–35.
262. Kasinathan RS, Morgan WM, Greenberg RM. *Schistosoma mansoni* express higher levels of multidrug resistance-associated protein 1 (SmMRP1) in juvenile worms and in response to praziquantel. Mol Biochem Parasitol. 2010;173:25–31.
263. Messerli SM, Kasinathan RS, Morgan W, Spranger S, Greenberg RM. *Schistosoma mansoni* P-glycoprotein levels increase in response to praziquantel exposure and correlate with reduced praziquantel susceptibility. Mol Biochem Parasitol. 2009;167:54–9.
264. Kasinathan RS, Sharma LK, Cunningham C, Webb TR, Greenberg RM. Inhibition or knockdown of ABC transporters enhances susceptibility of adult and juvenile schistosomes to praziquantel. PLoS Negl Trop Dis. 2014;8:e3265.
265. Abdulla M-H, Ruelas DS, Wolff B, Snedecor J, Lim K-C, Xu F, Renslo AR, Williams J, McKerrow JH, Caffrey CR. Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. PLoS Negl Trop Dis. 2009;3:e478.
266. Liang YS, Wang W, Dai JR, Li HJ, Tao YH, Zhang JF, Li W, Zhu YC, Coles GC, Doenhoff M. Susceptibility to praziquantel of male and female cercariae of praziquantel-resistant and susceptible isolates of *Schistosoma mansoni*. J Helminthol. 2010;84:202–7.
267. Liang YS, Coles GC, Dai JR, Zhu YC, Doenhoff MJ. Adult worm tegumental damage and egg-granulomas in praziquantel-

resistant and -susceptible *Schistosoma mansoni* treated in vivo. J Helminthol. 2002;76:327–33.
268. Hanallah S, El-Lakkany N, Mahmoud S, Mousa M, Botros S. Altered immunoglobulin isotype profile and anti-immature worm surface immunoglobulins in mice harboring a praziquantel-resistant *Schistosoma mansoni* isolate. Acta Pathol Microbiol Immunol Scand. 2003;111:1125–32.
269. Richards HC, Foster R. A new series of 2-aminomethyltetrahyd roquinoline derivatives displaying schistosomicidal activity in rodents and primates. Nature. 1969;222:581–2.
270. da Silva LC, Seabrook GR. Clinical trials with oral oxamniquine (UK 4271) for the treatment of mansonian schistosomiasis. Rev Inst Med Trop Sao Paulo. 1974;16:103–9.
271. Katz N, Coelho PM. Clinical therapy of schistosomiasis mansoni: the Brazilian contribution. Acta Trop. 2008;108:72–8.
272. Kramers PG, Gentile JM, Gryseels BJ, Jordan P, Katz N, Mott KE, Mulvihill JJ, Seed JL, Frohberg H. Review of the genotoxicity and carcinogenicity of antischistosomal drugs; is there a case for a study of mutation epidemiology? Report of a task group on mutagenic antischistosomals. Mutat Res. 1991;257:49–89.
273. Pica-Mattoccia L, Cioli D, Archer S. Binding of oxamniquine to the DNA of schistosomes. Trans R Soc Trop Med Hyg. 1989;83:373–6.
274. Beck L, Favre TC, Pieri OS, Zani LC, Domas GG, Barbosa CS. Replacing oxamniquine by praziquantel against *Schistosoma mansoni* infection in a rural community from the sugar-cane zone of Northeast Brazil: an epidemiological follow-up. Mem Inst Oswaldo Cruz. 2001;96(Suppl):165–7.
275. Khayyal MT, Girgis NL, Henry W. Effectiveness of a single dose of hycanthone orally in experimental schistosomiasis in hamsters. Bull World Health Organ. 1969;40:963–5.
276. Cioli D, Knopf PM. A study of the mode of action of hycanthone against *Schistosoma mansoni in vivo* and *in vitro*. Am J Trop Med Hyg. 1980;29:220–6.
277. Cioli D, Pica-Mattoccia L, Archer S. Drug resistance in schistosomes. Parasitol Today. 1993;9:162–6.
278. Cioli D, Pica-Mattoccia L, Moroni R. *Schistosoma mansoni*: hycanthone/oxamniquine resistance is controlled by a single autosomal recessive gene. Exp Parasitol. 1992;75:425–32.
279. Cioli D, Pica-Mattoccia L, Rosenberg S, Archer S. Evidence for the mode of antischistosomal action of hycanthone. Life Sci. 1985;37:161–7.
280. Pica-Mattoccia L, Archer S, Cioli D. Hycanthone resistance in schistosomes correlates with the lack of an enzymatic activity which produces the covalent binding of hycanthone to parasite macromolecules. Mol Biochem Parasitol. 1992;55:167–75.
281. Pica-Mattoccia L, Novi A, Cioli D. Enzymatic basis for the lack of oxamniquine activity in Schistosoma haematobium infections. Parasitol Res. 1997;83:687–9.
282. Krishna S, Bustamante AB, Haynes RK, Staines HM. Artemisinins: their growing importance in medicine. Trends Pharmacol Sci. 2008;29:520–7.
283. Ho WE, Peh HY, Chan TK, Wong WS. Artemisinins: pharmacological actions beyond anti-malarial. Pharmacol Ther. 2014;142:126–39.
284. Utzinger J, Xiao SH, Tanner M, Keiser J. Artemisinins for schistosomiasis and beyond. Curr Opin Investig Drugs. 2007;8:105–16.
285. Le WJ, You JQ, Mei JY. Chemotherapeutic effect of artesunate in experimental schistosomiasis. Acta Pharm Sin. 1983;18:619–21.
286. Xiao S, Tanner M, N'Goran EK, Utzinger J, Chollet J, Bergquist R, Chen M, Zheng J. Recent investigations of artemether, a novel agent for the prevention of schistosomiasis japonica, mansoni and haematobia. Acta Trop. 2002;82:175–81.
287. Zhai ZL, Jiao PY, Mei JY, Xiao SH. Glutathione inhibits the antischistosomal activity of artemether. Chin J Parasitol Parasitic Dis. 2002;20:212–5.
288. El-Bassiouni EA, Helmy MH, Saad EI, El-Nabi Kamel MA, Abdel-Meguid E, Hussein HS. Modulation of the antioxidant defence in different developmental stages of *Schistosoma mansoni* by praziquantel and artemether. Br J Biomed Sci. 2007;64:168–74.
289. Xiao SH, You JQ, Mei JY, Guo HF, Jiao PY, Sun HL, Yao MY, Feng Z. Effect of artemether on glucose uptake and glycogen content in *Schistosoma japonicum*. Acta Pharmacol Sin. 1997;18:363–7.
290. Shuhua X, Hotez PJ, Tanner M. Artemether, an effective new agent for chemoprophylaxis against shistosomiasis in China: its in vivo effect on the biochemical metabolism of the Asian schistosome. Southeast Asian J Trop Med Public Health. 2000;31:724–32.
291. Meshnick SR. Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol. 2002;32:1655–60.
292. Keiser J, Ingram K, Vargas M, Chollet J, Wang X, Dong Y, Vennerstrom JL. *In vivo* activity of aryl ozonides against *Schistosoma* species. Antimicrob Agents Chemother. 2012;56:1090–2.
293. Keiser J, Vargas M, Doenhoff MJ. Activity of artemether and mefloquine against juvenile and adult *Schistosoma mansoni* in athymic and immunocompetent NMRI mice. Am J Trop Med Hyg. 2010;82:112–4.
294. Hu SY, Liu J, Wang B, Hu ZD, Xiao CJ. The effect of artesunate in preventing the populations from *Schistosoma japonicum* infection during flood-control. Chin J Parasitol Parasit Dis. 2000;18:113–4.
295. Utzinger J, N'Goran EK, N'Dri A, Lengeler C, Xiao S, Tanner M. Oral artemether for prevention of *Schistosoma mansoni* infection: randomised controlled trial. Lancet. 2000;355:1320–5.
296. N'Goran EK, Utzinger J, Gnaka HN, Yapi A, N'Guessan NA, Kigbafori SD, Lengeler C, Chollet J, Shuhua X, Tanner M. Randomized, double-blind, placebo-controlled trial of oral artemether for the prevention of patent *Schistosoma haematobium* infections. Am J Trop Med Hyg. 2003;68:24–32.
297. Perez del Villar L, Burquillo FJ, Lopez-Aban J, Muro A. Systematic review and meta-analysis of artemisinin based therapies for the treatment and prevention of schistosomiasis. PLoS One. 2012;7:e45867.
298. Liu R, Dong HF, Guo Y, Zhao QP, Jiang MS. Efficacy of praziquantel and artemisinin derivatives for the treatment and prevention of human schistosomiasis: a systematic review and meta-analysis. Parasit Vectors. 2011;4:201.
299. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2009;361:455–67.
300. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, et al. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med. 2014;371:411–23.
301. Hua HY, Liang YS, Zhang Y, Wei JF, Guo HX. The sensitivity of artesunate against *Schistosoma japonicum* decreased after 10 years of use in China. Parasitol Res. 2010;107:873–8.
302. Liu R, Dong HF, Jiang MS. The sensitivity of artesunate against *Schistosoma japonicum* decreased after 10 years of use in China? Parasitol Res. 2012;110:1563–4.
303. Obonyo CO, Muok EM, Mwinzi PM. Efficacy of artesunate with sulfalene plus pyrimethamine versus praziquantel for treatment of *Schistosoma mansoni* in Kenyan children: an open-label randomised controlled trial. Lancet Infect Dis. 2010;10:603–11.
304. Van Nassauw L, Toovey S, Van Op den Bosch J, Timmermans JP, Vercruysse J. Schistosomicidal activity of the antimalarial drug, mefloquine, in *Schistosoma mansoni*-infected mice. Travel Med Infect Dis. 2008;6:253–8.
305. Keiser J, Chollet J, Xiao SH, Mei JY, Jiao PY, Utzinger J, Tanner M. Mefloquine-an aminoalcohol with promising antischistosomal properties in mice. PLoS Negl Trop Dis. 2009;3:e350.

306. Xiao SH. Mefloquine, a new type of compound against schistosomes and other helminthes in experimental studies. Parasitol Res. 2013;112:3723–40.
307. Keiser J, Silue KD, Adiossan LK, N'Guessan NA, Monsan N, Utzinger J, N'Goran EK. Praziquantel, mefloquine-praziquantel, and mefloquine-artesunate-praziquantel against *Schistosoma haematobium*: a randomized, exploratory, open-label trial. PLoS Negl Trop Dis. 2014;8:e2975.
308. Ingram K, Ellis W, Keiser J. Antischistosomal activities of mefloquine-related arylmethanols. Antimicrob Agents Chemother. 2012;56:3207–15.
309. Sulaiman SM, Ali HM, Homeida MM, Bennett JL. Efficacy of a new Hoffmann-La Roche compound (Ro 15-5458) against *Schistosoma mansoni* (Gezira strain, Sudan) in vervet monkeys (*Cercopithecus aethiops*). Trop Med Parasitol. 1989;40:335–6.
310. Sturrock RF, Bain J, Webbe G, Doenhoff MJ, Stohler H. Parasitological evaluation of curative and subcurative doses of 9-acridanone-hydrazone drugs against *Schistosoma mansoni* in baboons, and observations on changes in serum levels of anti-egg antibodies detected by ELISA. Trans R Soc Trop Med Hyg. 1987;81:188–92.
311. Pereira LH, Coelho PM, Costa JO, de Mello RT. Activity of 9-acridanone-hydrazone drugs detected at the pre-postural phase, in the experimental schistosomiasis mansoni. Mem Inst Oswaldo Cruz. 1995;90:425–8.
312. Metwally A, Abdel Hadi A, Mikhail EG, Abou Shadi O, Sabry H, el-Nahal H. Study of the efficacy of the new antischistosomal drug 10-[2-(diethylamino)ethyl]-9-acridanone-(thiazolidin-2-ylidene) hydrazone against an Egyptian strain of *S. mansoni* in mice. Arzneimittelforschung. 1997;47:975–9.
313. Guirguis FR. Efficacy of praziquantel and Ro 15-5458, a 9-acridanone-hydrazone derivative, against *Schistosoma haematobium*. Arzneimittelforschung. 2003;53:57–61.
314. Kamel G, Metwally A, Guirguis F, Nessim NG, Noseir M. Effect of a combination of the new antischistosomal drug Ro 15-5458 and praziquantel on different strains of *Schistosoma mansoni* infected mice. Arzneimittelforschung. 2000;50:391–4.
315. Stohler HR. RO 11-3128—a novel schistosomicidal compound. In: Proceedings of the 10th international congress of chemotherapy, vol. 1. Washington, DC: American Society for Microbiology; 1978.
316. Baard AP, Sommers DK, Honiball PJ, Fourie ED, du Toit LE. Preliminary results in human schistosomiasis with Ro 11-3128. S Afr Med J. 1979;55:617–8.
317. O'Boyle CA, Lambe R, Darragh A. Central effects in man of the novel schistosomicidal benzodiazepine meclonazepam. Eur J Clin Pharmacol. 1985;29:105–8.
318. Mahajan A, Kumar V, Mansour NR, Bickle Q, Chibale K. Meclonazepam analogues as potential new antihelmintic agents. Bioorg Med Chem Lett. 2008;18:2333–6.
319. Menezes CM, Rivera G, Alves MA, do Amaral DN, Thibaut JP, Noel F, Barreiro EJ, Lima LM. Synthesis, biological evaluation, and structure-activity relationship of clonazepam, meclonazepam, and 1,4-benzodiazepine compounds with schistosomicidal activity. Chem Biol Drug Des. 2012;79:943–9.
320. Olsen RW, Sieghart W. $GABA_A$ receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology. 2009;56:141–8.
321. Bender AS, Hertz L. Pharmacological characteristics of diazepam receptors in neurons and astrocytes in primary cultures. J Neurosci Res. 1987;18:366–72.
322. Thibaut JP, Monteiro LM, Leite LC, Menezes CM, Lima LM, Noel F. The effects of 3-methylclonazepam on *Schistosoma mansoni* musculature are not mediated by benzodiazepine receptors. Eur J Pharmacol. 2009;606:9–16.
323. Noel F, Mendonca-Silva DL, Thibaut JP, Lopes DV. Characterization of two classes of benzodiazepine binding sites in *Schistosoma mansoni*. Parasitology. 2007;134:1003–12.
324. Dufour V, Beech RN, Wever C, Dent JA, Geary TG. Molecular cloning and characterization of novel glutamate-gated chloride channel subunits from *Schistosoma mansoni*. PLoS Pathog. 2013;9:e1003586.
325. Pica-Mattoccia L, Ruppel A, Xia CM, Cioli D. Praziquantel and the benzodiazepine Ro 11-3128 do not compete for the same binding sites in schistosomes. Parasitology. 2008;135:47–54.
326. Sayed AA, Simeonov A, Thomas CJ, Inglese J, Austin CP, Williams DL. Identification of oxadiazoles as new drug leads for the control of schistosomiasis. Nat Med. 2008;14:407–12.
327. Vandewaa EA, Mills G, Chen GZ, Foster LA, Bennett JL. Physiological role of HMG-CoA reductase in regulating egg production by *Schistosoma mansoni*. Am J Physiol. 1989;257:R618–25.
328. Araujo N, Kohn A, Oliveira AA, Katz N. *Schistosoma mansoni*: the action of lovastatin on the murine model. Rev Soc Bras Med Trop. 2002;35:35–8.
329. Chen GZ, Foster L, Bennett JL. Antischistosomal action of mevinolin: evidence that 3-hydroxy-methylglutaryl-coenzyme a reductase activity in *Schistosoma mansoni* is vital for parasite survival. Naunyn Schmiedebergs Arch Pharmacol. 1990;342:477–82.
330. Rojo-Arreola L, Long T, Asarnow D, Suzuki BM, Singh R, Caffrey CR. Chemical and genetic validation of the statin drug target to treat the helminth disease, schistosomiasis. PLoS One. 2014;9:e87594.
331. Caffrey CR, Rohwer A, Oellien F, Marhofer RJ, Braschi S, Oliveira G, McKerrow JH, Selzer PM. A comparative chemogenomics strategy to predict potential drug targets in the metazoan pathogen, *Schistosoma mansoni*. PloS One. 2009;4:e4413.
332. Wang W, Li HJ, Qu GL, Xing YT, Yang ZK, Dai JR, Liang YS. Is there a reduced sensitivity of dihydroartemisinin against praziquantel-resistant *Schistosoma japonicum*? Parasitol Res. 2014;113:223–8.
333. Wang W, Li TY, Ji Y, Qu GL, Qian YL, Li HJ, Dai JR, Liang YS. Efficacy of artemether and artesunate in mice infected with praziquantel non-susceptible isolate of *Schistosoma japonicum*. Parasitol Res. 2014;113:925–31.
334. de Souza Dias LC, de Jesus PR, Deberaldini ER. Use of praziquantel in patients with schistosomiasis mansoni previously treated with oxamniquine and/or hycanthone: resistance of *Schistosoma mansoni* to schistosomicidal agents. Trans R Soc Trop Med Hyg. 1982;76:652–9.
335. Katz N, Rocha RS, de Souza CP, Coura Filho P, Bruce JI, Coles GC, Kinoti GK. Efficacy of alternating therapy with oxamniquine and praziquantel to treat *Schistosoma mansoni* in children following failure of first treatment. Am J Trop Med Hyg. 1991;44:509–12.
336. Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC. Drug resistance in veterinary helminths. Trends Parasitol. 2004;20:469–76.
337. Martin PJ, Le Jambre LF, Claxton JH. The impact of refugia on the development of thiabendazole resistance in *Haemonchus contortus*. Int J Parasitol. 1981;11:35–41.
338. Kenyon F, Greer AW, Coles GC, Cringoli G, Papadopoulos E, Cabaret J, Berrag B, Varady M, Van Wyck JA, Thomas E, et al. The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants. Vet Parasitol. 2009;164:3–11.
339. Vercruysse J, Levecke B, Prichard R. Human soil-transmitted helminths: implications of mass drug administration. Curr Opin Infect Dis. 2012;25:703–8.

340. Fairweather I, Boray JC. Mechanisms of fasciolicide action and drug resistance in *Fasciola hepatica*. In: Dalton JP, editor. Fasciolosis. Wallingford: CABI Publishing; 1999. p. 225–76.
341. Robles-Perez D, Martinez-Perez JM, Rojo-Vazquez FA, Martinez-Valladares M. Development of an egg hatch assay for the detection of anthelmintic resistance to albendazole in *Fasciola hepatica* isolated from sheep. Vet Parasitol. 2014;203:217–21.
342. Canevari J, Ceballos L, Sanabria R, Romero J, Olaechea F, Ortiz P, Cabrera M, Gayo V, Fairweather I, Lanusse C, et al. Testing albendazole resistance in *Fasciola hepatica*: validation of an egg hatch test with isolates from South America and the United Kingdom. J Helminthol. 2014;88:286–92.
343. Brennan GP, Fairweather I, Trudgett A, Hoey E, McCoy M, McConville M, Meaney M, Robinson M, McFerran N, Ryan L, et al. Understanding triclabendazole resistance. Exp Mol Pathol. 2007;82:104–9.
344. Ashrafi K, Barques MD, O'Neill S, Mas-Coma S. Fascioliasis: a worldwide parasitic disease of importance in travel medicine. Travel Med Infect Dis. 2014;12(6 Pt A):636–49.
345. Keiser J, Engels D, Buscher G, Utzinger J. Triclabendazole for the treatment of fascioliasis and paragonimiasis. Expert Opin Investig Drugs. 2005;14:1513–26.
346. Sanabria R, Ceballos L, Moreno L, Romero J, Lanusse C, Alvarez L. Identification of a field isolate of Fasciola hepatica resistant to albendazole and susceptible to triclabendazole. Vet Parasitol. 2013;193:105–10.
347. Coles GC, Stafford KA. Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole-resistant *Fasciola hepatica*. Vet Rec. 2001;148:723–4.
348. Coles GC, Rhodes AC, Stafford KA. Activity of closantel against adult triclabendazole-resistant *Fasciola hepatica*. Vet Rec. 2000;146:504.
349. Stitt AW, Fairweather I. *Fasciola hepatica*: tegumental surface changes in adult and juvenile flukes following treatment in vitro with the sulphoxide metabolite of triclabendazole (Fasinex). Parasitol Res. 1993;79:529–36.
350. Toner E, Brennan GP, Hanna RE, Edgar HW, Fairweather I. Tegumental surface changes in adult *Fasciola hepatica* in response to treatment in vivo with triclabendazole in the sheep host. Vet Parasitol. 2010;172:238–48.
351. Halferty L, Brennan GP, Hanna RE, Edgar HW, Meaney MM, McConville M, Trudgett A, Hoey L, Fairweather I. Tegumental surface changes in juvenile *Fasciola hepatica* in response to treatment in vivo with triclabendazole. Vet Parasitol. 2008;155:49–58.
352. Shareef PA, Brennan GP, McVeigh P, Khan MA, Morphew RM, Mousley A, Marks NJ, Saifullah MK, Brophy PM, Maule AG, et al. Time-dependent tegumental surface changes in juvenile *Fasciola gigantica* in response to triclabendazole treatment in goat. Acta Trop. 2014;136:108–17.
353. Stitt AW, Fairweather I. Spermatogenesis in *Fasciola hepatica*: an ultrastructural comparison of the effects of the anthelmintic, triclabendazole (Fasinex) and the microtubule inhibitor, tubulozole. Invertebr Reprod Dev. 1992;22:139–50.
354. Fetterer RH. The effect of albendazole and triclabendazole on colchicine binding in the liver fluke *Fasciola hepatica*. J Vet Pharmacol Ther. 1986;9:49–54.
355. Bennett JL, Kohler P. *Fasciola hepatica*: action in vitro of triclabendazole on immature and adult stages. Exp Parasitol. 1987;63:49–57.
356. Ryan LA, Hoey E, Trudgett A, Fairweather I, Fuchs M, Robinson MW, Chambers E, Timson DJ, Ryan E, Feltwell T, et al. *Fasciola hepatica* expresses multiple α- and β-tubulin isotypes. Mol Biochem Parasitol. 2008;159:73–8.
357. Stitt AW, Fairweather I, Mackender RO. The effect of triclabendazole ("Fasinex") on protein synthesis by the liver fluke, *Fasciola hepatica*. Int J Parasitol. 1995;25:421–9.
358. Wilkinson R, Law CJ, Hoey EM, Fairweather I, Brennan GP, Trudgett A. An amino acid substitution in *Fasciola hepatica* P-glycoprotein from triclabendazole-resistant and triclabendazole-susceptible populations. Mol Biochem Parasitol. 2012;186:69–72.
359. Savage J, Meaney M, Brennan GP, Hoey E, Trudgett A, Fairweather I. Effect of the P-glycoprotein inhibitor, R(+)-verapamil on the drug susceptibility of a triclabendazole-resistant isolate of *Fasciola hepatica*. Vet Parasitol. 2013;195:72–86.
360. Holmes SD, Fairweather I. *Fasciola hepatica*: motility response to metabolic inhibitors *in vitro*. Exp Parasitol. 1985;59:275–89.
361. Coles GC, Briscoe MG. Benzimidazoles and fluke eggs. Vet Rec. 1978;103:360–1.
362. Coles GC, Simpkin KG. Resistance of nematode eggs to the ovicidal activity of benzimidazoles. Res Vet Sci. 1977;22:386–7.
363. Schulman MD, Valentino D. *Fasciola hepatica*: effect of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide on glycolysis *in vitro*. Exp Parasitol. 1980;49:206–15.
364. Fairweather I, Holmes SD, Threadgold LT. *Fasciola hepatica*: motility response to fasciolicides *in vitro*. Exp Parasitol. 1984;57:209–24.
365. Martinez-Valladares M, Cordero-Perez C, Rojo-Vazquez FA. Efficacy of an anthelmintic combination in sheep infected with *Fasciola hepatica* resistant to albendazole and clorsulon. Exp Parasitol. 2014;136:59–62.
366. Reinemeyer CR, Hutchens DE, Eckblad WP, Marchiondo AA, Shugart JL. Dose-confirmation studies of the cestocidal activity of pyrantel pamoate paste in horses. Vet Parasitol. 2006;138:234–9.
367. Martin RJ, Puttachary S, Buxton SK, Verma S, Robertson AP. The Conqueror Worm: recent advances with cholinergic anthelmintics and techniques excite research for better therapeutic drugs. J Helminthol. 2014;29:1–11.
368. Martin RJ, Robertson AP, Buxton SK, Beech RN, Charvet CL, Neveu C. Levamisole receptors: a second awakening. Trends Parasitol. 2012;28:289–96.
369. Yue C, Lawrence S, Coles GC. Changing behaviour in the common horse tapeworm. Vet Rec. 2003;153:663–4.
370. Nunnari G, Pinzone MR, Gruttadauria S, Celesia BM, Madeddu G, Malaquarnera G, Pavone P, Cappellani A, Cacopardo B. Hepatic echinococcosis: clinical and therapeutic aspects. World J Gastroenterol. 2012;18:1448–58.
371. McManus DP, Gray DJ, Zhang W, Yang Y. Diagnosis, treatment, and management of echinococcosis. BMJ. 2012;344:e3866.
372. Morris DL, Taylor DH. *Echinococcus granulosus*: development of resistance to albendazole in an animal model. J Helminthol. 1990;64:171–4.
373. Pan D, Das S, Bera AK, Bandyopadhyay S, Bandyopadhyay S, De S, Rana T, Das SK, Suryanaryana VV, Deb J, et al. Molecular and biochemical mining of heat-shock and 14-3-3 proteins in drug-induced protoscolices of *Echinococcus granulosus* and the detection of a candidate gene for anthelmintic resistance. J Helminthol. 2011;85:196–203.
374. Kwa MSG, Veenstra JG, Roos MH. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. Mol Biochem Parasitol. 1994;63:299–303.
375. Townsend RB, Kelly JD, James R, Weston I. The anthelmintic efficacy of fenbendazole in the control of *Moniezia expansa* and *Trichuris ovis* in sheep. Res Vet Sci. 1977;23:385–6.
376. Mason P, Moffat J, Cole D, editors. Tapeworm in sheep revisited. In: Proceedings of the Society of sheep and beef cattle veterinarians; 2002; Wellington: New Zealand Veterinary Association.
377. Southwork J, Harvey C, Larson S. Use of praziquantel for the control of *Moniezia expansa* in lambs. N Z Vet J. 1996;44:112–5.
378. Coles GC. Sustainable use of anthelmintics in grazing animals. Vet Rec. 2002;151:165–9.
379. Gallella SD, Gunawardena GS, Karunaweera ND. *Bertiella studeri* infection: resistance to niclosamide. Ceylon Med J. 2004;49:65.

380. Sarti E, Schantz PM, Avila G, Ambrosio J, Medina-Santillan R, Flisser A. Mass treatment against human taeniasis for the control of cysticercosis: a population-based intervention study. Trans R Soc Trop Med Hyg. 2000;94:85–9.
381. Allan JC, Velasquez-Tohom M, Fletes C, Torres-Alvarez R, Lopez-Virula G, Yurrita P, Soto de Alfaro H, Rivera A, Garcia-Noval J. Mass chemotherapy for intestinal *Taenia solium* infection: effect on prevalence in humans and pigs. Trans R Soc Trop Med Hyg. 1997;91:595–8.
382. Gonzalez AE, Falcon N, Gavidia C, Garcia HH, Tsang VC, Bernal T, Romero M, Gilman RH. Time-response curve of oxfendazole in the treatment of swine cysticercosis. Am J Trop Med Hyg. 1998;59:832–6.
383. Garcia HH, Gonzalez AE, Gilman RH, Moulton LH, Verastegui M, Rodriguez S, Gavidia C, Tsang VC, Cysticercosis Working Group in Peru. Combined human and porcine mass chemotherapy for the control of *T. solium*. Am J Trop Med Hyg. 2006;74:850–5.
384. Newton CR, Preux PM, Singhi P. Parasitic disorders. Handb Clin Neurol. 2013;112:1139–52.
385. Brunetti E, Kern P, Vuitton DA, Writing Panel for the WHO-IWGE. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 2010;114:1–16.
386. Kern P. Clinical features and treatment of alveolar echinococcosis. Curr Opin Infect Dis. 2010;23:505–12.
387. Richter D, Richter J, Gruner B, Kratz E, Franz J, Kern P. *In vitro* efficacy of triclabendazole and clorsulon against the larval stage of *Echinococcus multilocularis*. Parasitol Res. 2013;112:1655–60.
388. World Health Organization. Accelerating work to overcome the global impact of neglected tropical diseases: a roadmap for implementation. Geneva: World Health Organization; 2012.
389. Curtale F, Mohamed MY, Youssef ZM. Comprehensive primary health care, a viable strategy for the elimination of schistosomiasis. Trans R Soc Trop Med Hyg. 2010;104:70–2.
390. Lelo AE, Mburu DN, Magoma GN, Mungai BN, Kihara JH, Mwangi IN, Maina GM, Kinuthia JM, Mutuku MW, Loker ES, et al. No apparent reduction in schistosome burden or genetic diversity following four years of school-based mass drug administration in Mwea, central Kenya, a heavy transmission area. PLoS Negl Trop Dis. 2014;8:e3221.
391. Utzinger J, Keiser J, Shuhua X, Tanner M, Singer BH. Combination chemotherapy of schistosomiasis in laboratory studies and clinical trials. Antimicrob Agents Chemother. 2003;47:1487–95.
392. World Health Organization. TDR BL3, 2008 report: lead discovery for infectious tropical diseases. Geneva: World Health Organization; 2009.
393. Ingram-Sieber K, Cowan N, Panic G, Vargas M, Mansour NR, Bickle QD, Wells TN, Spangenberg T, Keiser J. Orally active antischistosomal early leads identified from the open access malaria box. PLoS Negl Trop Dis. 2014;8:e2610.

Drug Resistance in Ectoparasites of Medical and Veterinary Importance

48

Kathryn A. Stafford and Gerald Christopher C. Coles

1 Introduction

Early insect control on animals relied on a combination of husbandry methods coupled with the use of mineral oils and tar distillates such as kerosene, phenols and cresol. While these proved highly effective insecticides, they were also comparatively toxic to the host. A number of inorganic compounds based on arsenic, boron and fluoride have also been used in insect control and the first reports of resistance were to these compounds. Where insecticides have been used extensively for the control of arthropods, resistance has inevitably followed. Resistance has even developed in one group of insects (*Cimex*) as a result of insecticide use against another group (mosquitoes). The introduction of DDT (dichlorodiphenyltrichloroethane) in 1939 led to its widespread use for insect control, which was most evident in the control of the malaria and typhus vectors during World War II where large amounts were used but within a few years accounts of resistance began to surface. The first reported case of control failure was to lime sulphur in 1914 by the San Jose scale, *Aspidiotus perniciosus*. Early reports of resistance were in agricultural crop pests; however by 1946 resistance had been recorded in the cattle tick, *Rhipicephalus* (formerly *Boophilus*) *microplus* and the blue tick *Rhipicephalus* (formerly *Boophilus*) *decoloratus*, in both cases to sodium arsenite dips. These early reports of resistance received very little attention until in 1946 DDT reportedly failed to control the housefly *Musca domestica* in both Sweden and Denmark. In 1947 failure of DDT to control the bedbug *Cimex lectularius* was reported from Hawaii and in 1951 the human body louse *Pediculus corporis* in Korea and Japan.

K.A. Stafford, B.Sc., M.Sc., Ph.D.
Department of Clinical Veterinary Sciences, University of Bristol, Langford House, Bristol, BS40 5DU, UK

G.C. Coles, M.A., Ph.D., Sc.D. (✉)
School of Veterinary Sciences, University of Bristol, Bristol, UK
e-mail: gerald.c.coles@bristol.ac.uk

Since these early reports of DDT failure, the product has been withdrawn from sale in the majority of countries due to its toxicity and has been replaced by newer generations of insecticides. Concerns over toxicity to both humans and wildlife, coupled with reports of product failure, lead to its widespread withdrawal during the 1970s. Since then, new classes of insecticides have been developed, but as with any drug, regular and indiscriminate use has led to the development of resistance to the majority of these products. Organophosphates target the enzyme acetylcholinesterase causing a termination in the nerve impulses. Resistance to OPs is usually associated with a point mutation and has been reported in a number of arthropod species such as the Ixodida and Diptera. The 1950s saw the development of the carbamate insecticides which are similar to organophosphates (OPs) in that they also target the enzyme acetylcholinesterase but they inhibit the enzyme by carbamylating the serine residue within the active site gorge. It is thought that the same mechanism of resistance as seen in OPs occurs in carbamate resistance which has been reported in Diptera and Phthiraptera. These were followed by the pyrethrins and pyrethroids, which due to their relative low toxicity to mammals are used widely in the control of fleas on pets and in the treatment of the human head louse.

The pyrethroids are classified into type 1 causing a repetitive discharge at the presynaptic nerve end and type 2 causing a toxic release of transmitter. Two mechanisms of resistance occur: an increase in the rate of metabolic detoxification and a change in target site sensitivity. As with other classes of insecticides, the widespread use of pyrethroids has led to the development of resistance in many classes of arthropods such as Ixodida and Phthiraptera. The IGRs disrupt the moulting process either by preventing the process of ecdysis or by the inhibition of chitin synthesis. Macrocyclic lactones block electrical activity in nerves by increasing the membranes conductance to chloride ions and the mechanism of resistance appears to involve changes in this transporter mechanism. Later classes of insecticide include: the insect growth regulators (IGRs), which are effective where an

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4_48

immediate kill is not essential since they act on the developmental stage of the insect larvae; the arylheterocycles phenylpyrazoles (fipronil); the chloronicotinyl nitroguanidines (imidacloprid); spinosad, which is derived from a bacterium called *Saccharopolyspora spinosa*; and fluolaner, a novel isoxazoline. All have been developed for flea control but in all probability have many additional uses.

2 Definition of Resistance

Resistance is defined by the World Health Organisation as 'the development of an ability in a strain of some organism to tolerate doses of a toxicant that would prove lethal to a majority of individuals in a normal population of the same species'. It has been suggested that a better definition would be 'a response of an organism or a population to a toxicant that enables the organism or population to withstand future toxicant exposures better, because gene amplification which may confer resistance does not require selection [1] and other individual responses to sub-lethal exposures are included' [2]. A very recent definition is given by Coles and Dryden: 'selection of a specific heritable trait (or traits) in a population of arthropods, due to that populations contact with a chemical, that results in a significant increase in the percentage of the population that will survive a standard dose of that chemical (or a closely related chemical in the case of cross resistance)' [3].

Whatever definition of resistance is used, monitoring field collected isolates for the presence of resistance is necessary for maintaining the efficacy of insecticides through resistance management. A simple test used for monitoring resistance is the contact test. Groups of insects are held, for established time periods, in tubes containing insecticide impregnated papers. At the termination of the given time period the numbers of live and dead individuals are counted. From this data the establishment of LD (lethal dose) values can be calculated. These are useful in monitoring resistance once it has reached high levels in any given population but are limited in detecting the emergence of resistance [4]. The introduction of the discriminating dose [5] to distinguish between resistant and susceptible individuals has proved more efficient than estimating resistance using regression lines [4]. The discriminating dose is defined as the dose which just kills 100 % of susceptible test insects within a given population. Any individuals from field collected isolates which survive at this dose are by definition resistant.

3 Mode of Action and Mechanisms of Resistance

There are a variety of neural transmitters and neural modulators present within insects. Neurotransmitters are chemical messengers released into the synaptic cleft where they have a temporary effect on the electrical potential of the postsynaptic membrane. Neuromodulators are released into the vicinity of the synapse where they modify synaptic transmission. A number of these, acetylcholine (Ach), γ-aminobutyric acid (GABA), glutamate, serotonin and octopamine, have been examined in detail. The neurotransmitter acetylcholine has been found to be the most important excitatory neurotransmitter present in the insect central nervous system. The receptors occur in at least two forms, nicotinic Ach and muscarinic Ach) [6, 7]. Glutamate is the principle excitatory transmitter found at the junctions of nerves and muscles while GABA is the principle inhibitory neurotransmitter at the nerve/muscle junction as well as the central nervous system.

3.1 The Organophosphates

Organophosphates target the enzyme acetylcholinesterase (AchE), a key enzyme in the nervous system. This enzyme is a glycosylated dimer which is attached to a membrane via a glycolipid anchor [8, 9]. In insects it terminates nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine. When targeted by organophosphates (OP), enzyme activity is inhibited by the serine residue within the active site gorge being covalently phosphorylated [10]. Resistance to OPs has been correlated with the overexpression of AchE [8] but is more usually associated with a point mutation which is frequently accompanied by a modification of the kinetic parameters of acetylcholine hydrolysis [11–15]. Resistance-associated mutations involving substitutions at key sites located within the active site gorge of the enzyme have a steric effect or alter the orientation of the active site residues [16]. However the isolation of AchE sequences from *R. microplus* indicates that an insensitive AchE phenotype does not result from an amino acid substitution in the AchE protein itself which would suggest the involvement of another mechanism [17].

3.2 The Carbamates

Carbamate insecticides target the same site as OPs, (the enzyme acetylcholinesterase) and inhibit the enzyme activity by carbamylating the serine residue within the active site gorge [10]. They are derivatives of carbamic acid and are relatively unstable compounds which break down in the environment within weeks. Hinkle et al. [18] observed higher AchE activity in two strains of the cat flea *Ctenocephalides felis*, one resistant to organophosphates while the second strain was resistant to the carbamate, propoxur, and suggests that the same mechanism of resistance is shared by the two insecticides.

3.3 The Pyrethroids

The pyrethroid insecticides are classified into type 1 and type 2 compounds. Type 1 compounds include DDT plus analogues and all pyrethroids containing descyano-3-phenoxybenzyl or other alcohols [19]. They cause a repetitive discharge at the presynaptic nerve end. Type 2 compounds contain an α-cyano-3-phenoxybenzyl alcohol and cause a toxic release of transmitter indicative of membrane depolarisation [19]. Under normal conditions the sodium current activates and deactivates within a few milliseconds but in the presence of pyrethroids this state is altered. Pyrethroids delay the deactivation of the sodium channel prolonging the open state and thus allowing a persistent inward current which results in repetitive firing and depolarisation of the nerve membrane.

There are two major mechanisms of pyrethroid resistance in insects. One is an increase in the rate of metabolic detoxification of the insecticide while the other is associated with changes in target site sensitivity.

3.4 The Insect Growth Regulators

As the name implies the IGRs cause a disruption in the moulting process. IGRs such as methoprene act as juvenile hormone mimics preventing the process of ecdysis while ones such as diflubenzuron inhibit the synthesis of chitin [20]. Since they act on the immature stage of the insect, they are widely used in environmental control but are of little use when the immediate control of adult insects is necessary. However, lufenuron is used to prevent viable egg production in the cat flea and both cyromazine and diclazuril are used to control blowfly strike in sheep. Structural changes in *Lucilia* cuticle after treatment with cyromazine suggest that the mechanism of action is not identical to diflubenzuron [21].

3.5 The Macrocyclic Lactones

The avermectins and milbemycins are a group of related macrocyclic lactones isolated from *Streptomyces* microorganisms. They block electrical activity in nerve and muscle preparations by increasing the membrane conductance to chloride ions causing ataxia and paralysis. Blocking occurs at the α-subunit of the glutamate-gated chloride channels in invertebrates. Probably due in part to its very widespread use to control nematodes in cattle, resistance has developed to ivermectin in the cattle tick *R. microplus* and been reported from Brazil, Mexico and Uruguay [22–24]. It was associated with an ABC transporter which was significantly upregulated in ivermectin-resistant female ticks [25].

3.6 The Arylheterocycles Phenylpyrazoles

Phenylpyrazole insecticides such as fipronil disrupt normal nerve functions by blocking the GABA-gated chloride channels of neurons in the central nervous system. The GABA receptors are responsible for inhibition of normal neural activity by preventing excessive stimulation of the nerves. Blocking of these receptors results in neural excitation and ultimately death. In houseflies resistance has been associated with microsomal oxidase and also esterase and was autosomally inherited, incompletely dominant and polygenic. In the USA an isolate of fleas from a field complaint case showed resistance to fipronil [26].

3.7 The Chloronicotinyl Nitroguanidines

Imidacloprid, thiacloprid and nitenpyram belong to this group of insecticides and are used as crop and structural pest insecticides and for flea control treatment. Imidacloprid binds to the nicotinergic acetylcholine receptors present on the postsynaptic membrane in the nervous system. This induces a slow depolarization in motoneuron cells from cockroach nerve cord preparations [27]. Although to date there has been no reported cases of resistance in ectoparasites to this insecticide, it has been found in the housefly in the USA [28].

3.8 Spinosad

Spinosad is produced by an actinomycete, *Saccharopolyspora spinosa*, and is one of a series of spinosyns [29]. The insecticide targets binding sites on nicotinic acetylcholine receptors that are different to other sites affected by insecticides. Resistance has been recorded from a field isolate of female *Musca domestica* [30] and in thrips is associated with a mutation in the acetylcholine receptor and also cytochrome P450 detoxification [31].

3.9 Isoxazolines

Isoxazolines are a novel class of insecticides that inhibit γ-aminobutyric acid gated chloride channels and L-glutamate-gated chloride channels [32]. They are active against cat fleas (*C. felis*), sheep blow fly larvae (*L. cuprina*), cattle ticks (*R. microplus*), the dog tick (*R. sanguineus*) and nymphs of *Ornithodorus moubata* [33].

3.10 New and Novel Approaches to Insect Control

The widespread development of resistance in many insect species to the available control chemicals has led to new and novel approaches being developed. The use of toxins from

alternative species such as scorpions, spiders and sea anemones [34, 35] is being developed with transgenic plants and recombinant baculoviruses being used as the delivery system [36] in crop pests. Naturally occurring plant products such as the coconut-derived emulsion shampoo for the control of the human head louse *Pediculus capitis* [37] have proven to be highly effective. Ozone is already recognised as an alternative fumigant in the control of stored product insects [38] and is being investigated as a method of control for insects that act as vectors for the transmission of both human and veterinary diseases.

4 The Development of Resistance in Individual Genera of Insects

4.1 Arachnida

4.1.1 Acari (Mites)

The acari are a subclass of the Arachnida containing two superorders: the anactinotrichida which contains the orders astigmata, prostigmata and oribatida and the actinotrichida which contains four orders, two of which are of medical importance, the mesostigmata and ixodida. The order astigmata contains the medically and veterinary important *Sarcoptes* and *Psoroptes* mites which cause scabies in humans and mange or scab in domestic animals in addition to various other allergic responses. The larval stages of three families of mites can act as vectors of scrub typhus, a rickettsial disease affecting humans while other families cause dermatitis in both humans and domestic animals. Treatment is traditionally with either topical applications of lindane or permethrin; however recently ivermectin has proved effective. Resistance has been described in Norwegian scabies to lindane [39], but none has been described in *Pthirus pubis*.

The order Prostigmata contains the demodex mites of which *D. canis* can cause demodectic mange in dogs whilst related species infect domesticated farm animals. The order Mesostigmata includes the suborder Dermanyssina which is of veterinary importance since this includes the chicken red mite *Dermanyssus gallinae*. Resistance has been recorded in *D. gallinae* to DDT, organophosphates and pyrethroid insecticides [40–42].

Early reports of resistance in *Psoroptes ovis* came from South America to the insecticide hexachlorocyclohexane (HCH) [43] and lindane plus diazinon [44]. Following UK deregulation of sheep scab resistance has been reported to the synthetic pyrethroid, flumethrin [45], and the organophosphate, propetamphos [46]. Ivermectin failed to control psoroptic mange in Belgium blue cattle in both Belgium [47] and the UK [48] while sarcoptes mites refractory to ivermectin have been reported from dogs [49].

4.1.2 Ixodida (Ticks)

The ixodida ticks can be divided into two groups: the argasidae or soft ticks and the ixodidae or hard ticks. Both feed on vertebrate blood and are major vectors in the spread of disease.

Resistance was first recorded in *R. microplus to* arsenic prior to 1940 and to DDT and other chlorinated hydrocarbons by the early 1950s [50]. By the late 1960s resistance to the organophosphorus acaricides and carbamates had also been reported [51]. In South America *R. microplus* resistance has been documented to organophosphates, synthetic pyrethroids and amitraz [52]. In 2001 Martins and Furlong [53] documented the first failure of injectable avermectin in the control of *R. microplus*.

Li et al. [54] reported resistance in *R. microplus* to the acaricides coumaphos and diazinon with a significant cross-resistance pattern between the two. Their results suggest an enhanced cytochrome P450 monooxygenase (cytP450)-mediated detoxification mechanism may exist in resistant strains in addition to the cytP450-mediated metabolic pathway that activates coumaphos. The failure of piperonyl butoxide (PBO) to synergise diazinon suggests a specific cytP450 involved in detoxification [54]. They concluded that resistance to coumaphos was likely to be conferred by a cytP450-mediated detoxification mechanism in addition to the mechanism of insensitive acetylcholinesterases.

Resistance continues to be documented to all available drugs especially in the tropical and suptropical cattle producing areas. Abbas et al. [55] give a detailed account of resistance to date.

4.2 Insecta

4.2.1 Diptera (Flies)

A number of diptera families contain species of medical and veterinary importance. This ranges from the nuisance bite of midges and stable flies through the transmission of diseases and various forms of myiasis. Myiasis is defined as the invasion of living tissue of animals by larvae of Diptera [56].

Ceratopogonidae (Biting Midges)

The *Ceratopogonidae* is a large family containing more than 60 genera and nearly 4000 species [57]. Most of the females within this family require a protein meal for maturation of the ovaries but this is only acquired from a blood meal taken from warm-blooded animals in four genera. The genera of *Culicoides* is the largest and is of veterinary importance in horses since the bite of *Culicoides* can lead to the development of culicoides hypersensitivity, more commonly referred to as sweet-itch. They are also important as vectors of arboviruses, blood-dwelling protozoa and filarial worms. Since people entering into areas of midges are more sensitive to their bite, control has been essential in certain parts of the world before an area can be opened up for tourism.

Despite this no reports of resistance have been recorded.

Psychodidae-Phlebotominae (Sandflies)

Around 700 species of phlebotominae have been described, found mainly in warmer climates. They are the intermediate host of leishmaniases and vectors of *Bartonella* and the viral disease, papatasi fever. Since their distribution is patchy and they are rarely present in sufficient numbers to reach pest proportions, control is conducted by house spraying, usually as part of malaria control. Resistance to DDT has been reported from across India [58–61] while elevated esterase and altered acetylcholinesterases, the mechanisms associated with resistance, have been isolated from populations from Sri Lanka [62]. Deltamethrin impregnated collars for dogs have recently been introduced to areas where canine leishmaniasis is widespread [63].

Simuliidae (Blackflies)

There are 24 genera of simuliidae of which four are of economic importance. The females of most simuliids require a blood meal for egg development to occur and they will feed off a variety of mammals including man. Eggs are laid in slow running water and therefore control is targeted towards this stage of the insect life cycle. Simuliidae are important as vectors in the spread of the filarial worm, *Onchocerca volvulus,* which causes onchocerciasis or river blindness. Resistance has been recorded in the larva of simuliidae to the insecticide permethrin [64]. Montagna et al. [65] report on the mechanisms of both pyrethroid and DDT resistance in populations of Patagonian *Simulium* where resistance was attributed to agricultural insecticide exposure.

Tabanidae (Horseflies, Deer Flies and Clegs)

There are more than 400 species of tabanidae organised into four sub-families, three of which are of economic importance since they are the vectors of three species of filarial worm and various viral diseases. In large numbers tabanids worry stock leading to a loss of production. While little effort has been made to control tabanidae species, Leprince et al. [66] describe the use of lambda-cyhalothrin impregnated ear tags for the control of *Tabanus fuscicostatus* on cattle.

Glossinidae (Tsetse Flies)

Tsetse flies are the biological vectors of pathogenic trypanosomes which cause sleeping sickness in humans and nagana in cattle. The breeding and feeding habits of tsetse make chemical control difficult and therefore most control is via visual and odour baited traps [67]. As a result the development of resistance has not been reported.

Muscidae and Fanniidae (Houseflies and Stableflies)

Musca domestica, the housefly, has a worldwide distribution and is important since it has been found to harbour over 100 different pathogens from helminths to viruses. Their role in the spread of disease is unclear. Greenberg [68] found a reduction in diarrhoeal infections due to *Shigella* but those due to *Salmonella* were unaffected following spraying with DDT. However because of their close contact with man chemical control has been extensively used. One of the earliest recorded incidences of DDT failure was to the housefly in Sweden and Denmark. Resistance has been recorded to the IGRs in *Musca domestica* in Turkey [69] and to cyromazine in the UK [70]. The stomoxyinae are haematophagous insects found worldwide. Both *Stomoxys calcitrans* and *Haematobia irritans* are of economic importance since their bite causes worry in livestock which can lead to reductions in milk yield and loss of condition. The control of *H. irritans* in the USA has primarily been based on the use of insecticides and therefore widespread resistance has resulted. The first reported cases of resistance was to the OP fenchlorphos in 1963 [70, 71]; however there had been unconfirmed reports of DDT failure as early as 1959 [72]. This was followed by reports of resistance to tetrachlorvinphos which was the first insecticide used in impregnated ear tags [73]. The late 1970s saw the use of pyrethroids for *H. irritans* control but reports of resistance soon followed [74–76]. Recent studies have shown resistance to diazinon, fenthion, ethion, pirimiphos-methyl and tetrachlorvinphos [77].

Calliphoridae (Blowflies)

The calliphoridae is a large family of over 1000 species. Two families are of medical and veterinary importance, the Chrysomyinae and Calliphorinae. The Calliphorinae are important since they are the agents of myiasis or the invasion of living tissue. *Cochliomyia hominivorax* is an obligatory agent of myiasis [53] and will attach to both animals and humans. Because of their life cycle they are ideally suited to control by non-chemical methods and therefore in the USA and Central America the screwworm fly has been eradicated through a controlled programme of sterile male release. Flies in the genus *Lucilia* and *Calliphora* are facultative agents of myiasis which can cause considerable economic loss as well as being a major welfare issue. Control is essential in the large sheep producing countries. Early control methods relied on plunge dip formulations of organochlorine compounds. Resistance was first recorded in Australia to the organophosphate insecticide in 1965 [78], to the carbamate group of insecticides in 1973 [79] and malathion in 1984 [80]. McKenzie [81, 82] reported resistance to dieldrin, diazinon and malathion all primarily due to allelic substitutions at a single genetic loci. Bioassays of field and laboratory populations [83] indicated no resistance to the pyrethroid deltamethrin despite its widespread use for lice control but resistance has been reported to cyromazine [84]. Because of the welfare issues involved with myiasis and the development of resistance, alternative methods of control are being evaluated using traps and targets.

Oestridae **(Gad Flies, Warble Flies and Stomach Bots)**
The Oestridae are divided into four groups or sub-families—Oestrinae, Hypodermatinae, Gasterophilinae and Cuterebrinae. The Oestrinae develop in the nasopharyngeal cavity of sheep, goats, equids and camels. The Hypodermatinae and Cuterebrinae are dermal parasites of cattle, rabbits and rodents, while the Gasterophilinae parasitise the alimentary tract of equids. Rich [85] described the infestation of sheep with *Oestrus ovis* as a relatively benign disease. There are no current reported cases of resistance. The warble flies *Hyperderma bovis* and *H. lineatum* are parasites of cattle which cause the phenomenon called 'gadding' and result in reduced weight gain and reduced milk production. *Gasterophilus* are stomach bots of equids that cause swelling around the point of attachment which in heavy infestations can cause chronic gastritis, loss of condition and in rare cases, perforation and death [86]. As with the Oestrinae there are no current reports of resistance.

4.2.2 Hemiptera (Bugs)

While a number of Hemiptera are blood sucking only two families are of medical importance, the Cimicidae and the Triatominae. Both are temporary ectoparasites of birds and mammals.

The two species of *Cimex* of medical importance are the bedbugs *C. lectularius* and *C. hemipterus*. Both parasitise on humans and chickens while *C. lectularius* also parasitises domestic animals. *C. lectularius* is distributed throughout both temperate and subtropical regions while *C. hemipterus* occurs in warmer tropical regions. While many have been implicated in the spread of disease, there is no scientific data to support this; however Hepatitis B antigens have been found to persist for up to 6 weeks and are present throughout this period in the faeces [87]. Lyons et al. [88] recorded the presence of HIV in *C. lectularius* for up to 1 h allowing for the possibility of mechanical transmission, but Lindsay [89] showed that bedbugs were not major routes for the distribution of Hepatitis virus and were therefore much less likely to transmit HIV since this is a much less virulent virus.

The medical importance of bedbugs is due to their irritating bite which can cause sleeplessness and they have recently undergone a resurgence thought to be due to increased travel, population management practices and the development of resistance [90]. A recent study found a 5200-fold increase in resistance to deltamethrin in bed bugs from Virginia with multiple mechanisms of resistance present within a single population [91].

By far the more important biting bugs medically are the triatominae as they act as vectors of Chagas' disease caused by *Trypanosoma cruzi*. The mature trypanosomes are found in the faeces of the bugs which are deposited on the skin while the bug feeds. Once triatominae are infected with *T. cruzi*, they remain so throughout their life which can extend for several years. Reports of resistance in triatominae are limited but testing of *T. infestans* populations in Bolivia indicates that most 'domestic' populations are resistant to deltamethrin and pyrethroid spraying in Argentina failed to control the Chagas disease vectors [92, 93].

4.2.3 Phthiraptera (Lice)

The phthiraptera comprise four groups. The Anoplura and Rhynchophthirina groups are blood sucking lice of mammals, while the Amblycera and Ischnocera groups are chewing lice which live off skin debris. Within the Anoplura group are the medically important *Pediculus capitis, P. humanus* and *Pthirus pubis* (the human head, body and pubic lice). *P. humanus* is important for its role as the vector in the spread of epidemic typhus (*Rickettsia prowazekii*) and relapsing fever (*Borrelia recurrentis*). The medical importance of *P. capitis* and *P. pubis* is due to the development of secondary infections following scratching.

Early reports of insecticide failure were in *P. humanus* to DDT following its extensive use in World War II. Resistance has been widely reported in *P. capitis* to synthetic pyrethroids [94–96] and the beginning of resistance to the insecticide carbaryl has been reported in the UK [96]. Resistance has also been recorded in the sheep louse (*Bovicola ovis*) in Southern Australia to synthetic pyrethroids [97]. Lee et al. [98] showed that the mechanism primarily involved in pyrethroid resistance in head lice is due to two point mutations in the para-orthologous sodium channel α-subunit possibly supplemented by oxidative metabolism as shown by synergism with PBO. Resistance in *B. ovis* has also been reported to the IGRs triflumuron and diflubenzuron [97].

4.2.4 Siphonaptera (Fleas)

The order Siphonaptera is a large one comprising over 2000 species and subspecies.

They are laterally compressed, hematophagous insects with a worldwide distribution. Two of the most important species are *C. felis* and *C. canis*, the cat and dog flea, [99] since both have a low host specificity and will parasitise both animals and man. Both species have been reported to be the intermediate host of *Dipylidium caninum* the dog tapeworm [100, 101] which is an occasional parasite of man [102]. More importantly for medical reasons, they are reported to be the transmitter of Friend Leukemia Virus [103], *Rickettsia typhi* [104] and *Yersinia pestis* [105–107]. In addition to their importance in the spread of diseases the bite of the flea can cause the condition flea allergy dermatitis in domestic pets.

Early reports of resistant fleas occurred in 1952 from the southern United States and by 1971 resistance was being recorded to the insecticides chlordane, dieldrin and hexachlorocyclohexane (HCH) [108]. Since then resistance has been recorded to bendiocarb, carbaryl, diazinon, malathion, propetamphos and propoxur [109] plus, cypermethrin,

D-phenothrin, fenvalerate, permethrin and resmethrin. The later generation of insecticides used for flea control, such as the arylheterocycles phenylpyrazoles, has as yet not been implicated in the development of resistance, but it is probably just a matter of time before they are.

5 Cross Resistance

Cross resistance is where an insect with resistance to one insecticide is able to survive exposure to a related insecticide. Examples of this are where insects display resistance to both lindane and dieldrin or parathion and malathion due to a common mode of insecticide action such as inhibition of acetylcholinesterase. If the same mechanism of resistance does not occur, then multiple resistance has developed and not cross resistance. While it can be understood that cross resistance develops between insecticides in the same class, cross resistance has also been recorded between different groups, e.g. the pyrethroid deltamethrin and the organophosphate fenthion in *Aedes aegypti* [110] and organophosphate resistance plus butacarb resistance in *Lucilia cuprina* [79].

6 Conclusion

In many ectoparasite species there is clearly a lack of knowledge regarding resistance mechanism, the genetics of resistance and possible changes in the biology associated with the development of resistance. There is an almost total lack of knowledge on the epidemiology of insecticide resistance even in such important insects as the cat flea. Despite this lack of knowledge, experience in the control of ectoparasites demonstrates that any widespread use of insecticides is very likely to result in the development of resistance. To combat this, treatments should either give 100 % control or substantial populations of ectoparasites need to be left untreated to ensure that parasites surviving treatment make only a small contribution to the next generation. Where ectoparasites are permanent residents on the host, this latter strategy will obviously not be acceptable on the grounds of welfare.

Therefore, monitoring for insecticide resistance is vital using tests based on discriminating doses or, where available, biochemical and/or molecular based tests.

Where resistance is found, alternative insecticides must be used or alternative non-insecticidal treatments must be developed. These may include the development of vaccines, use of target traps, the application of repellents and non-chemical control agents such as emulsions of oils to prevent insect respiration, fine silica dust to disturb water balance or changes in lighting patterns to disrupt feeding behaviour [111]. Ideally integrated pest management will evolve using a variety of strategies to control ectoparasites so that total reliance on chemical control is not necessary, thereby reducing the risk of resistance developing.

References

1. Devonshire AL, Field LM. Gene amplification and insecticide resistance. Annu Rev Entomol. 1991;36:1–23.
2. Bossard RL, Hinkle NC, Rust MK. Review of insecticide resistance in cat fleas (Siphonaptera: Pulicidae). J Med Entomol. 1989;35:415–22.
3. Coles GC, Dryden MW. Insecticide/acaricide resistance in fleas and ticks infesting dogs and cats. Parasit Vectors. 2014;7:8.
4. Roush RT, Miller GL. Considerations for design of insecticide resistance monitoring programs. J Econ Entomol. 1986;79:293–8.
5. Gunning RV, Easton CS, Greenup LR, Edge VE. Pyrethroid resistance in *Heliothis armiger* (Hübner) (Lepidoptera: Noctuidae) in Australia. J Econ Entomol. 1984;77:1283–7.
6. Sattelle DB. Acetylcholine receptors of insects. Adv Insect Physiol. 1980;15:215–315.
7. Venter JC, di Portio U, Robinson DA, Shreeve SM, Lai J, Kerlavage AR, Fracek Jr SP, Lentes KU, Fraser CM. Evolution of neurotransmitter receptor systems. Prog Neurobiol. 1988;30:105–69.
8. Fournier D, Bride JM, Poirie M, Berge JB, Plapp FW Jr. Insect glutathione transferases. Characteristics of the major forms from houseflies susceptible and resistant to insecticides. J Biol Chem. 1992;267:1840–5.
9. Chaabihi H, Fournier D, Fedon Y, Bossy JP, Ravallec M, Devauchelle G, Cerutti M. Biochemical characterization of *Drosophila melanogaster* acetylcholinesterase expressed by recombinant baculoviruses. Biochem Biophys Res Commun. 1994;203:734–42.
10. Corbett JR, Wright K, Baillie AC, editors. Insecticides acting elsewhere in the nervous system. II. Compounds that bind to the acetylcholine receptor. In: The biochemical mode of action of pesticides. 2nd ed. London: Academic Press; 1984. p. 159–62.
11. Mutero A, Pralavorio M, Bride JM, Fournier D. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. Proc Natl Acad Sci U S A. 1994;91:5922–6.
12. Zhu KY, Lee SH, Clark JM. A point mutation of acetylcholinesterase associated with azinphosmethyl resistance and reduced fitness in Colorado potato beetle. Pestic Biochem Physiol. 1996;55:100–8.
13. Kozaki T, Shono T, Tomita T, Kono Y. Fenitroxon insensitive acetylcholinesterases of the housefly, *Musca domestica* associated with point mutations. Insect Biochem Mol Biol. 2001;31:991–7.
14. Walsh SB, Dolden TA, Moores GD, Kristensen M, Lewis T, Devonshire AL, Williamson MS. Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance. Biochem J. 2001;359:175–81.
15. Devonshire AL, Heidari R, Bell KL, Campbell PM, Campbell DE, Odgers WA, Oakeshott JG, Russel RJ. Kinetic efficiency of mutant carboxylesterases implicated in organophosphate insecticide resistance. Pestic Biochem Physiol. 2003;76:1–13.
16. Vontas JG, Hejazi MJ, Hawkes NJ, Cosmidis N, LouKas I, Hemingway J. Resistance associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. Insect Mol Biol. 2002;11:329–38.
17. Baxter GD, Baxter SC. Acetylcholinesterase cDNA of cattle tick, *Boophilus microplus*: characterization and role in organophosphate resistance. Insect Biochem Mol Biol. 1998;28:581–9.
18. Hinkle NC, Koehler PG, Patterson RS. Residual effectiveness of insect growth regulators applied to carpet for control of cat flea (Siphonaptera: Pulicidae) larvae. J Econ Entomol. 1995;88:903–6.

19. Bloomquist JR. Ion channels as targets of insecticides. Annu Rev Entomol. 1996;41:163–90.
20. el-Gazzar LM, Koehler PG, Patterson RS, Milio J. Insect growth regulators: mode of action on the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). J Med Entomol. 1986;23:651–4.
21. Binnington KC. Ultrastructural changes in the cuticle of the sheep blow fly, induced by certain insecticides and biological inhibitors. Tissue Cell. 1985;17:131–40.
22. Klafke GM, Sabatini GA, de Albuquerque TA, Martins JR, Kemp DH, Miller RJ, Schumaker TT. Larval immersions tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. Vet Parasitol. 2006;142:386–90.
23. Fernádez-Salas A, Rodriguez-Vivas RI, Alonso-Diaz MA. First report of a *Rhipicephalus microplus* tick population multi-resistant to acaricides and ivermectin in the Mexican tropics. Vet Parasitol. 2012;183:338–42.
24. Castro-Janer E, Rifran L, González P, Niell C, Piaggio J, Gil A, Schumaker TT. Determination of the susceptibility of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) to ivermectin and fipronil by Larval Immersion Test (LIT) in Uruguay. Vet Parasitol. 2011;178:148–55.
25. Pohl PC, Klafke GM, Júnior JR, Martins JR, da Silva Vaz I Jr, Masuda A. ABC transporters as a multidrug detoxification mechanism in *Rhipicephalus* (*Boophilus*) *microplus*. Parasitol Res. 2012;111:2345–51.
26. Schenker R, Humbert-Droz E, Moyses EW, Yerly B. Efficacy of nitenpyram against a flea strain with resistance to fipronil. Suppl Comp Cont Educ Pract Vet. 2001;23:16–9.
27. Bai D, Lummis SCR, Leicht W, Breer H, Sattelle DB. Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neuron. Pestic Sci. 1991;33:137–204.
28. Kavi LA, Kaufmann PE, Scott JG. Genetics and mechanisms of imidacloprid resistance in house flies. Pestic Biochem Physiol. 2014;109:64–9.
29. Mertz FP, Yao RC. *Saccharopolyspora spinosa* sp. nov. isolated from soil collected in a sugar mill rum still. Int J System Bacteriol. 1990;40:34–9.
30. Markussen MD, Kristensen M. Spinosad resistance in female *Muisca domestica L.* from a field-derived population. Pest Manag Sci. 2012;68:75–82.
31. Xue BW, Narai Y, Nakano A, Kaneda T, Muirai T, Sonoda S. Spinosad resistance in melon thrips, *Thrips palmi*, is conferred by G275E mutation in α6 subunit of nicotinic acetyl choline receptor and cytochrome P450 detoxification. Pestic Biochem Physiol. 2014;112:51–5.
32. Gassel M, Wolf C, Noack S, Williams H, Ilg T. The novel isoxazoline ectoparasiticide fluralaner: selective inhibition of arthropod γ-aminobutyric acid and L-glutamate-gated chloride channels and insecticidal/acaricidal activity. Insect Biochem Mol Biol. 2014;45:111–24.
33. Asahi M, Kobayashi M, Matsui H, Nakahira K. Differential mechanisms of action of the novel γ-aminobutyric acid receptor antagonist ectoparasiticides fluralaner (A1443) and fipronil. Pest Manag Sci. 2015;71:91–5.
34. Gordon D, Karbat I, Ilan N, Cohen L, Kahn R, Gilles N, Dong K, Stühmer W, Tytgat J, Gurevitz M. The differential preference of scorpion α-toxins for insect or mammalian sodium channels: implications for improved insect control. Toxicon. 2007;49:452–72.
35. De Lima ME, Figueiredo SG, Pimenta AMC, Santos DM, Borges MH, Cordeiro MN, Richardson M, Oliveira LC, Stankiewicz M, Pelhate M. Peptides of arachnid venoms with insecticidal activity targeting sodium channels. Comp Biochem Physiol C Toxicol Pharmacol. 2007;146:264–79.
36. Bravo A, Gill SS, Soberón M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 2007;49:423–35.
37. Connolly M, Stafford K, Coles GC. Control of head lice with a coconut-derived emulsion shampoo. J Eur Acad Dermatol Venereol. 2009;23:67–9.
38. Hansen LS, Hansen P, Jensen KM. Lethal doses of ozone for control of all stages of internal and external feeders in stored products. Pest Manag Sci. 2012;68:1311–6.
39. Purvis RS, Tyring SK. An outbreak of lindane-resistant scabies treated successfully with permethrin 5% cream. J Am Acad Dermatol. 1992;25:1015–6.
40. Zeman P, Zelezny J. The susceptibility of the poultry red mite, *Dermanyssus gallinae* (De Geer, 1778), to some acaricides under laboratory conditions. Exp Appl Acarol. 1985;1:17–22.
41. Chauve C. The poultry red mite *Dermanyssus gallinae* (De Geer, 1778): current situation and future prospects for control. Vet Parasitol. 1998;79:239–45.
42. Beugnet F, Chauve C, Gauthey M, Beert L. Resistance of the red poultry mite to pyrethroids in France. Vet Rec. 1997;140:577–9.
43. Ault CN, Ruben AR, Miramón RE. Resistencia del *Psoroptes comunis* var. *ovis* frente al hexaclorociclohexano. Rev Med Vet. 1962;43:357–60.
44. Rosa WAJ, Lukovich R. Experiencia con cepas de *Psoroptes ovis* de Tres Arroyos, Baños con 87, 150 y 500 partes por millón de isomero gamma y con 0,1‰ de Diazinón. Rev Med Vet Argent. 1970;51:127–9.
45. Synge BA, Bates PG, Clark AM, Stephen FB. Apparent resistance of *P ovis* to flumethrin. Vet Rec. 1995;137:51.
46. Clark AM, Stephen FB, Cawley GD, Bellworthy SJ, Groves BA. Resistance of the sheep scab mite *Psoroptes ovis* to propetamphos. Vet Rec. 1996;139:451.
47. Lekimme M, Farnir F, Maréchal F, Losson B. Failure of injectable ivermectin to control psoroptic mange in cattle. Vet Rec. 2010;167:575–6.
48. Mitchell ES, Jones JR, Foster AP, Millar M, Milnes A, Williams J. Clinical features of psoroptic mange in cattle in England and Wales. Vet Rec. 2012;170:359.
49. Terada Y, Murayama N, Ikemura H, Morita T, Nagata M. *Sarcoptes scabiei* var *canis* refractory to ivermectin treatment in two dogs. Vet Dermatol. 2010;21:608–12.
50. Wharton RH, Norris KR. Control of parasitic arthropods. Vet Parasitol. 1980;6:135–64.
51. Shaw RD, Cook M, Carson RE Jr. Developments in the resistance status of the southern cattle tick to organophosphorus and carbamate insecticides. J Econ Entomol. 1968;61:1590–4.
52. Martins JR, Correa BL, Cereser VH. Acaricidal action of fluazuron, pour-on formulation, on the cattle tick *Boophilus microplus*. Pesqui Agropecu Gaucha. 1995;1:257–61.
53. Martins JR, Furlong J. Avermectin resistance in the cattle tick *Boophilus microplus* in Brazil. Vet Rec. 2001;149:64.
54. Li AY, Davey RB, Miller RJ, George JE. Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. J Med Entomol. 2003;40:482–190.
55. Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z. Acaricide resistance in cattle ticks and approaches to its management: The state of play. Vet Parasitol. 2014;203:6–20.
56. Kettle DS. Medical and veterinary entomology. 2nd ed. London: CAB International; 1995.
57. Wirth WW, Ratanaworabhan NC, Blanton WW. Synopsis of the genera of Ceratopogonidae (Diptera). Ann Parasitol Hum Comp. 1974;49:595–613.
58. Kaul SM, Sharma RS, Borgohain BK. D.D.T. resistance in *Sergentomyia shortti* (Diptera: Psychodidae) in Kamrup. Assam--first report in *Sergentomyia* genus. J Commun Dis. 1994;26:100–2.
59. Mukhopadhyay AK, Hati AK, Chakraborty S, Saxena NB. Effect of DDT on *Phlebotomus* sandflies in Kala-Azar endemic foci in West Bengal. J Commun Dis. 1996;28:171–5.

60. Kishor K, Kumar V, Kesari S, Bhattacharya SK, Das P. Susceptibility of *Phlebotomus argentipes*, a vector of visceral leishmaniasis in different foci in three states of India. J Vector Borne Dis. 2004;36:41–4.
61. Singh RK, Mittal PK, Dhiman RC. Insecticide susceptibility status of *Phlebotomus argentipes*, a vector of visceral leishmaniasis in different foci in three states of India. J Vector Borne Dis. 2012;49:254–7.
62. Surendran SN, Karunaratne SH, Adamsn Z, Hemingway J, Hawkes NJ. Molecular and biochemical characterization of a sand fly population from Sri Lanka: evidence for insecticide resistance due to altered esterases and insensitive acetylcholinesterase. Bull Entomol Res. 2005;95:371–80.
63. Maroli M, Mizzoni V, Siragusa C, D'Orazi A, Gradoni L. First field trial of the effect of deltamethrin-impregnated dog collars (Scalibor® protector band®) on canine leishmaniasis: evidence for an impact on the infection incidence. The 18th International Conference on the World Association for the Advancement of Veterinary Parasitology, Stresa, Italy, 26–30 August 2001; 2001, p. 203.
64. Hougard JM, Escaffre H, Darriet F, Lochouarn L, Riviere F, Back C. An episode of resistance to permethrin in larvae of *Simulium squamosum* (diptera: Simuliidae) from Cameroon, after 3½ years of control. J Am Mosq Control Assoc. 1992;8:184–6.
65. Montagna CM, Anguiano OL, Gauna LE, DE-D-Angelo AMP. Mechanisms of resistance to DDT and pyrethroids in Patagonian populations of *Simulium* blackflies. Med Vet Entomol. 2003;17:95–101.
66. Leprince DJ, Hribar LJ, Foil LD. Evaluation of the toxicity of lambda-cyhalothrin against horse flies (Diptera: Tabanidae) via bioassays and exposure to treated hosts. Bull Entomol Res. 1992;82:493–7.
67. Vale GA. Development of baits for tsetse flies (Diptera: Glossinidae) in Zimbabwe. J Med Entomol. 1993;30:831–42.
68. Greenberg B. Flies and disease. Sci Am. 1965;213:92–9.
69. Cetin H, Erler F, Yanikoglu A. Survey of insect growth regulator (IGR) resistance in house flies (*Musca domestica* L.) from southwestern Turkey. J Vector Ecol. 2009;34:329–37.
70. Bell HA, Robinson KA, Weaver RJ. First report of cyromazine resistance in a population of UK house fly (*Musca domestica*) associated with intensive livestock production. Pest Manag Sci. 2010;66:693–5.
71. Burns EC, Wilson BH. Field resistance of horn flies to the organic phosphate insecticide Ronnel. J Econ Entomol. 1963;56:718.
72. Burns EC, McCraine SE, Moody DW. Ronnel and Co-Ral for horn fly control on cable type back rubbers. J Econ Entomol. 1959;52:648–50.
73. Sheppard DC. Stirofos resistance in a population of horn flies. J Ga Entomol Soc. 1983;18:370–6.
74. Roush RT. Status of pyrethroid resistance. In: U.S. Proceedings 1985 Beltwide Cotton Production Resistance Conference; 1985. p. 126–7.
75. Barros AT, Saueressig TM, Gomes A, Koller WW, Furlong J, Girão ES, Pinheiro AC, Alves-Branco F de P, Sapper MF, Braga RM, Oliveira AA. Susceptibility of the horn fly, *Haematobia irritans* (Diptera: Muscidae) to insecticides in Brazil. Rev Bras Parasitol Vet. 2012;21:125–32.
76. Oyarzún MP, Li AY, Figueroa CC. High levels of insecticide resistance in introduced horn fly (Diptera: Muscidae) populations and implications for management. J Econ Entomol. 2011;104:258–65.
77. Barros ATM, Ottea J, Sanson D, Foil LD. Horn fly (Diptera: Muscidae) resistance to organophosphate insecticides. Vet Parasitol. 2001;96:243–56.
78. Shanahan GJ, Hart RJ. Changes in response of *Lucilia cuprina* Wied. to organophosphorus insecticides in Australia. Nature. 1966;212:1466–7.
79. Roxburgh NA, Shanahan GJ. Carbamate resistance in sheep blowfly, *Lucilia cuprina* (Wied). Vet Rec. 1973;93:467.
80. Hughes PB, Green PE, Reichmann KG. Specific resistance to malathion in laboratory and field populations of the Australian sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae). J Econ Entomol. 1984;77:1400–4.
81. McKenzie JA. Measuring fitness and intergenic interactions: the evolution of resistance to diazinon in *Lucilia cuprina*. Genetica. 1993;90:227–37.
82. McKenzie JA. Ecological and evolutionary aspects of insecticide resistance. Austin, TX: R. G. Landes – Academic Press; 1996.
83. Sales N, Levot GW, Hughes PB. Monitoring and selection of resistance to pyrethroids in the Australian sheep blowfly, *Lucilia cuprina*. Med Vet Entomol. 1989;3:287–91.
84. Levot GW. Cyromazine resistance detected in Australian sheep blowfly. Aust Vet J. 2012;90:433–7.
85. Rich GB. Post-treatment reactions in cattle during extensive field tests of systemic organophosphate insecticides. Can J Comp Med Vet Sci. 1965;29:30–7.
86. Blood DC, Radostits OM. Veterinary medicine – a textbook of the diseases of cattle, sheep, pigs and horses. London: Baillière-Tindall; 1989.
87. Wills W, London WT, Werner BG, Pourtaghva M, Larouze B, Millman I, Ogston W, Diallo S, Blumber BS. Hepatitis-B virus in bedbugs (*Cimex hemipterus*) from Senegal. Lancet. 1977;2:217–9.
88. Lyons SF, Jupp PG, Schoub BD. Survival of HIV in the common bedbug. Lancet. 1986;2:45.
89. Lindsay SW. The possibility of transmission of the human immuno-deficiency virus or hepatitis B virus by bedbugs or mosquitoes. Abstracts XIIIth International Congress for Tropical Medicine and Malaria Jomtien, Pattaya, Thailand, 29 November–4 December 1992, vol. 1; 1992. p. 61.
90. Davies TG, Field LM, Williamson MS. The re-emergence of the bed bug as a nuisance pest: implications of resistance to pyrethroids. Med Vet Entomol. 2012;26:241–54.
91. Adelman ZN, Kilcullen KA, Koganemaru R, Anderson MA, Anderson TD, Miller DM. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. PLoS One. 2011;6:e26228.
92. Depickère S, Buitrago R, Siñani E, Baune M, Monje M, Lopez R, Walechx E, Chavez T, Brenière SF. Susceptibility and resistance to deltamethrin of wild and domestic populations of *Triatoma infestans* (Reduviidae: Triatominae) in Bolivia; new discoveries. Mem Inst Oswaldo Cruz. 2012;107:1042–7.
93. Gurevitz JM, Gaspe MS, Enriquez GF, Vassena CV, Alvarado-Otequi JA, Provecho YM, Cueto GA, Picollo MI, Kitron U, Gürtler RE. Unexpected failures to control Chagas disease vectors with pyrethroid spraying in northern Argentina. J Med Entomol. 2012;49:1379–86.
94. Chosidow O, Brue C, Chastang C, Bouvet E, Izri MA, Rousset JJ, Monteny N, BastujiGarin S, Revuz J. Controlled study of malathion and d-phenothrin lotions for *Pediculus humanus* var *capitis*-infested schoolchildren. Lancet. 1994;344:1724–7.
95. Mumculoglu KY, Hemingway J, Miller J, Ioffe-uspensky I, Klaus S, Ben-Ishai F, Galun R. Permethrin resistance in the head louse *Pediculus capitis* from Israel. Med Vet Entomol. 1995;9:427–32.
96. Downs AMR, Stafford KA, Hunt LP, Ravenscroft JC, Coles GC. Widespread insecticide resistance in head lice to the over-the counter pediculocides in England, and the emergence of carbaryl resistance. Br J Dermatol. 2002;146:88–93.
97. James PJ, Cramp AP, Hook SE. Resistance to insect growth regulator insecticides in populations of sheep lice as assessed by a moulting disruption assay. Med Vet Entomol. 2008;22:326–30.
98. Lee SH, Yoon K-S, Williamson MS, Goodson SJ, Takano-Lee M, Edman JD, Devonshire AL, Clark JM. Molecular analysis of kdr-like resistance in permethrin-resistant strains of head lice, *Pediculus capitis*. Pestic Biochem Physiol. 2000;66:130–43.
99. Rust MK, Dryden MW. The biology, ecology and management of the cat flea. Annu Rev Entomol. 1997;42:451–73.

100. Yasuda F, Hashiguchi J, Nishikawa H, Watanabe S. Studies on the life-history of *Dipylidium caninum* (Linnaeus 1758). Bull Nippon Vet Zootech Coll. 1968;17:27–32.
101. Yutuc LM. The cat flea hitherto unknown to sustain the larva of *Dipylidium caninum* (Linnaeus 1758) from the Philippines. Philipp J Sci. 1968;97:285–9.
102. Service MW. A guide to medical entomology. Macmillan Tropical and Subtropical Medical Texts. London: McMillan; 1980. p. 127–35.
103. Rehacek J, Fischer RG, Luecke DH. Friend leukemia virus (FLV) activity in certain arthropods. II Quantitation infectivity determinations. Neoplasma. 1973;20:147–58.
104. Farhang-Azad A, Traub R, Sofi M, Wisseman CL Jr. Experimental murine typhus infection in the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). J Med Entomol. 1984;21:675–80.
105. Blanc G, Baltazard M. Revue chronologique sur la transmission et la conversation naturelles des typhus. III. Comportement des virus de la fievre boutonneuse et de la fievre pourpree chez les puces Xenopsylla cheopsis et Ctenocephalides canis. Arch Inst Pasteur Maroc. 1914;2:602–10.
106. Ioff I, Pokrovskaya M. Experiments with fleas of human dwellings as carriers of plague infection. Izv Gos Mikrobilo Inst Rostona-Donu. 1929;9:126–36.
107. Wheeler CM, Douglas JR. Transmission studies of sylvatic plague. Proc Soc Exp Biol Med. 1941;47:65–6.
108. Brown AWA, Pal R. Insecticide resistance in arthropods. 2nd ed. Geneva: WHO; 1971.
109. el-Gazzar LM, Milio J, Koehler PG, Patterson RS. Insecticide resistance in the cat flea (Siphonaptera: Pulicidae). J Econ Entomol. 1986;79:132–4.
110. Brengues C, Hawkes NJ, Chandre F, McCarroll L, Duchon S, Guillet P, Manguin S, Morgan JC, Hemingway J. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. Med Vet Entomol. 2003;17:87–94.
111. Stafford KA, Lewis PD, Coles GC. Preliminary study of intermittent lighting regimes for red mite (*Dermanyssus gallinae*) control in poultry house. Vet Rec. 2006;158:762–3.

Index

D.L. Mayers et al. (eds.), *Antimicrobial Drug Resistance*, DOI 10.1007/978-3-319-46718-4

D

E

T