Flavia Marinelli
Olga Genilloud *Editors*

Antimicrobials

New and Old Molecules in the Fight Against Multi-Resistant Bacteria

Springer

Antimicrobials

Flavia Marinelli · Olga Genilloud
Editors

Antimicrobials

New and Old Molecules in the Fight Against Multi-Resistant Bacteria

Springer

Editors
Flavia Marinelli
Department of Biotechnology
and Life Sciences
University of Insubria Varese and
The Protein Factory Research Center
Politecnico of Milano ICRM CNR Milano
and University of Insubria
Varese
Italy

Olga Genilloud
Fundación MEDINA
Parque Tecnológico Ciencias de la Salud
Granada
Spain

ISBN 978-3-642-39967-1 ISBN 978-3-642-39968-8 (eBook)
DOI 10.1007/978-3-642-39968-8
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013948855

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The positive impact of antibiotics in human health has been challenged in the past decade by the emergence and prevalence of antibiotic resistant pathogens either in the hospitals or in the community, requiring renovated efforts to identify and develop therapeutic alternatives. The current medical need to identify antibiotics with novel structures and unexploited mode of action is triggering the development of new strategies for the discovery of natural and synthetic molecules, providing new options in the never-ending battle against ever-evolving resistant bacteria.

The objective of this book is to present an updated review of the status of all major classes of antibiotics, especially focusing on most recent advances in already known chemical classes, including new analogs and semi-synthetic derivatives, as well as the recent new classes that have reached the clinic in the past years or are in clinical and preclinical development phases. This work is divided into two major sections covering both the clinical impact of bacterial pathogens and the current trends in antibiotic discovery and development.

The first section opens with a review by Davies (Chap. 1) on the origin and evolution of antibiotics emphasizing the need to understand their role in the environment and their chemical and biological evolution to successfully exploit their pharmaceutical potential. Rossolini et al. (Chap. 2) review the evolution of the clinical impact of Gram-positive pathogens, and especially the multiresistant ones, in health care-associated and community-acquired bacterial infections, whereas Paitan and Ron (Chap. 3) analyze the rising prevalence of resistant Gram-negative pathogens, including their various resistance mechanisms, prevalence, risk factors, as one of the major clinical problem given the lack of treatment options.

The second section contains a series of 13 chapters covering the status of different classes of antibiotics, including both novel candidates in development as well as mature compounds. The emergence of pan-resistant pathogens challenging the development of new β-lactams and the most recent advances in the understanding of the action of this family of antibiotics are accurately reviewed by Leemans et al. (Chap. 4). The chemical diversity of peptide antibiotics has been classified into five different classes of compounds. Glycopeptides are extensively described by Marcone and Marinelli (Chap. 5), whereas Baltz (Chap. 6) presents the specific characteristics of daptomycin and other related lipopeptides. Lantibiotics is another emerging family of peptides with no evident cross-resistance

with any of the major classes of antibiotics (Cortes, Chap. 7). Vaara reviews the status of old and new analogs of polymyxin against Gram-negative pathogens (Chap. 8), whereas Carter and McDonalds present the recent developments in the biosynthesis and medicinal chemistry of uridyl peptide antibiotics (Chap. 9). The recent development of new aminoglycosides within the review of traditional aminoglycosides by Kirst and Marinelli provides an extensive coverage of the evolution of this old class (Chap. 10). Similarly, the chapters on traditional macrolides (Kirst, Chap. 11) and tetracyclines (Genilloud and Vicente, Chap. 12) include recent progress in the development of semi-synthetic and synthetic analogs. The last four chapters include reviews on the class of oxazolidinones (Zappia et al., Chap. 13) with description of the antibacterial activity and chemistry of this synthetic new antibiotics, the development of actinonin and its analogs as peptide deformylase inhibitor (East, Chap. 15), the status of other smaller classes of protein synthesis inhibitors (Kirst, Chap. 14), and novel bacterial topoisomerase inhibitors (Pucci and Willes, Chap. 16).

The book concludes with an extended review by Genilloud and Vicente of recent strategies developed in the pharma and academic sectors to respond to emerging medical needs (Chap. 17), ranging from the use of selected old and new targets to novel screening approaches involving the implementation of alternative technologies and mode of action studies.

The editors thank the contribution of all authors, with a special mention of Herbert Kirst, who greatly supported in the preparation and revision of the last chapters ensuring the final completion of the work.

Flavia Marinelli
Olga Genilloud

Contents

Part I
Current Trends in Antibiotics, Pathogens and Medical Needs

Chapter 1
The Origin and Evolution of Antibiotics

Julian Davies

Abstract Microbes are the most prevalent living organisms in the biosphere; they constitute about 50 % of the Earth's weight. They are also the most prolific in terms of the production of antibiotics and other bioactive small molecules. This rich store of chemical diversity (termed the Parvome) provides an inexhaustible source of therapeutic agents that has barely been investigated. Devising new ways of harvesting these compounds is a major challenge that requires developing new insights into their origin and evolution and also predictions of their roles in chemical and biological evolution. Only with this information will it be possible to exploit their pharmaceutical potential to the full.

1.1 Introduction

The biosphere is populated with an enormous collection of low-molecular weight organic compounds with an extraordinary diversity of molecular structures produced by living organisms (the Parvome) (Davies and Ryan 2012). Although a significant proportion of these compounds may be products of the normal processes of biodegradation, the majority are made by defined, regulated biosynthetic pathways and are involved in many of the functions and interactions of cells, tissues, organs, and organisms (both positive and negative). These molecules have highly specific interactions with cellular targets (although very few have been identified), and may act both extra- and intracellularly. It is likely that all living beings make bioactive small molecules for these purposes. It has been suggested that the "central dogma of biology" is more than just the triumvirate of DNA, RNA, and protein and should include the wealth of bioactive small molecules

J. Davies (✉)
Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver BC V6T 1Z3, Canada
e-mail: jed@mail.ubc.ca

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_1,

(Schreiber 2005). They play essential, but as yet, largely unidentified roles in the maintenance of the biosphere.

One of the best recognized groups of bioactive small molecules are those that have antibiotic activity; they are produced principally by bacteria, fungi, plants, and sponges, but in all probability most living organisms, including humans and elephants, etc., make such molecules. They are critically important, not because of their number or distribution, but because of their demonstrated therapeutic properties. However, it is likely that, they represent only a small fraction of the Parvome.

The word "antibiotic" was coined by Selman Waksman (Strohl 1997) soon after the discovery of streptomycin in the early 1940s. This compound, together with penicillin (historically and therapeutically), presaged the most successful medical epoch ever, the age of antibiotics. Infectious diseases became curable and billions of humans have been saved from the historical and modern plagues.

Although Waksman's definition was convenient, it ignored the vast number of bioactive compounds without detectable therapeutic potential that are produced by living organisms. In reality, the word antibiotic defines a property or an activity and not a compound.

1.2 What's in a Name?

The word antibiotic is often incorrectly used. At the present time, almost any small molecule made by a microbe is termed an antibiotic: this is loose-thinking! What can these products be called when their functions are so broad and when they have such a wide range of structures, activities, and biochemical origins?

The name "secondary metabolite" has frequently been used for bioactive compounds; this term was employed originally with reference to plant products. The simplest definition comes from "Wikipedia:" "Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism."

It is true that antibiotic activity is most frequently detected when the logarithmic phase of growth begins to slow down, when a microorganism is no longer dividing exponentially (Campbell 1984). However, although antibiotic activity cannot be detected earlier, bioactive compounds could well be produced, at concentrations not detected by inhibition. This definition also seems somewhat derogatory, since the compounds produced play many critical roles and can in no sense be referred to as "secondary"! The fact is, secondary metabolites are mostly synthesized using primary metabolites as precursors. The timing of their production with respect to growth is of no real consequence.

The word "idiolyte" has also been used as a substitute for secondary metabolite; this refers to an association with a production phase late in microbial growth. This word has not really caught on, perhaps because of its use in immunology.

1.3 Many Sources, Uses, and Functions

Bioactive small molecules have many properties and applications in medicine, industry, agriculture, and other uses (Demain and Sanchez 2009); these go far beyond Waksman's definition which states that "an antibiotic is a chemical substance produced by a microorganism which has the capacity, in dilute solutions, to inhibit the growth of, or to kill other microorganisms." It was not realized at the time that, at even lower dilutions, antibiotics might actually stimulate growth or influence other biochemical functions in microorganisms. For example, the formation of biofilms.

In order to discuss the evolution of microbially produced bioactive compounds in the context of the evolution of the cell, it is essential to realize that bioactive small molecules can, and do have many sources and probably played numerous roles in the origins of living organisms.

Thus, the Parvome components have evolved to serve numerous ecological functions in different organisms and under many circumstances. The biosynthetic pathways for small molecules may share many commonalities and probably evolved in similar ways, but their roles in the organism that produces them are often intimately related to the lifestyle of the producer. Bioactive compounds may play different roles depending on their hosts. For example, the adrenergic hormones: these are relatively simple organic molecules that have specific functions depending on the circumstances. They have been characterized as hormones produced by and playing important roles in humans and animals, but recently they have been shown to affect the properties of bacterial populations.

Norepinephrine acts as a stress hormone and a neurotransmitter in animals, but also stimulates bacterial growth and enhances bacterial virulence functions. This catecholamine was one of the first compounds identified in studies of the growing discipline of microbial endocrinology (Freestone et al. 2008). Similar activities have been reported for other congeners of this family of compounds. Interestingly, the mammalian protein hormone insulin has been shown to enhance the growth of certain bacterial pathogens (Plotkin and Viselli 2000). These are good examples of the extensive biological plurality of functions exhibited by bioactive small molecules. This is especially true for bacterial products that were first characterized for their antibacterial activity and used therapeutically. When tested employing a range of assays and concentrations for their biological activity, most so-called antibiotics prove to have a surprising range of concentration-dependent biochemical activities (Davies et al. 2006). In addition, most antibiotics have multiple toxic side effects due to their ability to bind to specific human receptors of one kind or another. Yet, for our convenience, they remain labeled as antibiotics.

Biologically active compounds with a diversity of biological activities (including antibiosis) have been present in the biosphere for eons. For example, the lichens, which are ancient mutualistic associations of fungi/algae/bacteria produce a large number of bioactive low molecular weight compounds that may originate from any of the symbionts. Evidence suggests that they play roles in the

maintenance of the lichen structure, but many have antibacterial activity (and are called antibiotics) (Muller 2001). Plants are also prolific sources of bioactive small molecules with a huge range of functions and applications (Firn and Jones 2003).

The Parvome refers to the enormous diversity of organic compounds in the biosphere: these chemical entities must be essential but what roles do they play in their natural habitats? It is becoming increasingly obvious that microbes exist in all living organisms as communities or microbiomes (Banfield and Young 2009): Bioactive small molecules are likely involved in the establishment and maintenance of microbial communities through inter-species signaling activities. These broad ecological functions are not well understood and studies of their activities in situ are still in their infancy. It is unfortunate that the pervasive notion of small molecules as weapons of attack and defence has suppressed their recognition as ubiquitous agents of communication in biology.

1.4 How Old Are "Antibiotics"?

It does not make sense to discuss the evolution of antibiotics without some consideration of their origins. How old are these compounds and in what way and when, did their biosynthetic pathways evolve? This is distinct from the commercial evolution of antibiotics taking place at this time, driven by the competition between the pharmaceutical industry and resistant pathogens.

Calculations show that a biosynthetic pathway responsible for making a complex non-ribosomal peptide antibiotic (NRP) is at least one billion years old (Baltz 2010). The biosynthetic gene cluster for daptomycin is 128 kB in size (see Baltz, this volume). However, the precursor amino acids for their synthesis must have been present in the biosphere from earlier times. The NRPs include both protein-associated amino acids and other amino acids that have only been found in NRP structures. Thus, both protein and non-protein amino acids are very old and are thought to have been delivered to the Earth as organic components of meteorites. Meteorites have been shown to transport a number of different amino acids, both protein and NRP-associated (Pizzarello and Shock 2010) into the biosphere, and they likely played roles in prebiotic chemistry (van der Gulik et al. 2009). As an example, the components of the pharmaceutically important NRP antibiotic daptomycin with 10 different amino acids, including the rare 3-methylglutamic acid, have been detected in meteorites.

1.5 Antibiotic Myths

The notion that the antibiotic activities of small molecules are used as competitive weapons is mentioned frequently, but is largely unproven. After all, there are many natural products, chemically related to the compounds used in the clinic that have no antibiotic activity and could not have been identified in conventional screens.

Similarly, there is a long-held belief that Streptomycetes and related spore-forming Actinomycetes produce the majority of useful antibiotics. This also, is not correct: the large family of *Actinobacteria* are possibly the most fruitful microbes in terms of small molecule production (Miao and Davies 2010). Even the Pseudomonads and Firmicutes produce many bioactive small molecules; not all have demonstrated antibacterial or antiviral (phage) properties but many of the compounds play roles in pathogenesis and in various signaling processes. The eukaryotes also have their champions: the Fungi are rich in small molecule production and chemical diversity and have been exploited extensively by the pharmaceutical industry.

If the truth be known it is probable that all microbes, prokaryotic and eukaryotic, produce bioactive small molecules that may exhibit antibiotic activity under certain conditions. One defining feature is that all of these products are made by large and often complex, tightly regulated biosynthetic pathways. The gene clusters vary considerably: That for tetracycline (see Genilloud and Vincente, this volume) is around 30 kB and for pristinamycin (see Kirst, this volume), more than 200 kB.

Is chromosomal DNA of high G+C composition a prerequisite for small molecule production? A number of microbes with low G+C content (Firmicutes such as Staphylococci) are known to make non-ribosomal peptides but, in general, genomes with higher G+C content appear to have the greatest potential for small molecule production. There could be a reason for this: GC-rich genomes might be considered more "ancient."

Considering the evolution of bioactive molecules without having a clear idea of their true biological roles is difficult: in most cases their small molecule productivity appears to endow no specific selective advantage to the producing host. What roles might they have played in biochemical evolution? Until exhaustive small molecule screening and genome mining have been employed to investigate the microbial world, such questions will remain unanswered.

1.6 Mode of Action and Evolution of Targets

Assuming that the majority of bioactive small molecules are ancient (possibly as old as amino acids), what types of selection pressure determined their evolution? And what can be said about the development of their complex biosynthetic pathways? The evolution of the biosynthetic pathways for molecules such as daptomycin, tetracycline, and other well-known antibiotics is of great interest (Fischbach et al. 2008; Ridley et al. 2008). It is easy to say that they are old, but how did these complex genetic systems evolve and over what period of time? There has been much speculation over the evolution of "simple" biosynthetic pathways such as those for the protein amino acids (Teichman et al. 2001).

What are the benefits of small molecules to the producing organism? There are countless microbial natural products that cannot be detected using conventional

screening approaches. What are the evolved functions for this large number of fascinating molecules?

With respect to the process of chemical evolution, low molecular weight compounds (monomers) are likely to be ancient and were used as precursors to generate more complexity: peptides followed amino acids (van der Gulik et al. 2009). The same is true for the evolution of complex organelles found in cells: ribosomes and cell walls, for example. It is now generally accepted that "early" RNA was a ribozyme and this was the precursor of the protein-rich ribosome and other catalytic RNA structures (Noller 2012).

One can imagine that the primordial synthesis of simple polymers, such as peptides/proteins required that small molecule effectors bound to the catalytic RNA and so facilitated polymerization reactions. Under certain conditions, protein synthesis inhibitors can actually enhance peptide bond formation. Similar in vitro studies may mimic the primordial catalytic reactions of RNA. In an RNA world, activities and binding sites for effectors on the RNA could eventually become the binding sites for antibiotic inhibition in ribosomes (Davies et al. 1992). This suggests that structural relationships exist between small molecule binding sites on pro- and eukaryotic organelles such as ribosomes or nucleic acid synthesis complexes. Non-ribosomal peptides may have played roles as catalysts of primitive reactions by binding to nucleic acid fragments and enhancing the activity of ribozymes. They might have evolved into site-specific binding functions that led to their subsequent activity as inhibitors. Similar evolutionary transitions might have resulted in the formation (or conservation) of small molecule binding sites on human and animal hormone receptors (Catnach and Fairclough 1992).

Modern-day antibiotics have been shown to have a wide range of biological activities depending on the concentrations used. This phenomenon, referred to as hormesis (low concentration: positive effect, high concentration: negative effect) probably applied to all bioactive molecules throughout evolution (Kendig et al. 2010). Hormesis is the key to identifying true biological activity. It can be assumed that primordial bioactive molecules appeared in the environment at low concentrations and interacted with different target molecules/structures at concentrations well below inhibitory levels (before defined biosynthetic pathways had evolved). Many relics of these reactions remain: binding of low concentrations of antibiotics to the translation system can, under some circumstances, stimulate peptide bond formation. The peptidyl transferase reaction can be enhanced by some antibiotics. The same is true for nucleic acid processes, that required ribozymes: small molecules could have modulated their activity.

1.7 Parallel Chemical and Protein Evolution

Antibiotic "evolution" during the past 60 years has essentially been a synthetic chemical process. Almost all drugs have undergone successive rounds of chemical remodeling in efforts to overcome the appearance of pathogens with acquired

resistant to the current generation of antibiotics. This has been a typical "catch-22" situation. The mechanisms of resistance have been well characterized: inactivation/destruction of inhibitor, protection of target, secretion from cell, and others. The best-studied and most dramatic example, that of the β-lactam antibiotics, has seen the evolution by mutation and selection of over 1000 β-lactamase enzymes, each with subtle variations in active site (see Leemans et al. this volume). This has occurred in response to rounds of chemical improvements of the penicillin and cephalosporin antibiotics (Bush and Jacoby 2010).

1.8 Conclusions

Microbial small molecules are ancient, huge in number, and diverse in structure and function. This brief overview of the origins of bioactive small molecules leaves many unanswered questions, particularly with respect to evolutionary mechanisms. What are the evolved natural functions for these fascinating molecules? A discussion of the evolution of these compounds without having a clear sense of their true biological roles is difficult. How many different roles might they have? They did not evolve to challenge chemists, amuse biochemists or microbiologists, or to cure diseases that were absent on the Earth before the advent of man.

The following are recommendations for future studies:

(a) Low molecular weight organic molecules played important roles in the evolution of the biology of the cell. A better understanding of these processes will lead to the identification of new receptors and compounds that bind to them.
(b) Harvesting the Parvome, using genome mining and heterologous gene cluster expression, will revolutionize the pharmaceutical industry. There is no shortage of novel compounds!
(c) Studies of small molecule activity should focus on cell–cell signaling rather than on antagonistic activities.
(d) Creative studies on the activities of bioactive small molecules in microbiomes will aid in the understanding of all aspects of health and disease.

References

Baltz RH (2010) Genomics and the ancient origins of the daptomycin biosynthetic gene cluster. J. Antibiot (Tokyo) 63:506–511

Banfield JF, Young M (2009) Variety-the Splice of Life-in Microbial Communities. Science 326:1198–1199

Bush K, Jacoby GA (2010) Updated functional classification of β-lactamases. Antimicrob Agents Chemother 54:969–976

Campbell IM (1984) Secondary metabolism and microbial physiology. Adv Microb Physiol 25:1–60

Catnach SM, Fairclough PD (1992) Erythromycin and the gut. Gut 33:397–401

Davies J, von Ahsen U, Wank H, Schroeder R (1992) Evolution of secondary metabolite production: potential roles for antibiotics as prebiotic effectors of catalytic RNA reactions. In: Chadwick DJ and Whelan J (eds) Secondary metabolites: their function and evolution. Ciba Foundation symposium:171 Wiley, Chichester, pp 24–32

Davies J, Spiegelman GB, Yim G (2006) The world of subinhibitory antibiotic concentrations. Curr Opin Microbiol 9:1–9

Davies J, Ryan KS (2012) Introducing the Parvome: bioactive compounds in the microbial world. ACS Chem Biol; 7:252–9

Demain AL, Sanchez S (2009) Microbial drug discovery: 80 years of progress. J Antibiot (Tokyo) 62:5–16

Firn RD, Jones CG (2003) The origin of secondary metabolism-a unifying model. Mol Microbiol 37:989–994

Fischbach MA, Walsh CT, Clardy J (2008) The evolution of gene collectives: How natural selection drives chemical innovation. In: Proc Natl Acad Sci USA,105:4601–4608

Freestone PP, Sandrini SM, Haigh RD, Lyte M (2008) Microbial endocrinology: how stress influences susceptibility to infection. Trends Microbiol 16:55–64

Kendig EL, Le HH, Belcher SM (2010) Defining hormesis: evaluation of a complex concentration response phenomenon. International J of Toxicol 29:235–246

Miao V, Davies J (2010) *Actinobacteria:* the good, the bad, and the ugly. Antonie van Leeuwenhoek doi: 10.1007/s10482-010-9440-6

Muller K (2001) Pharmaceutically relevant metabolites from lichens. Appl Microbiol Biotechnol 56:9–16

Noller, HF (2012) Evolution of protein synthesis from an RNA world, Cold Spring Harb Perspect Biol, 4: a003681.

Pizzarello S and Shock E (2010) The organic composition of carbonaceous meteorites: the evolutionary story ahead of biochemistry. Cold Spring Harbor Perspect Biol, Mar 2010.

Plotkin BJ, Viselli SM (2000) Effect of insulin on microbial growth. Curr Microbiol 41:60–64

Ridley CP, Lee HY, Khosla C (2008) Evolution of polyketide synthases in bacteria. Proc Natl Acad Sci USA 105:4595–4600

Schreiber SL (2005) Small molecules: the missing link in the central dogma. Nat Chem Biol 1:64–66

Strohl WR (1997) Industrial antibiotics: today and the future. In: Strohl WR (ed) Biotechnology of Antibiotics, 1st edn. Marcel Dekker, New York

Teichman SA, Rison SCG, Thornton JM, Riley M, Gough J, Chothia C (2001) The evolution and structural anatomy of the small molecule metabolic pathways in *Escherichia coli*. J Mol Biol 311:693–708

van der Gulik P, Massar S, Gilis D, Buhrman H, Rooman M (2009) The first peptides: the evolutionary transition between prebiotic amino acids and early proteins. J Theor Biol 261:531–539

Chapter 2
Novel Infectious Diseases and Emerging Gram-Positive Multi-Resistant Pathogens in Hospital and Community Acquired Infections

Gian Maria Rossolini, Fabio Arena and Simona Pollini

Abstract Gram-positive pathogens are a major cause of healthcare-associated and community-acquired bacterial infections. Staphylococci (mostly *Staphylococcus aureus* but also coagulase-negative staphylococci), enterococci, streptococci, and *Clostridium difficile* are the most important species of clinical interest. Antibiotic resistance issues are common among Gram-positive pathogens, especially among staphylococci and enterococci. Methicillin-resistant *Staphylococcus aureus* (MRSA) and glycopeptide-resistant enterococci (GRE) are paradigms for difficult-to-treat multi-resistant pathogen capable of global-scale diffusion, with remarkable impact on morbidity, mortality, and healthcare-associated costs. MRSA, in particular, is the most relevant Gram-positive multi-resistant pathogen in terms of diffusion and overall clinical impact, being a leading cause for healthcare-associated infections worldwide, as well as an emerging cause of community-acquired infections that are often associated with novel MRSA strains. Resistance to anti-MRSA and anti-VRE drugs remains uncommon or exceptional among the respective species. However, invasive infections caused by MRSA strains resistant to glycopeptides, linezolid, or daptomycin, and by VRE strains resistant to linezolid or daptomycin have increasingly been reported, especially after prolonged

G. M. Rossolini (✉) · F. Arena · S. Pollini
Department of Medical Biotechnologies, University of Siena, Siena, Italy
e-mail: gianmaria.rossolini@unisi.it

F. Arena
e-mail: arena_fabio@hotmail.it

S. Pollini
e-mail: simona.pollini@unisi.it

G. M. Rossolini
Department of Experimental and Clinical Medicine, Section of Critical Care and Specialistic Medicines, University of Florence, Florence, Italy

G. M. Rossolini
Department of Laboratory Medicine, Clinical Microbiology and Virology Unit, Careggi University Hospital, Florence, Italy

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_2,

drug exposure, and a transferable resistance mechanism to linezolid and other antiribosomal agents has recently emerged among staphylococci and enterococci. This evolving scenario underscores the need for continuing efforts aimed at surveillance and control of infections caused by multi-resistant Gram-positives, and at the discovery and development of new drugs active against these pathogens.

2.1 Introduction

Gram-positive bacterial pathogens remain a very common cause for healthcare-associated infections (HCAIs) and for community-acquired infections, and represent a major target for antimicrobial chemotherapy. The most important Gram-positives of clinical interest are staphylococci, enterococci, streptococci, and *Clostridium difficile*, although other species (e.g., *Listeria* and corynebacteria) may also play a role in some settings. The spectrum of infections caused by Gram-positives is very broad, including skin and skin structure infections (SSSIs), upper and lower respiratory tract infections, bloodstream infections (BSIs) and endocarditis, surgical site infections (SSIs), bone and joint infections, diabetic foot infections, central nervous system infections, urinary tract infections (UTIs), and intestinal infections. Central venous catheters and other artificial devices are also a common site for Gram-positive infections, mostly caused by coagulase-negative staphylococci.

Antibiotic resistance issues are common among Gram-positive pathogens, especially among staphylococci and enterococci. Methicillin-resistant *Staphylococcus aureus* (MRSA) and glycopeptide-resistant enterococci (GRE) are well-known paradigms of difficult-to-treat Gram-positive multi-resistant pathogens capable of global-scale diffusion, which have attained high proportions in several epidemiological settings (see below). Resistance problems remain overall lower with streptococci and other Gram-positives, although relatively high proportions of penicillin- and/or macrolide-resistant pneumococci are reported in many countries (EARS-Net 2010; Linares et al. 2010; Darabi et al. 2010).

The scope of this chapter is to provide an overview of the most important multi-resistant Gram-positive pathogens emerging as causes of HCAIs and community-acquired infections, i.e., MRSA and GRE, and to briefly discuss some aspects related with *Clostridium difficile* infection.

2.2 Methicillin-Resistant *S. aureus* as a Cause of Hospital- and Community-Acquired Infections

Among Gram-positives, methicillin-resistant *S. aureus* (MRSA) is by far the most relevant resistant pathogen, being a leading cause for SSSIs, BSIs, and hospital-acquired pneumonia (HAP) worldwide (Boucher and Corey 2008). MRSA strains have acquired a *mecA* gene encoding a peculiar penicillin-binding protein (PBP),

named PBP2a, which is not inhibited by methicillin, oxacillin, and other conventional β-lactams available for clinical use (see Leemans et al., this volume), and can take over the functions of the resident staphylococcal PBPs (Fuda et al. 2004). Thus, expression of PBP2a results in clinical resistance to those compounds (which are normally the first choice for treatment of *S. aureus* infections), and anti-MRSA antibiotics (which are often more toxic and expensive, see below) become the mandatory treatment option (Welte and Pletz 2010).

Overall, MRSA poses a global healthcare challenge affecting both industrialized and low-income countries. Proportions of MRSA infections can reach values higher than 50–60 % of *S. aureus* infections in some settings (Stefani et al. 2012), although with significant geographical and institutional differences which largely depend on the efficacy of infection control practices adopted at the nationwide or local level. In Europe, for instance, the proportion of MRSA among invasive isolates of *S. aureus* was reported to vary between 0.5 and 52 % in different countries, according to the most recent data from the EARS-Net surveillance system (EARS-Net 2010). In some countries (e.g., the United Kingdom and France) the enforcement of strict infection control strategies has apparently been successful in curbing the dissemination of MRSA in recent years (EARS-Net 2010; Johnson et al. 2012). However, MRSA proportions continue to be very high in several countries, and MRSA remains one of the leading multi-resistant pathogens in terms of clinical burden (EARS-Net 2010; Kock et al. 2010).

MRSA infections were originally detected in the 1960s (i.e., soon after introduction of methicillin in clinical practice) and their epidemiology has undergone significant evolution during the past decades. Initially, these infections emerged as typical hospital-acquired infections (HA-MRSA infections), and exhibited a remarkable ability at spreading both in acute-care hospitals and in long-term care facilities where strict infection control practices were not enforced (DeLeo and Chambers 2009; Kock et al. 2010). The mortality rate associated with invasive HA-MRSA infections varies considerably between different settings, but in some cases may exceed 50 % (Klevens et al. 2007; Kock et al. 2010). In the mid-2000s, in the United States, it was calculated that the yearly in-hospital mortality attributable to MRSA infections was overall comparable with the mortality associated with HIV/AIDS, viral hepatitis and tuberculosis taken together (Boucher and Corey 2008). A recent European study has confirmed the substantial clinical burden associated with MRSA BSIs in terms of mortality rates and length of hospital stay (De Kraker et al. 2011), underscoring the impact and the public health relevance of this resistant pathogen. HAP caused by MRSA also represents a major clinical challenge, with high mortality rates particularly in ventilated patients (Kollef et al. 2005; Welte and Pletz 2010). Since recent global-scale surveillance data indicate that *S. aureus* is the leading cause of HAP in the United States and Europe, being associated with approximately one-third to one-fourth of cases, respectively (Jones 2010), this further underscores the impact of MRSA in hospital-acquired infections of the lower respiratory tract. Spreading of HA-MRSA typically follows a clonal pattern. A limited number of very successful HA-MRSA clonal complexes (CCs) have disseminated internationally, with CC5 and

CC8 being the most prevalent worldwide and CC22, CC30 and CC45 being less frequently detected and limited to specific areas (Stefani et al. 2012).

More recently, MRSA infections have also emerged as community-associated (CA) infections (CA-MRSA infections) (DeLeo et al. 2010). Unlike HA-MRSA infections, CA-MRSA infections are often encountered among young and otherwise healthy subjects lacking the risk factors that are typically associated with HA-MRSA infections (i.e., long hospitalization periods, prolonged antimicrobial therapy, chronic cardiovascular, and pulmonary diseases, diabetes) (Liu et al. 2011). SSSIs are the most common presentation of CA-MRSA infections (approximately 90 % of all clinical manifestations), with many of them being mild to moderate (DeLeo et al. 2010; Skov et al. 2012). However, CA-MRSA may also cause severe infections, such as necrotising cellulitis or fasciitis and necrotising pneumonia, associated with high mortality rates (up to 75 % in case of necrotizing pneumonia) (Li et al. 2011). Noteworthy, most of the CA-MRSA strains involved in severe infections necrotising infections produce potent cytotoxins, such as the Panton–Valentine leukocidin, the a-hemolysin or the α-type phenol-soluble modulins, which are believed to play an important role in the pathogenesis of these infections (David and Daum 2010). CA-MRSA has experienced a remarkable diffusion in North America, while these infections have remained overall less common in Europe, although with an increasing trend (Otter and French 2010). CA-MRSA also disseminates with a clonal pattern, but a higher diversity has been observed in the population structure, with clonal complexes differing in different geographic areas and some being quite characteristic of specific areas or continents. For instance, while CC1 and CC8 are mostly detected among CA-MRSA from the United States and Canada, ST80 appears to circulate in Europe (DeLeo et al. 2010; Rolo et al. 2012). Unlike HA-MRSA strains, which usually exhibit complex multi-resistant phenotypes including non β-lactam agents (e.g., fluoroquinolones, macrolides, and lincosamides, see Leemans et al.; Pucci and Wiles; Kirst, this volume), CA-MRSA strains often remain susceptible to these drugs, and this peculiar resistance profile, together with the presence of certain classes of SCC*mec* elements carrying the *mecA* gene (e.g., SCC*mec*IV types and SCC*mec*V), have been regarded as biological markers for CA-MRSA strains (David and Daum 2010). However, in recent years, the spread of CA-MRSA clones in the hospital setting and the movement of typical HA-MRSA clones (such as CC5) in the opposite direction has increasingly been reported (Campanile et al. 2012; David and Daum 2010; Maree et al. 2007; Otter and French 2011; Song et al. 2011; Valsesia et al. 2010), blurring the original distinction between CA-MRSA and HA-MRSA infections and making typical CA-MRSA clones a potential cause for HA infections.

Since the early 2000s, livestock-associated (LA) MRSA infections in humans were also reported, caused by MRSA strains of CC398 which are commonly found among pigs and cattle (Crombe et al. 2012; Porrero et al. 2012; Schaumburg et al. 2012; van Cleef et al. 2011). LA-MRSA infections caused by CC398 strains have mostly been reported from Europe and only sporadically from Asia and the United States (Monecke et al. 2011). These infections appear to be common only in

individuals having frequent contact with livestock and living in geographical areas with high density of farms (van Cleef et al. 2011), and may range from mild SSSIs to severe infections such as BSIs, endocarditis, pneumonia, and necrotising fasciitis (Mammina et al. 2010; Soavi et al. 2010; van der Mee-Marquet et al. 2011). Recent studies indicate that LA-MRSA is not significantly spreading into hospital settings in Europe, and that invasive infections are quite uncommon (Grundmann et al. 2010; Wassenberg et al. 2011).

The most popular options for MRSA infections include vancomycin, teicoplanin (see Marcone and Marinelli, this volume), linezolid (see Zappia et al., this volume), and daptomycin (see Baltz, this volume). Tigecycline (see Genilloud and Vicente, this volume) is also very active against MRSA, while telavancin (a new lipoglycopeptide, see Marcone and Marinelli, this volume) and ceftaroline (a new cephalosporin endowed with high binding affinity to PBP2a, see Leemans et al., this volume) have been the most recent additions in the repertoire of anti-MRSA drugs. Moreover, a number of novel anti-MRSA agents of various classes are found at various developmental stages of the pipeline (e.g., dalbavancin, oritavancin, razupenem, omadacycline, and nemonoxacin) (Hait et al. 2011; Kihara et al. 2008; Li et al. 2010; Zhanel et al. 2010; see Marcone and Marinelli; Leemans et al.; Genilloud and Vicente; Pucci and Wiles, this volume).

Vancomycin and teicoplanin (see Marcone and Marinelli, this volume) are normally considered the first choice for infections caused by MRSA, although with some limitations related with slow bactericidal activity, potential toxicity (especially for vancomycin), and individual pharmacokinetic variability which mandates for therapeutic drug monitoring at least in severe infections (Liu et al. 2011). Despite an increased use in clinical practice since almost three decades (due to the global emergence of MRSA), resistance to glycopeptides has remained very uncommon among MRSA strains. In fact, *S. aureus* has evolved two mechanisms of glycopeptide resistance, of which one is mediated by chromosomal mutations that alter the cell wall structure and physiology limiting the access of glycopeptides to the D-ala-D-ala target in peptidoglycan precursors, while the other is mediated by acquisition of a *van* gene cluster which is responsible for the synthesis of modified peptidoglycan precursors with reduced affinity for glycopeptides. The former mechanism has been described since the late 1990s (Hiramatsu et al. 1997) and is associated with a moderate increase in MIC values (usually up to 4–8 mg/L for vancomycin, the so-called VISA phenotype) (Howden et al. 2010). In some cases the VISA phenotype is only expressed by a subpopulation in a background of susceptible bacterial cells (the so-called hVISA phenotype) (Howden et al. 2010). The emergence of VISA and hVISA strains appear to be typically associated with prolonged exposure to glycopeptides, and such strains are often recovered from patients with vancomycin treatment failure (Bae et al. 2009; Howden et al. 2010; Khatib et al. 2011). Indeed, isolates exhibiting the VISA phenotype have been identified belonging to many epidemic MRSA clonal lineages, including the hospital acquired ST5 and ST8 (Gardete et al. 2008; Hageman et al. 2008; Howe et al. 2004), but their overall proportions has remained low and significant epidemic diffusion has not been observed. Several mutations associated with the

VISA phenotype have been characterised (Gardete et al. 2012; Howden et al. 2010), and it has been demonstrated how the stepwise accumulation of mutations can lead first to the hVISA phenotype and that to a homogeneous VISA phenotype (Neoh et al. 2008). Noteworthy, mutations involved in the resistance phenotype can also be responsible for the repression of some virulence-related properties (such as the quorum sensing regulator Agr, the α-type phenol-soluble modulins, α-hemolysin and protein A), which may help the resistant bacteria to evade the host immune system (Gardete et al. 2012) but could also be associated with reduced fitness and poor in vivo survival (McCallum et al. 2006) accounting for the low propensity to epidemic diffusion exhibited by VISA strains.

Glycopeptide resistance mediated by acquisition of a *van* gene cluster is typically associated with higher MICs (vancomycin MICs are usually >16 mg/L; the so-called VRSA phenotype). This resistance mechanism was first detected in an MRSA strain isolated in 2002 in the United States (Bartley 2002) and raised considerable concern. However, only a few additional VRSA isolates have been reported thus far, including 11 isolates from the United States (Sievert et al. 2008, http://www.cdc.gov/HAI/settings/lab/vrsa_lab_search_containment.html), one from India (Saha et al. 2008) and 1 from Iran (Aligholi et al. 2008), showing no propensity to cross-transmission and epidemic diffusion, and in no case VRSAs were involved in severe bacteremic infections (most isolates were from infected ulcers or wounds, or simply colonizers). This was likely due to a fitness defect associated with the modified cell wall structure. In fact, competition experiments between an MRSA recipient of CC5 (a lineage prone to the acquisition of resistance traits) and its isogenic VRSA transconjugant revealed that, in the absence of vancomycin, the transconjugant had a significant fitness disadvantage (Kos et al. 2012). GRE were the most likely source of the *van* operon found in VRSA strains, as suggested by the similarity of their genetic contexts and by results of in vitro and in vivo transfer experiments (Perichon and Courvalin 2009). Indeed, in many cases of VRSA isolation, a GRE had also been co-isolated from the patient (Perichon and Courvalin 2009).

The most recent anti-MRSA drugs may offer advantages in terms of pharmacokinetic properties, clinical efficacy, and/or reduced toxicity and usually retain activity against glycopeptide non-susceptible MRSA strains (with the exception of daptomycin, which exhibit reduced activity against some VISA strains (Yang et al. 2010). Linezolid (see Zappia et al., this volume) is the most popular anti-MRSA option (in alternative to glycopeptides) due to oral bioavailability and improved clinical outcomes reported in some infections such as nosocomial pneumonia (Wunderink et al. 2012) and complicated SSSIs (Itani et al. 2010).

Linezolid resistance is still very uncommon among staphylococci, with susceptibility rates close to 100 % among MRSA, and slightly lower (98 %) among methicillin-resistant CNS (Flamm et al. 2012; Jones et al. 2009; Ross et al. 2011). Resistance to linezolid can be due to mutational modification of the ribosomal target (23S rRNA or L3 and L4 ribosomal proteins) (Long and Vester 2012), and in case of rRNA mutations can increase in a stepwise manner with the accumulation of mutated copies of the 23S rRNA genes in the bacterial chromosome

(Besier et al. 2008). This type of resistance has mostly been reported following prolonged exposure to the drug (e.g., in osteomyelitis or in cystic fibrosis patients (Benefield et al. 2012; Endimiani et al. 2011), while resistant strains do not exhibit significant propensity for cross-transmission and spreading (Long and Vester 2012). A transferable resistance mechanism to linezolid, mediated by ribosomal methylation via the plasmid-encoded Cfr protein, has also been detected in MRSA and in methicillin-resistant coagulase-negative staphylococci (Bongiorno et al. 2010; Bonilla et al. 2010; Long et al. 2006; Morales et al. 2010; Sanchez-Garcia et al. 2010). The ribosomal modification carried out by the Cfr protein is associated with resistance to several anti-ribosomal agents including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (the $PhLOPS_A$ phenotype), suggesting that Cfr production could be co-selected by different antimicrobial agents used both in clinical and in veterinary practice (Long et al. 2006; see Kirst; Zappia et al., this volume). The emergence of the *cfr* gene in MRSA is a matter of major concern, since Cfr-positive MRSA strains may exhibit high linezolid MICs (up to 64 mg/L) and their potential for cross-transmission and causing nosocomial outbreaks with invasive infections (e.g., ventilator-associated pneumonia and BSIs) has been documented (Morales et al. 2010; Sanchez-Garcia et al. 2010).

Also daptomycin resistance (see Baltz, this volume) is very uncommon among MRSA, although some VISA strains may exhibit reduced susceptibility to this drug. Resistance is achieved via accumulation of multiple chromosomal mutations contributing to the increase in MIC values (Mishra et al. 2009; Yang et al. 2009). Some of these mutations, affecting cell-wall thickness, are apparently involved in cross-resistance with glycopeptides and account for the reduction of daptomycin activity against VISA strains (Cafiso et al. 2012; Yang et al. 2009). However, mutations that alter the cell surface charge (e.g., mutations in *yycFG* and *mprF,* and mutations that upregulate the *dltABCD* operon) were also found to be associated with decreased susceptibility to daptomycin (Yang et al. 2009, 2010), underscoring the notion that resistance to daptomycin can be achieved by multiple mechanisms. Daptomycin-resistant MRSA strains are usually selected following prolonged exposure to the drug (e.g., in osteomyelitis and orthopedic prosthesis infections) (Enoch et al. 2007) and thus far have not shown propensity to cross-transmission and epidemic diffusion.

Resistance to telavancin (see Marcone and Marinelli, this volume) and ceftaroline (see Leemans et al., this volume) has not been reported from clinical infections. However, prolonged in vitro exposure of MRSA to subinhibitory concentrations of telavancin resulted in the selection of mutants with televancin MICs of 2 mg/L (Kosowska-Shick et al. 2009), while the presence of multiple mutations in PBP2a from some MRSA isolates can result in decreased binding affinity of ceftaroline, with increased MIC values (1–4 mg/L) (Mendes et al. 2012). Altogether, these findings suggest that resistance to these new molecules could arise by mutation in a stepwise manner.

2.3 Infections Caused by Glycopeptide-Resistant Enterococci

Enterococci are gut commensals that can act as opportunistic pathogens and are a leading cause for HCAIs including UTIs, BSIs and endocarditis, SSIs, complicated intra-abdominal infections, and infections of catheters and other medical devices (Malani et al. 2002). *Enterococcus faecalis* and *Enterococcus faecium* are the two most relevant species, although infections by unusual species, such as *Enterococcus gallinarum*, have occasionally been described (Contreras et al. 2008).

Enterococci are intrinsically resistant to many antibiotics and exhibit a remarkable ability to acquire resistance to anti-enterococcal agents. From the clinical perspective, the most important resistance issue is represented by acquired resistance to glycopeptides, which are the drugs of choice for enterococcal infections caused by ampicillin-resistant strains, which are now quite prevalent (Arias et al. 2012; EARS-Net 2010; Hidron et al. 2008).

Acquired glycopeptide resistance is due to the synthesis of a modified peptidoglycan target with reduced affinity to glycopeptides following the acquisition of a set of genes (*van* genes) that encode the several functions required for modified peptidoglycan biosynthesis (Reynolds and Courvalin 2005). Several variants of such gene clusters have been discovered (e.g., *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, *vanN*) that can be associated with variable glycopeptide resistance phenotypes and are often carried on transposable elements such as *Tn1546* (Lebreton et al. 2011; Raynolds and Courvalin 2005; Sujatha et al. 2012; Xu et al. 2010; see Marcone and Marinelli, this volume).

Glycopeptide resistance in enterococci was originally reported in the late 1980s (Uttley et al. 1989) and has undergone a global diffusion during the past two decades, especially in *E. faecium*. In the United States, a remarkable dissemination of GRE has been observed, with proportions of up to 60 % reported among *E. faecium* isolates from BSIs (Deshpande et al. 2007). In Europe, the proportion of GRE is quite variable depending on the country (from 2 to 35 % for invasive isolates of *E. faecium*), and mixed trends (increasing or decreasing) have been reported in different countries (EARS-Net 2010 report).

Molecular epidemiology has identified a lineage of *E. faecium* belonging in CC17 as the leading cause of infections, and outbreaks caused by this pathogen have been reported worldwide (Willems et al. 2005). Strains of this lineage have adapted to the hospital niches and acquired virulence genes (e.g., esp_{Efm} and hyl_{Efm}) (Billström et al. 2008; Leavis et al. 2004), and are usually resistant to penicillins and often to glycopeptides.

Very few options (and not all of them approved) are available for treating infections caused by GRE, including linezolid (see Zappia et al., this volume), tigecycline (see Genilloud and Vicente, this volume), daptomycin (see Baltz, this volume), and quinupristin-dalfopristin (only for *E. faecium* strains, see Kirst, this volume).

Resistance to linezolid, which is the most popular option for GRE infections, is still uncommon among enterococci but has been increasingly reported since 2002 (Auckland et al. 2002; Gonzales et al. 2001; Ntokou et al. 2012). Resistance is usually due to ribosomal target modification following mutations of the 23S rRNA or, less frequently, of the L3 and L4 ribosomal proteins (Prystowsky et al. 2001; Saager et al. 2008). Mutants are often selected from susceptible strains following prolonged drug exposure (Rahim 2003; Swoboda 2005), but linezolid-resistant strains have also been isolated from patients with no previous exposure to the drug, indicating that these strains have some potential for cross-transmission and dissemination even in the absence of antibiotic pressure (Schutle et al. 2008). Most recently, the plasmid-encoded Cfr ribosomal methylase, which mediates transferable resistance to linezolid and other anti-ribosomal agents in staphylococci (see above), has also been detected in a linezolid-resistant strain of *E. faecalis* isolated from a patient that had received long-term linezolid treatment (Diaz et al. 2012). This observation highlights that transferable linezolid resistance mediated by Cfr could also spread among enterococci.

Enterococcal strains non susceptible to daptomycin remain relatively rare but have been reported since 2003, mostly among GREs (Kelesidis et al. 2011). Resistance can occur either in isolates exposed to prolonged drug treatment or in isolates from patients with no previous exposure to daptomycin (Lesho et al. 2006; Kelesidis et al. 2012), suggesting the possibility of cross-transmission and dissemination even in the absence of antibiotic pressure. Various mutations, either in a regulatory system involved in the cell envelope response to antibiotics (liaFSR) or in genes encoding proteins involved in phospholipid metabolism have been associated with daptomycin resistance (Arias et al. 2011; Munita et al. 2012; see Baltz, this volume), but the mechanism of resistance remains unclear. Currently, there are no known transferable determinants that confer resistance to daptomycin.

Concerning tigecycline (see Genilloud and Vicente, this volume), resistance is very uncommon among enterococcal isolates (Bérenger et al. 2011; Hope et al. 2010; Zhao et al. 2012). However, emergence of strains with increased tigecycline MICs during therapy has been occasionally documented (Werner et al. 2008), and the recent detection of enterococcal isolates with reduced susceptibility to tigecycline in different reservoirs, including animals for food consumption, suggests that selection of tigecycline-resistant isolates by antibiotics other than tigecycline might occur in non-clinical settings (Freitas et al. 2011).

2.4 Issues with *Clostridium difficile* Infections

Clostridium difficile infection (CDI) is a leading cause of nosocomial diarrhea and one of the most relevant HCAIs worldwide, with a significant burden on inpatients morbidity and mortality (Miller et al. 2011). During recent years, CDI has shown increasing trends in incidence and severity in many countries. For instance, data from US vital records indicate that the number of death certificates with

enterocolitis due to *C. difficile* listed as the primary cause of death increased from 793 in 1999 to 7483 in 2008 (Lessa et al. 2012). This dramatic increase in the incidence and severity of *C. difficile* infections has largely been attributed to emergence and global spread of hypervirulent epidemic strains, such as BI/NAP1/027 (McDonald et al. 2005). Dissemination of these strains has apparently been promoted, at least in part, by the overuse of some very popular drugs, such as the fluoroquinolones (see Pucci and Wiles), to which these hypervirulent strains are resistant.

Resistance to first-line drugs for CDI, including metronidazole for treatment of mild to moderate cases and vancomycin for treatment of severe episodes, has been reported but remains rare and is not regarded as a major clinical problem (Huang et al. 2009; Shah et al. 2010). However, a reduced response to these standard treatments and a relatively high incidence of recurrences (up to 20–25 %) have been reported (Louie et al. 2011; Tenover et al. 2012).

Rifaximin is a non-absorbable rifamycin derivative characterized by potent activity against *C. difficile* (Hecht et al. 2007; Shah et al. 2010), considered as an alternative regimen in refractory CDI and in recurrences after successful treatment with vancomycin. Recently, it has been suggested that rifaximin could also be considered as a first-line agent for mild CDI cased (Rubin et al. 2011). Resistance to rifamycins, which occurs by mutational amino acid substitutions in the β-subunit of the bacterial RNA polymerase, is overall uncommon but has occasionally been reported at high rates (Curry et al. 2009; Huang et al. 2009), and the possibility of resistance should be considered especially in patients previously exposed to rifampin or rifaximin (O'Connor et al. 2008).

Fidaxomicin is a new macrocyclic antibiotic that targets RNA polymerase, specifically developed for treatment of CDI. Fidaxomicin has potent activity against *C. difficile*, including "hyperepidemic" strains, while exhibiting a narrow spectrum of activity with low interference on the commensal microbiota and reaching high concentrations in the gut in absence of systemic absorption (Baines et al. 2008). Resistance to fidaxomicin has been described after in vitro exposure (Baines et al. 2008), but was found to be very uncommon in clinical trials (Goldstein et al. 2011). Resistance associated with mutations in *rpoB* and *rpoC* genes, encoding the β and β' subunits of bacterial RNA polymerase respectively, but these mutants are not cross-resistant to rifamycins nor cross-resistance with fidaxomycin has been reported for rifampin-resistant mutants (Babakhani et al. 2011; Baines et al. 2008).

2.5 Conclusions

Microbial drug resistance has become a public health problem of global dimension. Resistance issues affect both Gram-positive and Gram-negative pathogens. Although multi-resistant Gram-negatives are now emerging as a major clinical challenge due to the dramatic shortage of new treatment options available against

these pathogens, the overall burden caused by multi-resistant Gram-positives, and of MRSA in particular, remains of primary importance. In fact, MRSA rates continue to be high in most settings, and infections caused by MRSA and VRE strains resistant to the principal backup drugs (linezolid, daptomycin and—for MRSA—glycopeptides) have increasingly been reported. This dynamic resistance scenario, together with the ability of MRSA to evolve different epidemiological patterns (e.g., CA-MRSA and, most recently, LA-MRSA) underscores the need for continuing efforts aimed at surveillance and control of infections caused by multi-resistant Gram-positives, and at the discovery and development of new drugs active against these pathogens.

References

Aligholi M, Emaneini M, Jabalameli F, Shahsavan S, Dabiri H, Sedaght H (2008) Emergence of high-level vancomycin-resistant *Staphylococcus aureus* in the Imam Khomeini Hospital in Tehran. Med Princ Pract 17:432–434

Arias CA, Panesso D, McGrath DM, Qin X, Mojica MF, Miller C, Diaz L, Tran TT, Rincon S, Barbu EM, Reyes J, Roh JH, Lobos E, Sodergren E, Pasqualini R, Arap W, Quinn JP, Shamoo Y, Murray BE, Weinstock GM (2011) Genetic basis for in vivo daptomycin resistance in enterococci. N Engl J Med 365:892–900

Arias CA, Mendes RE, Stilwell MG, Jones RN, Murray BE (2012) Unmet needs and prospects for oritavancin in the management of vancomycin-resistant enterococcal infections. Clin Infect Dis 54(Suppl 3):S233–S238

Auckland C, Teare L, Cooke F, Kaufmann ME, Warner M, Jones G, Bamford K, Ayles H, Johnson AP (2002) Linezolid-resistant enterococci: report of the first isolates in the United Kingdom. J Antimicrob Chemother 50:743–746

Babakhani F, Gomez A, Robert N, Sears P (2011) Killing kinetics of fidaxomicin and its major metabolite, OP-1118, against *Clostridium difficile*. J Med Microbiol 60:1213–1217

Bae IG, Federspiel JJ, Miro JM, Woods CW, Park L, Rybak MJ, Rude TH, Bradley S, Bukovski S, de la Maria CG, Kanj SS, Korman TM, Marco F, Murdoch DR, Plesiat P, Rodriguez-Creixems M, Reinbott P, Steed L, Tattevin P, Tripodi MF, Newton KL, Corey GR, Fowler VG (2009) Heterogeneous vancomycin-intermediate susceptibility phenotype in bloodstream methicillin-resistant *Staphylococcus aureus* isolates from an international cohort of patients with infective endocarditis: prevalence, genotype, and clinical significance. J Infect Dis 200:1355–1366

Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, Kuijper EJ, Wilcox MH (2008) Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. J Antimicrob Chemother 62:1046–1052

Bartley J (2002) First case of VRSA identified in Michigan. Infect Control Hosp Epidemiol 23:480

Benefield RJ, Hinde GK, Abolnik IZ (2012) Osteomyelitits due to linezolid-resistant *Staphylococcus epidermidis*. Clin Infect Dis 54:1216–1217

Berenger R, Bourdon N, Auzou M, Leclercq R, Cattoir V (2011) In vitro activity of new antimicrobial agents against glycopeptide-resistant *Enterococcus faecium* clinical isolates from France between 2006 and 2008. Med Mal Infect 41:405–409

Besier S, Ludwig A, Zander J, Brade V, Wichelhaus TA (2008) Linezolid resistance in *Staphylococcus aureus*: gene dosage effect, stability, fitness costs, and cross-resistances. Antimicrob Agents Chemother 52:1570–1572

Billstrom H, Lund B, Sullivan A, Nord CE (2008) Virulence and antimicrobial resistance in clinical *Enterococcus faecium*. Int J Antimicrob Agents 32:374–377

Bongiorno D, Campanile F, Mongelli G, Baldi MT, Provenzani R, Reali S, Lo RC, Santagati M, Stefani S (2010) DNA methylase modifications and other linezolid resistance mutations in coagulase-negative staphylococci in Italy. J Antimicrob Chemother 65:2336–2340

Bonilla H, Huband MD, Seidel J, Schmidt H, Lescoe M, McCurdy SP, Lemmon MM, Brennan LA, Tait-Kamradt A, Puzniak L, Quinn JP (2010) Multicity outbreak of linezolid-resistant *Staphylococcus epidermidis* associated with clonal spread of a cfr-containing strain. Clin Infect Dis 51:796–800

Boucher HW, Corey GR (2008) Epidemiology of methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis 46(Suppl 5):S344–S349

Cafiso V, Bertuccio T, Spina D, Purrello S, Campanile F, Di PC, Purrello M, Stefani S (2012) Modulating activity of vancomycin and daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. PLoS ONE 7:e29573

Campanile F, Bongiorno D, Falcone M, Vailati F, Pasticci MB, Perez M, Raglio A, Rumpianesi F, Scuderi C, Suter F, Venditti M, Venturelli C, Ravasio V, Codeluppi M, Stefani S (2012) Changing Italian nosocomial-community trends and heteroresistance in *Staphylococcus aureus* from bacteremia and endocarditis. Eur J Clin Microbiol Infect Dis 31:739–745

Contreras GA, DiazGranados CA, Cortes L, Reyes J, Vanegas S, Panesso D, Rincon S, Diaz L, Prada G, Murray BE, Arias CA (2008) Nosocomial outbreak of *Enteroccocus gallinarum*: untaming of rare species of enterococci. J Hosp Infect 70:346–352

Crombe F, Willems G, Dispas M, Hallin M, Denis O, Suetens C, Gordts B, Struelens M, Butaye P (2012) Prevalence and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* among pigs in Belgium. Microb Drug Resist 18:125–131

Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, Pasculle AW, Harrison LH (2009) High frequency of rifampin resistance identified in an epidemic *Clostridium difficile* clone from a large teaching hospital. Clin Infect Dis 48(4):425–429

Darabi A, Hocquet D, Dowzicky MJ (2010) Antimicrobial activity against *Streptococcus pneumoniae* and *Haemophilus influenzae* collected globally between 2004 and 2008 as part of the Tigecycline Evaluation and Surveillance Trial. Diagn Microbiol Infect Dis 67:78–86

David MZ, Daum RS (2010) Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 23:616–687

de Kraker ME, Wolkewitz M, Davey PG, Koller W, Berger J, Nagler J, Icket C, Kalenic S, Horvatic J, Seifert H, Kaasch AJ, Paniara O, Argyropoulou A, Bompola M, Smyth E, Skally M, Raglio A, Dumpis U, Kelmere AM, Borg M, Xuereb D, Ghita MC, Noble M, Kolman J, Grabljevec S, Turner D, Lansbury L, Grundmann H; BURDEN Study Group (2011) Clinical impact of antimicrobial resistance in European hospitals: excess mortality and length of hospital stay related to methicillin-resistant *Staphylococcus aureus* bloodstream infections. Antimicrob Agents Chemother 55:1598–1605

DeLeo FR, Chambers HF (2009) Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era. J Clin Invest 119:2464–2474

DeLeo FR, Otto M, Kreiswirth BN, Chambers HF (2010) Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet 375:1557–1568

Deshpande LM, Fritsche TR, Moet GJ, Biedenbach DJ, Jones RN (2007) 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 58(2):163–170

Diaz L, Kiratisin P, Mendes RE, Panesso D, Singh KV, Arias CA (2012) Transferable plasmid-mediated resistance to linezolid due to *cfr* in a human clinical isolate of *Enterococcus faecalis*. Antimicrob Agents Chemother 56:3917–3922

EARS-Net (2010) Annual report of the European Antimicrobial Resistance Surveillance Network. European Centre for Disease Prevention and Control. http://www.ecdc.europa.eu/en/publications/Publications/1111_SUR_AMR_data.pdf.pdf

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 (2011) Emergence of linezolid-resistant *Staphylococcus aureus* after prolonged treatment of cystic fibrosis patients in Cleveland, Ohio. Antimicrob Agents Chemother 55:1684–1692

Enoch DA, Bygott JM, Daly ML, Karas JA (2007) Daptomycin. J Infect 55:205–213

Flamm RK, Farrell DJ, Mendes RE, Ross JE, Sader HS, Jones RN (2012) 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. doi:10.1016/j.diagmicrobio.2012.05.012

Freitas AR, Novais C, Correia R, Monteiro M, Coque TM, Peixe L (2011) Non-susceptibility to tigecycline in enterococci from hospitalised patients, food products and community sources. Int J Antimicrob Agents 38:174–176

Fuda C, Suvorov M, Vakulenko SB, Mobashery S (2004) The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. J Biol Chem 279:40802–40806

Gardete S, Aires-De-Sousa M, Faustino A, Ludovice AM, de Lencastre H (2008) Identification of the first vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) isolate from a hospital in Portugal. Microb Drug Resist 14:1–6

Gardete S, Kim C, Hartmann BM, Mwangi M, Roux CM, Dunman PM, Chambers HF, Tomasz A (2012) Genetic pathway in acquisition and loss of vancomycin resistance in a methicillin resistant *Staphylococcus aureus* (MRSA) strain of clonal type USA300. PLoS Pathog 8:e1002505

Goldstein EJ, Citron DM, Sears P, Babakhani F, Sambol SP, Gerding DN (2011) Comparative susceptibilities to fidaxomicin (OPT-80) of isolates collected at baseline, recurrence, and failure from patients in two phase III trials of fidaxomicin against *Clostridium difficile* infection. Antimicrob Agents Chemother 55:5194–5199

Gonzales RD, Schreckenberger PC, Graham MB, Kelkar S, DenBesten K, Quinn JP (2001) Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. Lancet 357:1179

Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, Friedrich AW (2010) Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med 7:e1000215

Hageman JC, Patel J, Franklin P, Miscavish K, McDougal L, Lonsway D, Khan FN (2008) Occurrence of a USA300 vancomycin-intermediate *Staphylococcus aureus*. Diagn Microbiol Infect Dis 62:440–442

Hait H, Arbeit R, Molnar D, Noel GJ, Tanaka SK (2011) In a phase 2 complicated skin and soft tissue infections (cSSTI) trial outcomes assessed early in the course of therapy were consistent with outcomes assessed 10–17 days after completing therapy for patients treated with either omadacycline (OMC; PTK796) or linezolid. 21st European congress of clinical microbiology and infectiousdiseases (ECCMID)/27th international congress of chemotherapy (ICC)

Hecht DW, Galang MA, Sambol SP, Osmolski JR, Johnson S, Gerding DN (2007) In vitro activities of 15 antimicrobial agents against 110 toxigenic *Clostridium difficile* clinical isolates collected from 1983 to 2004. Antimicrob Agents Chemother 51:2716–2719

Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK (2008) 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 29:996–1011

Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother 40:135–136

Hope R, Mushtaq S, James D, Pllana T, Warner M, Livermore DM (2010) Tigecycline activity: low resistance rates but problematic disc breakpoints revealed by a multicentre sentinel survey in the UK. J Antimicrob Chemother 65:2602–2609

Howden BP, Davies JK, Johnson PD, Stinear TP, Grayson ML (2010) Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. Clin Microbiol Rev 23:99–139

Howe RA, Monk A, Wootton M, Walsh TR, Enright MC (2004) Vancomycin susceptibility within methicillin-resistant *Staphylococcus aureus* lineages. Emerg Infect Dis 10:855–857

Huang H, Weintraub A, Fang H, Nord CE (2009) Antimicrobial resistance in *Clostridium difficile*. Int J Antimicrob Agents 34:516–522

Itani KM, Dryden MS, Bhattacharyya H, Kunkel MJ, Baruch AM, Weigelt JA (2010) Efficacy and safety of linezolid versus vancomycin for the treatment of complicated skin and soft-tissue infections proven to be caused by methicillin-resistant *Staphylococcus aureus*. Am J Surg 199:804–816

Johnson AP, Davies J, Guy R, Abernethy J, Sheridan E, Pearson A, Duckworth G (2012) Mandatory surveillance of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia in England: the first 10 years. J Antimicrob Chemother 67:802–809

Jones RN (2010) Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. Clin Infect Dis 51(Suppl 1):S81–S87

Jones RN, Kohno S, Ono Y, Ross JE, Yanagihara K (2009) ZAAPS International Surveillance Program (2007) for linezolid resistance: results from 5591 Gram-positive clinical isolates in 23 countries. Diagn Microbiol Infect Dis 64:191–201

Kelesidis T, Humphries R, Uslan DZ, Pegues DA (2011) Daptomycin nonsusceptible enterococci: an emerging challenge for clinicians. Clin Infect Dis 52:228–234

Kelesidis T, Humphries R, Uslan DZ, Pegues D (2012) De novo daptomycin-nonsusceptible enterococcal infections. Emerg Infect Dis 18:674–676

Khatib R, Jose J, Musta A, Sharma M, Fakih MG, Johnson LB, Riederer K, Shemes S (2011) Relevance of vancomycin-intermediate susceptibility and heteroresistance in methicillin-resistant *Staphylococcus aureus* bacteraemia. J Antimicrob Chemother 66:1594–1599

Kihara R, Yanagihara K, Morinaga Y, Araki N, Nakamura S, Seki M, Izumikawa K, Kakeya H, Yamamoto Y, Tsukamoto K, Kamihira S, Kohno S (2008) Potency of SMP-601, a novel carbapenem, in hematogenous murine bronchopneumonia caused by methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus*. Antimicrob Agents Chemother 52:2163–2168

Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 298:1763–1771

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 TA, Witte W, Friedrich AW (2010) Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. Euro Surveill 15:19688

Kollef MH, Shorr A, Tabak YP, Gupta V, Liu LZ, Johannes RS (2005) Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia. Chest 128:3854–3862

Kos VN, Desjardins CA, Griggs A, Cerqueira G, Van TA, Holden MT, Godfrey P, Palmer KL, Bodi K, Mongodin EF, Wortman J, Feldgarden M, Lawley T, Gill SR, Haas BJ, Birren B, Gilmore MS (2012) 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. doi:10.1128/mBio.00112-12

Kosowska-Shick K, Clark C, Pankuch GA, McGhee P, Dewasse B, Beachel L, Appelbaum PC (2009) Activity of telavancin against staphylococci and enterococci determined by MIC and resistance selection studies. Antimicrob Agents Chemother 53:4217–4224

Leavis H, Top J, Shankar N, Borgen K, Bonten M, van Embden J, Willems RJ (2004) A novel putative enterococcal pathogenicity island linked to the esp virulence gene of *Enterococcus faecium* and associated with epidemicity. J Bacteriol 186:672–682

Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V (2011) D-Ala-D-Ser VanN-type transferable vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother 55:4606–4612

Lesho EP, Wortmann GW, Craft D, Moran KA (2006) De novo daptomycin nonsusceptibility in a clinical isolate. J Clin Microbiol 44:673

Lessa FC, Gould CV, McDonald LC (2012) Current status of *Clostridium difficile* infection epidemiology. Clin Infect Dis 55(Suppl 2):S65–S70

Li CR, Li Y, Li GQ, Yang XY, Zhang WX, Lou RH, Liu JF, Yuan M, Huang P, Cen S, Yu LY, Zhao LX, Jiang JD, You XF (2010) In vivo antibacterial activity of nemonoxacin, a novel non-fluorinated quinolone. J Antimicrob Chemother 65:2411–2415

Li HT, Zhang TT, Huang J, Zhou YQ, Zhu JX, Wu BQ (2011) Factors associated with the outcome of life-threatening necrotizing pneumonia due to community-acquired *Staphylococcus aureus* in adult and adolescent patients. Respiration 81:448–460

Linares J, Ardanuy C, Pallares R, Fenoll A (2010) Changes in antimicrobial resistance, serotypes and genotypes in *Streptococcus pneumoniae* over a 30-year period. Clin Microbiol Infect 16:402–410

Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, Kaplan SL, Karchmer AW, Levine DP, Murray BE, Rybak J, Talan DA, Chambers HF (2011) Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children: executive summary. Clin Infect Dis 52:285–292

Long KS, Vester B (2012) Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob Agents Chemother 56:603–612

Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B (2006) The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. Antimicrob Agents Chemother 50:2500–2505

Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, Gorbach S, Sears P, Shue YK (2011) Fidaxomicin versus vancomycin for *Clostridium difficile* infection. N Engl J Med 364:422–431

Malani PN, Kauffman CA, Zervos MJ (2002) Enterococcal disesase, epidemiology, and treatment. In: Gilmore MS, Clewell DB, Courvalinv P, Dunny GM, Murray BE (eds) The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington D.C

Mammina C, Cala C, Plano MR, Bonura C, Vella A, Monastero R, Palma DM (2010) Ventilator-associated pneumonia and MRSA ST398, Italy. Emerg Infect Dis 16:730–731

Maree CL, Daum RS, Boyle-Vavra S, Matayoshi K, Miller LG (2007) Community-associated methicillin-resistant *Staphylococcus aureus* isolates causing healthcare-associated infections. Emerg Infect Dis 13:236–242

McCallum N, Karauzum H, Getzmann R, Bischoff M, Majcherczyk P, Berger-Bachi B, Landmann R (2006) In vivo survival of teicoplanin-resistant *Staphylococcus aureus* and fitness cost of teicoplanin resistance. Antimicrob Agents Chemother 50:2352–2360

McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, Johnson S, Gerding DN (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med 353:2433–2441

Mendes RE, Tsakris A, Sader HS, Jones RN, Biek D, McGhee P, Appelbaum PC, Kosowska-Shick K (2012) Characterization of methicillin-resistant *Staphylococcus aureus* displaying increased MICs of ceftaroline. J Antimicrob Chemother 67:1321–1324

Miller BA, Chen LF, Sexton DJ, Anderson DJ (2011) Comparison of the burdens of hospital-onset, healthcare facility-associated *Clostridium difficile* infection and of healthcare-associated infection due to methicillin-resistant *Staphylococcus aureus* in community hospitals. Infect Control Hosp Epidemiol 32:387–390

Mishra NN, Yang SJ, Sawa A, Rubio A, Nast CC, Yeaman MR, Bayer AS (2009) Analysis of cell membrane characteristics of in vitro-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53:2312–2318

Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M, Chow H, Ip M, Jatzwauk L, Jonas D, Kadlec K, Kearns A, Laurent F, O'Brien FG, Pearson J, Ruppelt A, Schwarz S, Scicluna E, Slickers P, Tan HL, Weber S, Ehricht R (2011) A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS ONE 6:e17936

Morales G, Picazo JJ, Baos E, Candel FJ, Arribi A, Pelaez B, Andrade R, De la Torre MA, Fereres J, Sanchez-Garcia M (2010) Resistance to linezolid is mediated by the cfr gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. Clin Infect Dis 50:821–825

Munita JM, Panesso D, Diaz L, Tran TT, Reyes J, Wanger A, Murray BE, Arias CA (2012) Correlation between mutations in *liaFSR* of *Enterococcus faecium* and MIC of daptomycin: revisiting daptomycin breakpoints. Antimicrob Agents Chemother 56:4354–4359

Neoh HM, Cui L, Yuzawa H, Takeuchi F, Matsuo M, Hiramatsu K (2008) Mutated response regulator *graR* is responsible for phenotypic conversion of *Staphylococcus aureus* from heterogeneous vancomycin-intermediate resistance to vancomycin-intermediate resistance. Antimicrob Agents Chemother 52:45–53

Ntokou E, Stathopoulos C, Kristo I, Dimitroulia E, Labrou M, Vasdeki A, Makris D, Zakynthinos E, Tsakris A, Pournaras S (2012) Intensive care unit dissemination of multiple clones of linezolid-resistant *Enterococcus faecalis* and *Enterococcus faecium*. J Antimicrob Chemother 67:1819–1823

O'Connor JR, Galang MA, Sambol SP, Hecht DW, Vedantam G, Gerding DN, Johnson S (2008) Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. Antimicrob Agents Chemother 52:2813–2817

Otter JA, French GL (2010) Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Europe. Lancet Infect Dis 10:227–239

Otter JA, French GL (2011) Community-associated meticillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. J Hosp Infect 79:189–193

Perichon B, Courvalin P (2009) VanA-type vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53:4580–4587

Porrero MC, Wassenaar TM, Gomez-Barrero S, Garcia M, Barcena C, Alvarez J, Saez-Llorente JL, Fernandez-Garayzabal JF, Moreno MA, Dominguez L (2012) Detection of methicillin-resistant *Staphylococcus aureus* in Iberian pigs. Lett Appl Microbiol 54:280–285

Prystowsky J, Siddiqui F, Chosay J, Shinabarger DL, Millichap J, Peterson LR, Noskin GA (2001) Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. Antimicrob Agents Chemother 45:2154–2156

Rahim S, Pillai SK, Gold HS, Venkataraman L, Inglima K, Press RA (2003) Linezolid-resistant, vancomycin-resistant *Enterococcus faecium* infection in patients without prior exposure to linezolid. Clin Infect Dis 36:E146–E148

Reynolds PE, Courvalin P (2005) Vancomycin resistance in enterococci due to synthesis of precursors terminating in D-alanyl-D-serine. Antimicrob Agents Chemother 49:21–25

Rolo J, Miragaia M, Turlej-Rogacka A, Empel J, Bouchami O, Faria NA, Tavares A, Hryniewicz W, Fluit AC, de Lencastre H (2012) High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One 7:e34768

Ross JE, Farrell DJ, Mendes RE, Sader HS, Jones RN (2011) Eight-year (2002–2009) summary of the linezolid (Zyvox(R) annual appraisal of potency and spectrum; ZAAPS) program in European countries. J Chemother 23:71–76

Rubin DT, Sohi S, Glathar M, Thomas T, Yadron N, Surma BL (2011) Rifaximin is effective for the treatment of *Clostridium difficile*-associated diarrhea: results of an open-label pilot study. Gastroenterol Res Pract 2011:106978

Saager B, Rohde H, Timmerbeil BS, Franke G, Pothmann W, Dahlke J, Scherpe S, Sobottka I, Heisig P, Horstkotte MA (2008) Molecular characterisation of linezolid resistance in two vancomycin-resistant (VanB) *Enterococcus faecium* isolates using pyrosequencing. Eur J Clin Microbiol Infect Dis 27:873–878

Saha B, Singh AK, Ghosh A, Bal M (2008) Identification and characterization of a vancomycin-resistant *Staphylococcus aureus* isolated from Kolkata (South Asia). J Med Microbiol 57:72–79

Sanchez-Garcia M, De la Torre MA, Morales G, Pelaez B, Tolon MJ, Domingo S, Candel FJ, Andrade R, Arribi A, Garcia N, Martinez SF, Fereres J, Picazo J (2010) Clinical outbreak of linezolid-resistant *Staphylococcus aureus* in an intensive care unit. JAMA 303:2260–2264

Schaumburg F, Kock R, Mellmann A, Richter L, Hasenberg F, Kriegeskorte A, Friedrich AW, Gatermann S, Peters G, von Eiff C, Becker K (2012) Population dynamics among methicillin resistant *Staphylococcus aureus* in Germany during a 6-year period. J Clin Microbiol. doi:10.1128/JCM.01174-12

Schulte B, Heininger A, Autenrieth IB, Wolz C (2008) Emergence of increasing linezolid-resistance in enterococci in a post-outbreak situation with vancomycin-resistant *Enterococcus faecium*. Epidemiol Infect 136:1131–1133

Shah D, Dang MD, Hasbun R, Koo HL, Jiang ZD, DuPont HL, Garey KW (2010) *Clostridium difficile* infection: update on emerging antibiotic treatment options and antibiotic resistance. Expert Rev Anti Infect Ther 8:555–564

Sievert DM, Rudrik JT, Patel JB, McDonald LC, Wilkins MJ, Hageman JC (2008) Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002–2006. Clin Infect Dis 46:668–674

Skov R, Christiansen K, Dancer SJ, Daum RS, Dryden M, Huang YC, Lowy FD (2012) Update on the prevention and control of community-acquired meticillin-resistant *Staphylococcus aureus* (CA-MRSA). Int J Antimicrob Agents 39:193–200

Soavi L, Stellini R, Signorini L, Antonini B, Pedroni P, Zanetti L, Milanesi B, Pantosti A, Matteelli A, Pan A, Carosi G (2010) Methicillin-resistant *Staphylococcus aureus* ST398, Italy. Emerg Infect Dis 16:346–348

Song JH, Hsueh PR, Chung DR, Ko KS, Kang CI, Peck KR, Yeom JS, Kim SW, Chang HH, Kim YS, Jung SI, Son JS, So TM, Lalitha MK, Yang Y, Huang SG, Wang H, Lu Q, Carlos CC, Perera JA, Chiu CH, Liu JW, Chongthaleong A, Thamlikitkul V, Van PH (2011) Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. J Antimicrob Chemother 66:1061–1069

Stefani S, Chung DR, Lindsay JA, Friedrich AW, Kearns AM, Westh H, Mackenzie FM (2012) Meticillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. Int J Antimicrob Agents 39:273–282

Sujatha S, Praharaj I (2012) Glycopeptide resistance in Gram-positive cocci: a review. Interdiscip Perspect Infect Dis 2012:781679

Swoboda S, Fritz S, Martignoni ME, Feldhues RA, Hoppe-Tichy T, Buchler MW, Geiss HK (2005) Varying linezolid susceptibility of vancomycin-resistant *Enterococcus faecium* isolates during therapy: a case report. J Antimicrob Chemother 56:787–789

Tenover FC, Tickler IA, Persing DH (2012) Antimicrobial-resistant strains of *Clostridium difficile* from North America. Antimicrob Agents Chemother 56:2929–2932

Uttley AH, George RC, Naidoo J, Woodford N, Johnson AP, Collins CH, Morrison D, Gilfillan AJ, Fitch LE, Heptonstall J (1989) High-level vancomycin-resistant enterococci causing hospital infections. Epidemiol Infect 103:173–181

Valsesia G, Rossi M, Bertschy S, Pfyffer GE (2010) Emergence of SCC*mec* type IV and SCC*mec* type V methicillin-resistant *Staphylococcus aureus* containing the Panton-Valentine leukocidin genes in a large academic teaching hospital in central Switzerland: external invaders or persisting circulators? J Clin Microbiol 48:720–727

van Cleef BA, Monnet DL, Voss A, Krziwanek K, Allerberger F, Struelens M, Zemlickova H, Skov RL, Vuopio-Varkila J, Cuny C, Friedrich AW, Spiliopoulou I, Pászti J, Hardardottir H, Rossney A, Pan A, Pantosti A, Borg M, Grundmann H, Mueller-Premru M, Olsson-Liljequist

B, Widmer A, Harbarth S, Schweiger A, Unal S, Kluytmans JA (2011) Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg Infect Dis 17:502–505

van der Mee-Marquet N, Francois P, Domelier-Valentin AS, Coulomb F, Decreux C, Hombrock-Allet C, Lehiani O, Neveu C, Ratovohery D, Schrenzel J, Quentin R (2011) Emergence of unusual bloodstream infections associated with pig-borne-like *Staphylococcus aureus* ST398 in France. Clin Infect Dis 52:152–153

Wassenberg MW, Bootsma MC, Troelstra A, Kluytmans JA, Bonten MJ (2011) Transmissibility of livestock-associated methicillin-resistant *Staphylococcus aureus* (ST398) in Dutch hospitals. Clin Microbiol Infect 17:316–319

Welte T, Pletz MW (2010) Antimicrobial treatment of nosocomial meticillin-resistant *Staphylococcus aureus* (MRSA) pneumonia: current and future options. Int J Antimicrob Agents 36:391–400

Werner G, Gfrorer S, Fleige C, Witte W, Klare I (2008) Tigecycline-resistant *Enterococcus faecalis* strain isolated from a German intensive care unit patient. J Antimicrob Chemother 61:1182–1183

Willems RJ, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJ (2005) Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis 11:821–828

Wunderink RG, Niederman MS, Kollef MH, Shorr AF, Kunkel MJ, Baruch A, McGee WT, Reisman A, Chastre J (2012) Linezolid in methicillin-resistant *Staphylococcus aureus* nosocomial pneumonia: a randomized, controlled study. Clin Infect Dis 54:621–629

Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M (2010) *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob Agents Chemother 54:4643–4647

Yang SJ, Kreiswirth BN, Sakoulas G, Yeaman MR, Xiong YQ, Sawa A, Bayer AS (2009) Enhanced expression of *dltABCD* is associated with the development of daptomycin nonsusceptibility in a clinical endocarditis isolate of *Staphylococcus aureus*. J Infect Dis 200:1916–1920

Yang SJ, Nast CC, Mishra NN, Yeaman MR, Fey PD, Bayer AS (2010) Cell wall thickening is not a universal accompaniment of the daptomycin nonsusceptibility phenotype in *Staphylococcus aureus*: evidence for multiple resistance mechanisms. Antimicrob Agents Chemother 54:3079–3085

Zhanel GG, Calic D, Schweizer F, Zelenitsky S, Adam H, Lagace-Wiens PR, Rubinstein E, Gin AS, Hoban DJ, Karlowsky JA (2010) New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. Drugs 70:859–886

Zhao C, Sun H, Wang H, Liu Y, Hu B, Yu Y, Sun Z, Chu Y, Cao B, Liao K, Lei J, Hu Z, Zhang L, Zhang X, Xu Y, Wang Z, Chen M (2012) Antimicrobial resistance trends among 5608 clinical Gram-positive isolates in China: results from the Gram-positive cocci resistance surveillance program (2005–2010). Diagn Microbiol Infect Dis 73:174–181

Chapter 3
Gram-Negative Pathogens: Overview of Novel and Emerging Resistant Pathogens and Drugs

Yossi Paitan and Eliora Z. Ron

Abstract The rising prevalence of multiresistant Gram-negative bacterial infections has become a major clinical problem, as currently such infections comprise the majority of untreatable bacterial infections. The variety of resistance and transfer mechanisms and their rapid spread among Gram-negative bacteria constitutes a global infection control challenge, and an urgent need for development of new antimicrobials. Unfortunately, infection rates with multiresistant nonfermenters, such as *Pseudomonas aeruginosa, Acinetobacter baumannii*, or extended-spectrum β-lactamase producing *Enterobacteriaceae* and carbapenem resistant *Enterobacteriaceae* such as *Klebsiella pneumoniae,* are growing progressively while the pace of antibiotic drug development has slowed considerably during the last decade. This chapter reviews the main emerging Gram-negative resistant pathogens, their various resistance mechanisms, prevalence, risk factors, and summarizes the novel drugs being developed against them.

3.1 Introduction

Antibacterial therapy, introduced only about 70 years ago, has since saved millions of lives and its use led to a major reduction of premature death from bacterial infection. This reduction led to the assumption that pathogenic bacteria and the high mortality due to infectious diseases would be a thing of the past. Unfortunately, soon after introduction of antibiotics reports concerning emergence of resistance was published. The first report was published by Abraham and Chain

Y. Paitan
Clinical Microbiology Laboratory, Meir Medical Center, Kfar Saba, Israel

E. Z. Ron (✉)
Department of Molecular Microbiology and Biotechnology, Tel Aviv University and MIGAL Galil Research Center, Kiriat Shmone, Israel
e-mail: eliora@post.tau.ac.il

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_3,

(1940) describing an enzyme, named penicillinase, which is able to degrade benzylpenicillin, conferring resistance to penicillin. Since then, antimicrobial resistance has become the most important factor in the field of infectious diseases.

Antibiotic resistance mechanisms, which appear *de novo* or are transmitted among medically important bacteria, are well studied and described in many reviews. These mechanisms involve detoxification of the antibiotic molecule or mutations in the designated target. Antibiotic molecules can be (i) destroyed, (ii) modified to be less efficient, or (iii) removed by efflux pumps. Antibiotic targets can be (i) enzymatically modified, (ii) rendered insensitive by mutations, (iii) protected by a barrier, and (iv) replaced. Recent studies indicate that not all resistance mechanisms can be explained by the conceptual antibiotics–bacteria (targets) interactions, i.e., population level resistance mechanisms (Lee et al. 2010). It is now apparent that interspecies and intraspecies horizontal gene transfer of both Gram-negative and Gram-positive bacteria is the dominant process for achieving multiresistant bacteria. The selective pressure of antimicrobial use in hospitals, community, and agriculture comprises the engine driving this process.

Emerging antibiotic resistance is recognized as one of the most significant public health problems of the last few decades. Mortality rates due to multidrug-resistant bacterial infections are high. Klevens et al. (2007) estimated that in 2002, 1.7 million healthcare-associated infections occur each year in American hospitals and were associated with about 99,000 deaths. This represents a huge increase from previous estimation which reported that in 1992 about 13,300 people died from hospital-acquired infection (NIAID 2006). It is estimated that in the EU, Iceland, and Norway about 37,000 patients died as a direct outcome of a hospital-acquired infection each year, and an additional 111,000 died as an indirect outcome of the hospital-acquired infection (ECDC 2008), and about 25,000 patients died from an infection with a multidrug-resistant bacteria; two-thirds being due to Gram-negative bacteria (ECDC/EMEA Joint Working Group 2009). Moreover, multidrug bacterial infection rates are growing progressively while the pace of antibiotic drug development has slowed considerably during the last decade. The emerging Gram-negative resistant pathogens, the mechanisms of resistance, and an overview of novel drugs against them are the scope of this chapter.

3.2 MDR, XDR and PDR Pathogens, and Resistance Mechanisms

The terms multidrug resistance (MDR), extensively drug resistance (XDR), and pan-drug resistance (PDR) are increasingly used in the biomedical literature to describe highly resistant bacteria. MDR is defined as nonsusceptibility to at least one agent in three or more antimicrobial classes, XDR is defined as nonsusceptibility to at least one antimicrobial agent in all but two or less antimicrobial classes and PDR is defined as nonsusceptibility to all agents in all antimicrobial

classes. It should be noted that only in 2012 internationally acknowledged definitions of the terms were established (Magiorakos et al. 2012); therefore reports published before 2012 using these terms should be examined for the exact definitions used, before comparison is made as different definitions have been used (Falagas et al. 2008).

Presently, the most frequent MDR bacteria are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp.* which therefore got the phrase 'ESKAPE' following their first initial (Rice 2008), several reports add *Clostridium difficile* or other *Enterobacteriaceae* (Peterson 2009). In spite of their somewhat current lower prevalence, it is well recognized that Gram-negative PDR, MDR, and XDR infections create an emerging threat to hospitalized patients with a significant impact on length of hospitalization, mortality, and cost (Maragakis 2010; Giske et al. 2008). The rising crisis of multidrug-resistant Gram-negative bacteria has led to the emergence of salvage therapy using colistin, an older polymyxin, known to have neurotoxicity and nephrotoxicity (Rice 2006; Kallel et al. 2006, see Vaara, this volume). However, there are already reports describing isolates of several Gram-negative bacteria that are resistant to all available antibiotics, including polymyxins (Falagas et al. 2005, 2008).

The resistance of Gram-negative bacteria to different antimicrobials is due to the development of several mechanisms. These include modifying enzymes, β-lactamases, porin mutations, efflux pumps, and binding site-specific mutations. The knowledge of resistance mechanisms and patterns is highly important to achieve good clinical outcome, an adequate administration of antimicrobials, and for the development of new antimicrobial compounds.

3.2.1 Resistance to β-Lactam Antibiotics

Production of β-lactamase enzymes is the most common, significant, and efficient mechanism of resistance to β-lactam antibiotics (see Leemans et al., this volume). Many enzymes have been described, encoded either by chromosomal genes or by movable elements such as plasmids and transposons. β-lactamases hydrolyze β-lactam antibiotics, they include penicillinases, cephalosporinases, AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs), oxacillinases (OXA), and carbapenemases such as the *K. pneumoniae* carbapenemases (KPCs) as described in Table 3.1. Several classification schemes for β-lactamases have been proposed as displayed in Table 3.1, the Bush-Jacoby-Medeiros classification is based on functional similarities (Bush et al. 1995; Bush and Jacoby 2010) and the Ambler classification on their amino acid sequences, (Ambler 1980). The Ambler classification divides the β-lactamases into four classes (A, B, C, and D). In brief, class A include broad-spectrum β-lactamases, ESBLs, and carbapenemases. Class B are metallo-β-lactamases. Class C are AmpC β-lactamases, and class D are oxacillinases. The spread of AmpC β-lactamases and

Table 3.1 Summary of β-lactamase classification schemes

Ambler class	Bush-Jacoby-Medeiros group	Active site	Functional characteristics	Inhibition by clavulanate	Main representative enzyme families/types
A	2a, 2b, 2be, 2br, 2ber, 2c, 2ce, 2e and 2f	Serine	Penicillinases, cephalosporinases and carbapenemases with diverse hydrolytic properties	Yes, except 2br and 2ber, variable in 2f	TEM, SHV, CTX-M, KPC, SME, PER, VEB, GES, CARB-3, RTG-4, PSE-1, PC1, IMI-1, CepA
B (B1, B2, B3)	3a, 3b	Metallo (Zn^{2+})	Broad-spectrum carbapenemases inhibited by EDTA	No	VIM, IMP, IND, CcrA, L1, CAU-1, GOB-1, FEZ-1, CphA, Sfh-1, NDM-1
C	1, 1e	Serine	Cephalosporinases	No	CMY, *E. coli* AmpC, P99, ACT-1, FOX-1, MIR-1, GC1
D	2d, 2de, 2df	Serine	Oxacillinases, cephalosporinases, and carbapenemases	Yes	OXA

Based on Bush et al. (1995); Ambler (1980); Bush and Jacoby (2010)

KPCs in *Enterobacteriaceae* is linked to plasmid-mediated transfer of genes encoding AmpC (Livermore and Woodford 2006) or KPC (Walther-Rasmussen and Hoiby 2007). MBL spreading was linked to both plasmid-mediated transfer and integron-mediated transfer, while ESBLs spreading has been attributed to several processes, including clonal expansion, mutational events, and plasmid-mediated transfer (Livermore and Woodford 2006).

A second resistance mechanism to β-lactam antibiotics is through alterations in the enzymes target (Spratt 1994, see Leemans et al., this volume). β-lactam antibiotics exert their activity by binding antagonistically to penicillin-binding proteins (PBPs). A PBPs with a reduced affinity for β-lactams results in resistant phenotype.

An additional resistance mechanism is the downregulation of antibiotic intake either by changes in cellular permeability or by up-regulation of efflux systems. In *P. aeruginosa*, upregulation of efflux system results in various resistance phenotypes (Livermore 2002; Bonomo and Szabo 2006) and reduced susceptibility to carbapenems is achieved by mutations suppressing the expression of the porin OprD, which carbapenems enter through. Derepression of efflux system in *A. baumannii* results in a reduced susceptibility and resistance to several antibiotics (Bonomo and Szabo 2006).

3.2.2 Resistance to Aminoglycosides

Aminoglycosides (see Kirst and Marinelli, this volume) inhibit protein synthesis by binding to 16S rRNA and by disrupting the integrity of the cell membrane (Shakil et al. 2008). Resistance mechanisms responsible to aminoglycosides include: target modification, efflux pumps overexpression, and aminoglycosides modifying enzymes (Dozzo and Moser 2008).

3.2.3 Resistance to Tigecycline

Tigecycline (see Genilloud and Vicente, this volume), the first FDA approved glycylcycline antibiotic, inhibits the 30S ribosomal subunit. Upregulation of efflux pumps is the most important mechanism conferring resistance to tigecycline in both *Enterobacteriaceae* and nonfermenters (Keeney et al. 2007; Hornsey et al. 2010).

3.2.4 Resistance to Fluoroquinolones

Fluoroqinolones (see Pucci and Wiles, this volume) act by binding to DNA gyrase and topoisomerase IV. Plasmid-mediated resistance occurs through acetylation,

efflux pumps, and expression of resistance proteins (Qnr), which protect the targets from inhibition (Drlica and Malik 2003). Chromosomal resistance mechanisms include upregulated expression of efflux pumps and target mutations in the *gyrA/gyrB* for DNA gyrase and *parC/parE* for topoisomerase IV (Drlica and Malik 2003).

3.2.5 Resistance to Colistin

Colistin belongs to the polymyxins antibacterial class (see Vaara, this volume). It acts by binding to the lipid A of the bacterial lipopolysaccharide, leading to the disintegration of the bacterial membranes (Falagas et al. 2010). Bacterial cell membrane changes, such as a decrease in the content of lipopolysaccharides, specific outer membrane proteins and Mg^{2+} and Ca^{2+}, account for resistance (Mortensen et al. 2009).

3.2.6 Resistance to Phosphonic Acid Derivatives

Fosfomycin is a phosphonic acid derivative which inhibits an enzyme involved in the first step of bacterial cell wall synthesis. Due to its molecular structure, mode of action and its target, resistance to fosfomycin is rarely reported (Eschenburg et al. 2005). Resistance may be related to either mutations in the uptake and transport mechanisms or the presence of a fosfomycin-modifying enzyme eliminating its antibacterial activity (Beharry and Palzkill 2005; Wachino et al. 2010; Hou et al. 2012).

These various resistance mechanisms present in Gram-negative *Enterobacteriaceae* and nonfermenters pathogens arise often in the same strains, resulting in a MDR, XDR, and PDR phenotypes. Such strains are isolated most often among *Pseudomonas, Acinetobacter,* and *Klebsiella* species and less frequently in other *Enterobacteriaceae.*

3.3 Emerging Gram-Negative Resistant Pathogens

3.3.1 Pseudomonas aeruginosa

P. aeruginosa is considerably the most important nonfermenter frequently implicated in healthcare-associated infections (HAIs). Although it rarely colonizes healthy humans (Speert et al. 1993), it is responsible for multiple types of infections including pneumonia, urinary tract infection (UTI), bacteremia, and wound

infections. *P. aeruginosa* infections are often associated with invasive devices, mechanical ventilation, catheters, surgery wounds, and burns (Rice 2006) particularly in immunocompromised, immuno-incompetent, and intensive care unit patients (Giamarellou and Kanellakopoulou 2008). *P. aeruginosa* can be recovered from sink drains, vegetables, moist surfaces, and even antiseptic solutions, therefore representing a major concern to hospitals. In addition, *P. aeruginosa* is the predominant respiratory pathogen in cystic fibrosis (CF) patients (Govan and Deretic 1996). Many CF isolates are multiresistant due to repeated rounds of antibiotic selection pressure, hypermutability, and co-selecting resistance which facilitate resistance development (Oliver et al. 2000; Henrichfreise et al. 2007). The overall prevalence of *P. aeruginosa* infections has remained stable during 1986–2003; however, the prevalence of resistant *P. aeruginosa* isolates had increased considerably in 2003 compared to 1998 (NNIS 2004), and third-generation cephalosporins, quinolones, and imipenem resistance rates increased by 20, 9, and 15 %, respectively.

3.3.1.1 Antibiotic Resistance Mechanisms

The resistance of *P. aeruginosa* cannot be dissociated from its virulence. The type III secretion system is an important component of its virulence; it is used to translocate cytotoxins into the host cells, which can damage host tissues and inhibit phagocytosis (Urbanowski et al. 2005). The biofilm formation ability of *P. aeruginosa* also increases its virulence and resistance (Rice 2006). *P. aeruginosa* has two main intrinsic resistance mechanisms: several multidrug powerful efflux pump systems, and an inducible chromosomal AmpC β-lactamase. Many antibiotics including aminoglycosides (see Kirst and Marinelli, this volume), fluoroquinolones (see Pucci and Wiles, this volume), monobactams, extended-spectrum penicillins, cephalosporins (see Leemans et al., this volume) and colistin (see Vaara, this volume) overcome these intrinsic resistance mechanisms. However, acquired resistance to all of these antibiotics was developed; these include several powerful efflux pumps (Aeschlimann 2003), impermeability through slowness porin channels (Rice 2006), enzymatically inactivation of antibiotics (Rice 2006), and mutational resistance, as described in Table 3.2. The constantly and continuously evolving resistances of *P. aeruginosa* and the continuing antibiotic pressure have led eventually to the development of PDR isolates with convergence of several resistance mechanisms (Bonomo and Szabo 2006).

3.3.1.2 Risk Factors for Infections and Resistance Development

As prevalence of drug resistant *P. aeruginosa* is on the rise, many studies have focused on the risk factors associated with the occurrence of MDR *P. aeruginosa.* Several studies reported the isolation of MDR *P. aeruginosa* strains after prolonged use of antipseudomonal agents (Zavascki et al. 2006; Georges et al. 2006).

Table 3.2 General types and mechanisms of antibiotic resistance in *P. aeruginosa*. Multiple mechanisms are often present in MDR, XDR and PDR isolates

Mechanism type	Antibiotics	Main enzyme, proteins, genes, and mutation targets
Enzyme-mediated resistance		
β-lactamases	Cephalosporins, carbapenems	OXA, Metallo-β-lactamases (IMP, VIM, SPM, GIM)
	Penicillins	PSE-1, PSE-4
	Third-generation cephalosporins, piperacillin	AmpC
16S rRNA methylases	Aminoglycosides (high-level resistance)	*rmtA, rmtB, rmtC, rmtD, armA, npmA*
Aminoglycoside-modifying enzymes	Aminoglycosides	Acetyltransferases, nucleotidyltransferases, phosphotransferases
Target mutations		
Topoisomerases II and IV	Fluoroquinolones	*gyrA, gyrB, parC, parE*
Drug permeability and efflux pumps		
Over-expression multi-drug efflux pumps (up-regulation or mutation of *mexR* repressor gene)	β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, sulfonamides, and macrolides	MexAB-OprM, MexCD-OprJ, MexEF-OprN
Porin deletions	Meropenem, imipenem	OprD
Membrane changes		
Lipopolysaccharide modifications	Colistin, aminoglycosides	*pmrA, pmrB* and *pohP*, OprH over-expression

Based on Tam et al. (2010); Livermore (2002); Bonomo and Szabo (2006); Falagas et al. (2010)

In addition, history of *P. aeruginosa* colonization or infection in chronic obstructive pulmonary disease (Ohmagari et al. 2005), mechanical ventilation and length of hospitalization (Arruda et al. 1999), malignant disease, intensive care units (ICU) hospitalization, and confined to bed (Aloush et al. 2006; Paramythiotou et al. 2004) have all been considered as risk factors for MDR *P. aeruginosa* infection.

3.3.2 Acinetobacter baumannii

A. baumanni has emerged as a worrying pathogen globally and is frequently connected to HAIs. It is an opportunistic pathogen, difficult to treat, considered to be a key nosocomial outbreak causing pathogen, as it is common in ICU and difficult to eradicate due to its resistant to drying. *A. baumanni* was even isolated from computer keyboards and from a bed rail 9 days after an infected patient was discharged (Peleg and Paterson 2006). It is intrinsically resistant to many antimicrobials (Navon-Venezia et al. 2005) and particularly attacks immunocompromised, immunocompetent, and patients with invasive devices (Abbo et al. 2005). *A. baumanni* has been implicated in UTI, bacteremia, ventilator-associated pneumonia (VAP), wounds, soft tissue infractions, and catheter-associated infections (Rice 2006). Sporadic cases of endocarditis, meningitis, osteomyelitis, and arthritis continuous ambulatory peritoneal dialysis peritonitis have been reported (Bergogne-Berezin and Towner 1996).

Since the early 1980s, hospital outbreaks of *A. baumannii* infections in the United States, Europe, and other parts of the world have been reported (Bergogne-Berezin and Towner 1996). Several of which were associated with resistant strains susceptible only to polymyxins (see Vaara, this volume) and ampicillin-sulbactam (Go et al. 1994; Jones et al. 2004; Lolans et al. 2006; Mahgoub et al. 2002, see Leemans et al., this volume). In most cases, one epidemic strain was predominantly detected in a given epidemiological location. Dissemination of a multiresistant *A. baumannii* occurs on a national and international scale (Da Silva et al. 2007), and by airline travel (Peleg et al. 2006; Naas et al. 2007). There are also reports of MDR and PDR *A. baumannii* in Latin America, Africa, Australia, Asia, and the Middle East (Iredell et al. 2007; Gales et al. 2006). Several studies demonstrated significant trends in the emergence of multidrug-resistant *Acinetobacter* strains. In the United States, the National Nosocomial Infection Surveillance system reported that there is a significant increases in *Acinetobacter* strains resistant to amikacin, ceftazidime, and imipenem (5–20 %, 25–68 %, and 0–20 %, respectively) among strains collected from 1986 to 2003 (Gaynes and Edwards 2005).

Carbapenem resistance in *A. baumannii* is a present issue worldwide (see Leemans et al., this volume). The MYSTIC report from 48 European hospitals, indicated that in 2002–2004 just 73.1 and 69.8 % of *A. baumannii* isolates were susceptible to meropenem and imipenem, respectively and that susceptibility to

ceftazidime, ciprofloxacin (see Pucci and Wiles, this volume), and gentamicin (see Kirst and Marinelli, this volume), was also very low, with 32.4, 34.0, and 47.6 % being susceptible respectively (Unal and Garcia-Rodriguez 2005).

Often tigecycline (see Genilloud and Vicente, this volume) and colistin (see Vaara, this volume) are the only available antimicrobials against XDR *A. baumannii* infections. Unfortunately, resistance to both tigecycline (Hornsey et al. 2010; Chang et al. 2012) and polymyxins (Beno et al. 2006; Gales et al. 2006; Garcia-Penuela et al. 2006) has been reported. Resistant to tigecycline and polymyxins represents a major concern as many XDR isolates are susceptible only to these antibiotics or only to polymyxins. In Germany, a surveillance study which included 215 *A. baumannii* isolates, reported that 6 % were resistant to tigecycline and 2.8 % were resistant to colistin (Seifert et al. 2006). Gales et al. (2006) reported 1.7 % of polymyxin B-nonsusceptible *Acinetobacter* spp. worldwide. For a detailed review on *A. baumannii* readers is referred to an excellent review by Peleg et al. (2008). A significant contribution to the epidemiology of *A. baumannii* infections in the United States and the United Kingdom is the homecoming of military personnel from Iraq or Afghanistan (CDC 2004; Scott et al. 2007). These isolates were mostly multidrug resistant, being resistant to piperacillin-tazobactam cephalosporins and fluoroquinolones and only 10 % were nonsusceptible to carbapenems (Scott et al. 2007).

3.3.2.1 Antibiotic Resistance Mechanisms

A. baumannii comprises a remarkable array of antimicrobial intrinsic and acquired resistance mechanisms (Poirel and Nordmann 2006) as summarized in Table 3.3. The intrinsic mechanisms include a chromosomally encoded AmpC cephalosporinases (Bou and Martinez-Beltran 2000) and multidrug efflux pumps, conferring resistant to a wide variety of antimicrobial agents. Acquired mechanisms include a variety of Ambler class D OXA carbapenemase and Ambler class B MBLs (Poirel and Nordmann 2006, see Leemans et al., this volume). Additional enzymatic mechanisms include aminoglycoside-modifying enzymes such as acetyltransferases, nucleotidyltransferases, and phosphotransferases (Nemec et al. 2004, see Kirst and Marinelli, this volume). Nonenzymatic mechanisms include changes in outer membrane proteins (OMPs) (Siroy et al. 2005), multidrug efflux pumps (Magnet et al. 2001), alterations in the affinity or expression of penicillin-binding proteins (Obara and Nakae 1991), 16S rRNA methylation (Lee et al. 2006), DNA gyrase or topoisomerase IV (Vila et al. 1997), and ribosomal protection (Ribera et al. 2003) as outlined in Table 3.3.

3.3.2.2 Risk Factors for Infections and Resistance Development

The risk factors for acquisition of an *A. baumannii* MDR infection have been studied extensively, but it is difficult to distinguish between colonization and infection. In the majority of studies, antibiotic use was the most common risk

Table 3.3 General types and mechanisms of antibiotic resistance in *A. baumannii*. Multiple mechanisms are often present in MDR, XDR and PDR isolates

Mechanism type	Antibiotics	Main enzyme, proteins, genes, and mutation targets
Enzyme mediated resistance		
β-lactamases	Cephalosporins, carbapenems	OXA, Metallo-β-lactamases, Class A ESBLs
	Penicillins	Narrow-spectrum β-lactamases (TEM-1, TEM-2)
	Third-generation cephalosporins	AmpC
16S rRNA methylases	Aminoglycosides (high-level resistance)	*armA*
Aminoglycoside-modifying enzymes	Aminoglycosides	Acetyltransferases, nucleotidyltransferases, phosphotransferases
Ribosomal protection		
Ribosomal protection	Tetracyclines and glycylcyclines	*tet(M)*
Target Mutations or alteration		
Altered penicillin-binding proteins	Imipenem and other penicillins	24-kD PBP
Topoisomerases II and IV	Fluoroquinolones	*gyrA, parC*
Drug permeability and efflux pumps		
Multidrug efflux pumps	β-lactams, carbapenems, aminoglycosides, fluoroquinolones, tetracyclines, sulfonamides, macrolides, and tigecycline	AdeABC, AdeM, *tet(A), tet(B), adeB*
Porin changes	Meropenem, imipenem	CarO, 22-, 33-, 43-, 47-, 44-, and 37-kDa OMPs, HMP-AB, OmpW
Membrane changes		
Lipopolysaccharide modifications	Colistin, aminoglycosides	Unknown

Based on Peleg et al. (2008)

factor identified, in particular third-generation cephalosporins and carbapenems. The second most common risk factor was mechanical ventilation (Falagas and Kopterides 2006), followed by invasive procedures, instrumentation, ICU hospitalization and its length, length of hospitalization, perior hospitalizations, nursing home residence, illness severity, and recent surgeries (Mahgoub et al. 2002; Falagas and Kopterides 2006; Medina et al. 2007; Maragakis and Perl 2008). In addition, environmental contamination, including medical equipment, furniture and other surfaces, contaminated hands of health workers was associated to outbreaks.

3.3.3 Enterobacteriaceae and Klebsiella pneumoniae

Members of the *Enterobacteriaceae* family are Gram-negative, facultative anaerobic rods, or coccobacilli. They are widespread throughout the environment; many are commonly recognized nosocomial pathogens and are very frequently isolated from many types of human clinical specimens. *Enterobacteriaceae* comprised about 50 % of all isolates from hospital acquired infections and 80 % from all Gram-negative hospital isolates (Karlowsky et al. 2003). *E. coli* is the third most frequently isolated pathogen from human clinical specimens. *E. coli*, *Enterobacter* spp. *Klebsiella* spp. and *Proteus mirabilis* are the third, sixth, eighth, and tenth most common agents of bloodstream infections (Diekma et al. 2002; Fluit et al. 2001). Among nosocomial pneumonia causing agents, *E. coli*, *Enterobacter* spp. *K. pneumoniae* and *Serratia* spp. are the third, fourth, sixth, and seventh most common pathogens (Fluit et al. 2001). In 2000, *Klebsiella* spp. accounted for 58, 23, and 20 % of UTIs in North America, Europe and Latin America, respectively (Farrel et al. 2003; Gordon et al. 2003). Many studies have reported that mortality associated with XDR *Enterobacteriaceae* infections, mostly with ESBL and carbapenem-resistant isolates was high (Schwaber et al. 2008, see Leemans et al., this volume). Infections by PDR *Enterobacteriaceae* are still uncommon; however, they were associated with high mortality 33.3 % among 28 patients with PDR infections in Greece according to Falagas et al. (2008).

A few years after the introduction of ampicillin, plasmid-mediated resistance by SHV and TEM β-lactamases evolved and became a major clinical problem. In 1968, Datta (1969) reported that 17 % of *E. coli* isolates, originated from faeces of patients admitted for elective surgery in London, were resistant to ampicillin. In the late 1980s, the introduction of third-generation extended spectrum cephalosporins, and their wide use against *Enterobacteriaceae* infections has been the force and selective pressure for the development of ESBL resistance. Mutants in both the SHV and TEM β-lactamases genes were described and the resistance spread among different *Enterobacteriaceae*, particularly among *Klebsiella* spp. and *E. coli*, marking the beginning of the ESBL resistance era (Jacoby and Medeiros 1991). Such mutants were able to hydrolyze third-generation

cephalosporins and monobacts, and have spread over the next 20 years worldwide mainly in the hospital environment.

Indeed, until 2000, most ESBL isolates were hospital *Klebsiella* spp. with TEM and SHV mutants; however the movement of a broad-spectrum β-lactamase gene, coding for the CTX-M β-lactamase from a *Kluyvera* spp. onto a broad host range plasmids, has caused a much more serious problem, the proliferation and spread of the ESBLs CTX-M enzymes worldwide (Canton and Coque 2006). More than 80 different variants of CTX-M enzymes have been reported and disseminated in different countries. However, some genotypes, such as CTX-M-15 and CTX-M-14 are considered to be very successful enzymes and have spread globally. Most ESBL producers *Enterobacteriaceae* are also multiresistant. Livermore et al. (2008) reported that in the UK more than 80 % and more than 40 % of the ESBL positive *E. coli* from bacteraemias are resistant to fluoroquinolones and gentamicin, respectively. The clinical implication is that the rates of inappropriate empiric therapy of bacteraemias may increase. A longer delay in the initiation of an effective antibacterial therapy among patients with an ESBL positive bacteraemia has been associated with a 1.85-fold higher mortality rates (Schwaber and Carmeli 2007).

3.3.3.1 Antibiotic Resistance Mechanisms

Chromosomal AmpC β-lactamases hyperproduction and the production of extended-spectrum β-lactamases (ESBLs) are the main mechanisms by which *Enterobacteriaceae* become multiresistant (see Leemans et al., this volume). There are three major groups of ESBLs, the TEM family consists more than 160 members, the SHV family consists more than 110 members, and the CTX-M family consists more than 80 members which are extensively disseminated globally. Different CTX-M ESBL enzymes variants are prevalent in different countries; however CTX-M enzymes are not very common in the USA, the SHV and TEM ESBL types are dominant with only several reports of CTX-M enzymes reported (Moland et al. 2009). ESBL-producing bacteria are often also resistant to fluoroquinolones (see Pucci and Wiles, this volume) through mutations in *gyrA* (Robicsek et al. 2006).

The growing prevalence of multi-resistant ESBL-producing *Enterobacteriaceae*, which are often resistant to all currently available antimicrobials, led to a wide use with carbapenems as the drug of choice against ESBL infections. This use has been the selective pressure for the development of carbapenem resistance. Enzyme carbapenem resistance is mediated by Ambler class A carbapenemases such as KPC, Ambler class B MBL carbapenemases such as VIM, IMP, and the new emerging NDM-1 and Ambler class D OXA type carbapenemases. The majority of these enzymes is carried on plasmids and therefore represents a major concern. The KPC carbapenemase have spread predominantly among *K. pneumoniae* both in the USA, Israel, Greece, and several other countries (Bratu et al. 2007; Leavitt et al. 2007). XDR *Enterobacteriaceae*, such as many KPC producing *K. pneumoniae,* are

often susceptible only to polymyxins (see Vaara, this volume) and gentamicin (see Kirst and Marinelli, this volume).

3.3.3.2 Risk Factors for Infections and Resistance Development

As *Enterobacteriaceae*, PDR infections are still uncommon; there are not a lot of studies regarding the risk factors for infections. A matched case–control study indicated that antibiotic exposure, particularly, quinolones and anti-pseudomonal penicillins, was a risk factor for the development of carbapenem-resistant isolates (Falagas et al. 2007). Schwaber et al. (2008) identified ICU stay, recipient of antibiotics (particularly fluoroquinolones), and poor functional status as independent risk factors for carbapenem-resistant *K. pneumoniae* isolations. Information regarding risk factors of PDR is limited. Daikos et al. (2007) reported that long hospital stay and considerable use of colistin were associated with the infection of a PDR *K. pneumoniae* (MBL positive, colistin-resistant) in ICU patients.

3.3.3.3 *Klebsiella pneumoniae*

Among the *Enterobacteriaceae* family, *K. pneumoniae* is the species with the highest rates of carbapenem resistance and has been recognized as the most problematic and the one most likely to be MDR, XDR, or PDR. Infections by KPC and NDM-1 producing *K. pneumoniae* are an increasing clinical problem worldwide, and an infection control challenge. The first KPC producing *K. pneumoniae* isolate was reported in 2001 in North Carolina (Yigit et al. 2001), and has since became the most prevalent carbapenem resistance mechanism in the USA (Lee et al. 2009). KPCs, encoded by the bla_{KPC} gene, are located within Tn3-type transposon, Tn4401, which enables its insertion into diverse plasmids of Gram-negative bacteria, explaining its interspecies and geographic dissemination. Although first reported in 2001, it took only a few years for KPC producing *K. pneumoniae* to spread and cause several hospital outbreaks (Bratu et al. 2005) and consequently have spread throughout the USA and worldwide. In 2009, the United States Centers for Disease Control and Prevention (CDC) agency released a report about KPC producing bacteria suggesting the term Carbapenem-Resistant *Enterobacteriaceae* (CRE) as a more accurate term, as KPC is produced not only in *K. pneumoniae* but also in other *Enterobacteriaceae* (CDC 2009). This CDC report indicated that the overall prevalence of carbapenem resistance among *K. pneumoniae* isolates rose from about 1 % in 2000 to 8 % In 2007 (CDC 2009). The first outbreak of KPC producing *K. pneumoniae* out of the USA was in Israel (Leavitt et al. 2007). KPC producing *K. pneumoniae* are now endemic in both Israel and Greece, and were reported in many other countries including China, Brazil, the United Kingdom, Sweden, Norway, Colombia, India, Italy, Finland,

and several other countries. A molecular analysis study of KPC producing *K. pneumoniae* revealed that only few fit lineages are responsible for the dissemination of the bla_{KPC} gene. A single dominant strain, multilocus sequence type 258 (ST258) accounted for about 70 % of the isolates mostly harboring KPC-3. A second fit strain, ST14, was also detected from isolates originating in several facilities in the Midwestern states (Kitchel et al. 2009).

Infections caused by KPC producing *K. pneumoniae* are associated with high mortality rates of 47–66 % (Bratu et al. 2005; Patel et al. 2008; Borer et al. 2009) frequent treatment failures, and increased length of hospitalization and cost (CDC 2009). Reported risk factors for infections include, previous treatment with antibiotics, transplantation, long hospital stays, mechanical ventilation, advanced age, severity of illness, and probably also previous carbapenem use (Bratu et al. 2005; Patel et al. 2008). Presently, the therapeutic options for KPC producing bacterial infections are mostly polymyxins and tigecycline. Information regarding the optimal use of these antibiotics, as combination therapy or monotherapy is limited. Lee et al. (2009) reported that 3 of 12 patients treated with polymyxin monotherapy developed polymyxin resistance during treatment, while none of four cases treated with polymyxin and tigecycline combination therapy developed resistance. Although aminoglycoside resistance is increasing among KPC producing bacteria, the use of gentamicin in susceptible strains was found to be effective. Fortunately, most isolates of the KPC producing *K. pneumoniae* successful ST258 clone, which accounts to the majority of KPC producing *K. pneumoniae* infections, remain sensitive to gentamicin (Livermore 2009).

The most recently recognized carbapenemase enzyme is the New Delhi metallo-β-lactamase (NDM) (Yong et al. 2009). Since its discovery at 2008, it has been reported worldwide, mostly in patients epidemiologically linked to the Indian subcontinent, where it is endemic. The bla_{NDM} gene is located on a plasmid enabling its dissemination between bacteria, and it has spread worldwide due to travel and "medical tourism" (Yong et al. 2009; Kumarasamy et al. 2010). The gene has moved from India and Pakistan to the USA, the United Kingdom, Canada, Japan, Belgium, Netherlands, France, Turkey, Spain, Taiwan, Singapore, Kenya, Australia, South Africa, the Sultanate of Oman, and many other countries worldwide.

NDM-1 is a novel broad-spectrum carbapenemase that confers resistance to carbapenems and all other β-lactam antibiotics, with the exception of aztreonam. However, most NDM-1-producers also produce aztreonam hydrolyzing-β-lactamases making these pathogens resistant to all β-lactams, and nearly all antibiotics, severely limiting options for treatment. As the emergence of NDM-1 producing bacteria is very recent, there is no data regarding risk factors for infections apart from being epidemiologically linked to the Indian subcontinent or receiving medical care in India. Like in the case of KPS producing bacteria, the medical options are limited. Only polymyxins and glycylcyclines show good in vitro activity against NDM-1 producers but colistin-resistant NDM-1 producing isolates have also been reported (Kumarasamy et al. 2010).

3.4 The Complexity of the Resistance Problem

The current state in the field of resistance, antimicrobials, and antibacterial therapy implies that we are at the end of the "antibiotics era" approaching the beginning of the post antibiotic era. The resistance crises are undoubtedly not limited to the clinicians and clinical microbiologists. The challenge is a global challenge, a complex problem requiring the collaboration and combined efforts of governments, academia, agricultural and pharmaceutical industry workers, microbiologists, ecologists, policy makers and politicians, health care specialists, and probably all the public. It is a global concern as each one of us has the probability to get infected with a bacterial pathogen resistant to antibiotic therapy. Currently, we are able to treat the majority of infections, but the rising prevalence of *Enterobacteriaceae* susceptible only to carbapenems (see Lemans et al., this volume), tigecycline (see Genilloud and Vicente, this volume) and polymyxins (see Vaara, this volume) is worrying, and there is a growing concern about carbapenem resistance caused by KPC and NDM. In the case of MDR, and XDR nonfermenters, only tigecycline and polymyxins are the last active agents. This unavoidable circumstance emphasizes the urgent need for the development of new antimicrobials against multiresistant Gram-negative bacteria.

3.4.1 Novel Antimicrobials

Antibiotic therapy for emerging multiresistant bacterial infections is limited. There are several suggested alternatives to antimicrobial therapy such as phage therapy (Abedon et al. 2011) and passive immunization (Keller and Stiehm 2000); however their practical use is still limited. The conventional treatment still relies on the discovery and development of new and more efficient antibiotics. This can be done by modification of existing antibiotics, identification and discovery of new antibiotics from unexplored ecological niches, exploring uncultivated bacteria by metagenomics (MacNeil et al. 2001), drug engineering such as dual target activities hybrid antibiotics (Thomas et al. 2011), and most important identifying new antibiotic targets in pathogenic bacteria for new antimicrobials.

3.4.2 Novel β-Lactam and β-Lactamase Inhibitors

Overcoming resistance to β-lactams may result from the development of novel antibiotic compounds or development of new β-lactamase inhibitors to protect them from the action of ESBLs and MBLs.

3.4.2.1 Novel Cephalosporins

CXA-101, a novel cephalosporin in development, to be used as a single agent or with tazobactam (Takeda et al. 2007; Moya et al. 2010), it has potent activity against resistant *P. aeruginosa* and possibly also against several ESBL producing *Enterobacteriaceae* such as *E. coli* and *K. pneumoniae* (Titelman et al. 2011, see Leemans et al., this volume).

3.4.2.2 Novel Carbapenems

Carbapenems are important antibacterial agents often used to treat MDR Gram-negatives as they are stable to hydrolysis by many ESBLs. Various potent carbapenems are at different stages of development (see Leemans et al., this volume). Doripenem which has recently been marketed (Chahine et al. 2010) and is somewhat equivalent to imipenem but confers higher clinical cure rates with *P. aeruginosa* infections. Tomopenem (CS-023), with activity against *P. aeruginosa* and other different hospital pathogens (Koga et al. 2008). Razupenem (PZ-601), with activity against multidrug-resistant Gram-positive and Gram-negative including ESBL producers (Livermore et al. 2009). Biapenem, which has been approved in Japan for respiratory infections and UTI, and is active against both Gram-positive and Gram-negative bacteria including several ESBL producing *Enterobacteriaceae* (Jia et al. 2010; Gomi et al. 2011). Penipenep/betamipron, which has been approved in Asia for respiratory infections and UTI, has comparable activity to imipenem/cilastatin against Gram-negative bacteria (Goa and Noble 2003). Tebipenem pivoxil, an oral carbapenem with some activity against *E. coli*, *K. pneumoniae* and *Acinetobacter* spp. (Bassetti et al. 2009). Additional carbapenems are at different stages of development or clinical trials which include Sulopenem, ME1036, SM-197436, SM-232721, and SM-232724; however their activity against Gram-negative MDR bacteria is still questionable.

3.4.2.3 Novel Monobactams

BAL30072 is a novel dihydroxypyridone monobactam derived from tigemonam which is active against multiresistant Gram-negative *Bacilli*, including carbapenem-resistant strains, MBL producing strains, and MDR *P. aeruginosa* and *Acinetobacter* spp. isolates (Mushtaq et al. 2010; Page et al. 2010, see Leemans et al., this volume).

3.4.2.4 Novel *β*-Lactamase Inhibitors

The combination of β-lactam/β-lactamase inhibitors potentates the action of the β-lactam by protecting it from hydrolysis. They can be classified as β-lactam or non β-lactam according to their structure (see Leemans et al., this volume).

Inhibitors with a β-lactam structure: BLI-489, which is an imidazole-substituted 6-methylidene-penem with activity against class A, C, and D β-lactamases including ESBL (Shahid et al. 2009). Ro 48–1220, an 2β-alkenyl penem sulfones, which inhibits class A and C β-lactamases (Tzouvelekis et al. 1997). Several oxapenems β-lactamase inhibitors (AM-112-AM-115) which display activity against class A, C, and D enzymes (Jamieson et al. 2003). BAL 30376 is a novel triple combination composed of: a siderophore monobactam BAL19764; a novel bridged monobactam, BAL 29880, which inhibits class C β-lactamases; and clavulanic acid, which inhibits many class A and some class D β-lactamases. It is active against most ESBL, AmpC, and carbapenemase-mediated resistance in *Enterobacteriaceae*; however it is inactive against strains with KPC carbapenemases (Page et al. 2011; Livermore et al. 2010). Several other inhibitors with a β-lactam structure such as 4-phenyl cyclic phosphate against class A and C enzymes (Kaur et al. 2003), C3-methylene-modified penicillin sulfones potent against class C enzymes (Buynak et al. 2005) and LK-157 against a wide range of serine β-lactamases but not against CTX-M (Shahid et al. 2009) are in development.

Inhibitors with a nonβ-lactam structure: ME1071 (CP3242), a maleic acid derivative, is a novel specific inhibitor for metallo-beta-lactamases (MBL) which inhibits IMP-1 and VIM-2 MBLs in *P. aeruginosa*, and also *E. coli*, *K. pneumoniae*, *S. marcescens*, and *A. baumanii* (Shahid et al. 2009; Ishii et al. 2010). MK-7655 is a novel agent active against class A and C carbapenemases, and was used in combination with imipenem against KPC-2 producer *K. pneumoniae* and possibly also MBL producing *P. aeruginosa* (Hirsch et al. 2010). NXL104 inhibits β-lactamases through the formation of a stable covalent carbamoyl linkage (Shahid et al. 2009). It is has been tested in combination with ceftazidime, ceftriaxone, cefepime, meropenem, mecillinam, and several other β-lactamase and restored susceptibility *E. coli* and *K. pneumoniae* isolates producing class A ESBLs and class C β-lactamases and also ESBL/AmpC co-expressing *E. coli.* (Lagacé-Wiens 2011). In other studies, it was active against multidrug-resistant nonfermenters including AmpC enzymes in *P. aeruginosa* when combined to ceftazidime (Curcio 2011), and CTX-M producing *Enterobacteriaceae* when combined to ceftazidime or cefotaxime (Shahid et al. 2009).

3.4.3 Novel Non β-Lactam Antibiotic Compounds

Development of non β-lactam new drugs can be divided into compounds belonging to known class of antimicrobial agents such as tetracyclines, aminoglycosides, polymyxins and fluoroquinolones, or to compounds which represent new classes of antimicrobials.

3.4.3.1 Compounds Belonging to Known Class of Antimicrobial Agents

Several novel polymyxins derivatives and analogs such as CB-128, 804 which has activity against colistin susceptible and resistant isolates of *E. coli, K. pneumoniae, A. baumannii,* and *P. aeruginosa* are currently in clinical development (Quale et al. 2011, see Vaara, this volume). NAB739 and NAB740 are two synthetic polymyxin derivatives, which retain equal antibacterial activity of polymyxin B but may be less nephrotoxic (Vaara et al. 2008).

TP-434 is a broad-spectrum fluorocycline (synthetic tetracycline) targeting the protein cellular machinery, with activity against MDR Gram-negatives and Gram-positive bacteria expressing major antibiotic resistance mechanisms including tetracycline-specific efflux and ribosomal-protection except *P. aeruginosa* (Grossman et al. 2012; Xiao et al. 2012, see Genilloud and Vivente, this volume).

Plazomicin (ACHN-490) is a novel aminoglycoside resistant to plasmid-mediated aminoglycoside-modifying enzymes. It is active against *P. aeruginosa* and carbapenem resistant MDR *Enterobacteriaceae* except NDM-1 mediated resistance (Livermore et al. 2011; Landman et al. 2011, see Kirst and Marinelli, this volume).

Omadacycline (PTK-0796) is a novel aminomethyl tetracycline which evades efflux-mediated tetracycline resistance and has now completed Phase II clinical trials (Sutcliffe 2011; Török et al. 2010, see Genilloud and Vicente, this volume).

Sitafloxacin hydrate is a new broad-spectrum oral fluoroquinolone which is active against many Gram-positive, Gram-negative, and anaerobic clinical isolates, including strains resistant to other fluoroquinolones. Sitafloxacin was found to be active against clinical isolates of *K. pneumoniae* including ciprofloxacin resistant strains and ESBL producing strains, *E. cloacae, P. aeruginosa*, and some activity against quinolone-resistant strains of *A. baumannii* (Anderson 2008, see Pucci and Wiles, this volume).

Finafloxacin is a novel fluoroquinolone exhibiting broad-spectrum activity against Gram-positive and Gram-negative bacteria. Its activity is enhanced under slightly acidic conditions (pH 5.0–6.5) (see Pucci and Wiles, this volume). Finafloxacin activity was higher than the activity of ciprofloxacin, levofloxacin, and moxifloxacin against *E. coli* strains expressing known fluoroquinolone resistance determinants alone and in combinations, especially at pH 5.8 (Emrich et al. 2010).

Iclaprim is a diaminopyrimidine which acts as a dihydrofolate reductase (DHFR); it has a comparable activity to trimethoprim against *Enterobacteriaceae* (Schneider et al. 2003).

3.4.3.2 Compounds Which Represent New Classes of Antimicrobials

Finding new targets for the development of new antimicrobials is an additional way to fight against the "resistance crises" (see Genilloud and Vincente, this volume). New targets such as DNA interacting agents, new protein synthesis inhibitors, lipopolysaccharide synthesis, specific essential enzymes, specific

antigens, and antimicrobial peptides may become important to the treatment of MDR, XDR, and PDR bacteria.

GSK2251052 (AN3365) is a novel boron antibiotic which is an aminoacyl-tRNA synthetase inhibitor and therefore inhibits protein synthesis for the treatment of multidrug-resistant Gram-negative bacterial including *P. aeruginosa* infections (Sutcliffe 2011).

LpxC is a deacetylase involved in Gram-negatives lipopolysaccharide synthesis and is a new target for antimicrobial agents. LpxC inhibitors are active against MDR *P. aeruginosa* and MDR *E. coli* (Talbot 2010; Mansoor et al. 2011).

MBX 1162 is a bis-indole antibiotic, which interacts with the DNA, inhibiting DNA and RNA synthesis, and induction of the SOS response. It was found to be in vitro active against MDR *A. baumannii* and ESBL-producing *K. pneumoniae* and several Gram-positive bacteria including MRSA and VRE (Butler et al. 2010).

Mastoparan, melittin, and indolicidin are new antimicrobial peptides which showed good activity against both colistin-susceptible and colistin-resistant *A. baumannii*. (Vila-Farres et al. 2012). They belong to a growing group of antimicrobials and host defense peptides with promising possible application and clinical relevance (Peters et al. 2010; Hancock and Sahl 2006).

KB001 is a high affinity antibody targeting the *P. aeruginosa* PcrV protein of the type III secretion system. It is blocking *P. aeruginosa* virulence mechanism by blocking it ability to deliver exotoxins leading to reduced pathogenicity (Baer et al. 2009).

3.5 Conclusions

In this review we discussed bacterial resistance to antibiotics, the current serious situation and the few attempts to overcome the problem. Focus was on the medical needs for facing infections by Gram-negative pathogens. The acute dangerous situation that exists today may bring us back to the time before 1930, when bacterial infections had no cure. If this happens, the prophecy of Louis Pasteur will be fulfilled and “microbes will have the last word”.

References

Abbo A, Navon-Venezia S, Hammer-Muntz O et al (2005) Multidrug-resistant A*cinetobacter baumannii*. Emerg Infect Dis 11:22–29

Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM (2011) Phage treatment of human infections. Bacteriophage 1:66–85

Abraham EP, Chain E (1940) An enzyme from bacteria able to destroy penicillin. Nature 146:837

Aeschlimann JR (2003) The role of multidrug efflux pumps in the antibiotic resistance of *Pseudomonas aeruginosa* and other Gram-negative bacteria. Pharmacother 23:916–924

Aloush V, Navon-Venezia S, Seigman-Igra Y et al (2006) Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. Antimicrob Agents Chemother 50:43–48

Ambler RP (1980) The structure of β-lactamases. Philos Trans R Soc Lond B Biol Sci 289:321–331

Anderson DL (2008) Sitafloxacin hydrate for bacterial infections. Drugs Today (Barc) 44:489–501

Arruda EA, Marinho IS, Boulos M et al (1999) Nosocomial infections caused by multiresistant *Pseudomonas aeruginosa*. Infect Control Hosp Epidemiol 20:620–623

Baer M, Sawa T, Flynn P et al (2009) An engineered human antibody fab fragment specific for *Pseudomonas aeruginosa* PcrV antigen has potent antibacterial activity. Infect Immun 77:1083–1090

Bassetti M, Nicolini L, Esposito S, Righi E, Viscoli C (2009) Current status of newer carbapenems. Curr Med Chem 16:564–575

Beharry Z, Palzkill T (2005) Functional analysis of active site residues of the fosfomycin resistance enzyme FosA from *Pseudomonas aeruginosa*. J Biol Chem 280:17786–17791

Beno P, Krcmery V, Demitrovicova A (2006) Bacteraemia in cancer patients caused by colistin-resistant Gram-negative bacilli after previous exposure to ciprofloxacin and/or colistin. Clin Microbiol Infect 12:497–498

Bergogne-Berezin E, Towner KJ (1996) *Acinetobacter* spp. as a nosocomial pathogen: microbiological, clinical, and epidemiological features. Clin Microbiol Rev 9:148–165

Bonomo RA, Szabo D (2006) Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin Infect Dis 43(Suppl 2):S49–S56

Borer A, Saidel-Odes L, Riesenberg K et al (2009) Attributable mortality rate for carbapenem-resistant *Klebsiella pneumoniae* bacteremia. Infect Control Hosp Epidemiol 30:972–976

Bou G, Martinez-Beltran J (2000) Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC beta-lactamase in *Acinetobacter baumannii*. Antimicrob Agents Chemother 44:428–432

Bratu S, Brooks S, Burney S et al (2007) Detection and spread of *Escherichia coli* possessing the plasmid-borne carbapenemase KPC-2 in Brooklyn, New York. Clin Infect Dis 44:972–975

Bratu S, Landman D, Haag R et al (2005) Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City. Arch Intern Med 165:1430–1435

Bush K, Jacoby GA (2010) Updated functional classification of beta-lactamases. Antimicrob Agents Chemother 54:969–976

Bush K, Jacoby GA, Medeiros AA (1995) A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 39:1211–1233

Butler MM, Williams JD, Peet NP et al (2010) Comparative in vitro activity profiles of novel bis-indole antibacterials against Gram-positive and Gram-negative clinical isolates. Antimicrob Agents Chemother 54:3974–3977

Buynak JD, Ghadachanda VR, Vogeti L, Zhang H, Chen H (2005) Synthesis and evaluation of 3-(carboxymethylidene)- and 3-(carboxymethyl) penicillinates as inhibitors of beta-lactamase. J Org Chem 70:4510–4513

Canton R, Coque TM (2006) The CTX-M β-lactamase pandemic. Curr Opin Microbiol 9:466–475

CDC (2004) *Acinetobacter baumannii* infections among patients at military medical facilities treating injured U.S. service members, 2002–2004. MMWR Morb Mortal Wkly Rep 53:1063–1066

CDC (2009) Guidance for control of infections with carbapenem-resistant or carbapenemase-producing *Enterobacteriaceae* in acute care facilities. Morb Mortal Wkly Rep 58:256–260

Chahine EB, Ferrill MJ, Poulakos MN (2010) Doripenem: a new carbapenem antibiotic. Am J Health Syst Pharm 67:2015–2024

Chang KC, Lin MF, Lin NT, Wu WJ, Kuo HY, Lin TY, Yang TL, Chen YC, Liou ML (2012) Clonal spread of multidrug-resistant *Acinetobacter baumannii* in eastern Taiwan. J Microbiol Immunol Infect 45:37–42

Curcio D (2011) Activity of a novel combination against multidrug-resistant nonfermenters: ceftazidime plus NXL104. Expert Rev Anti Infect Ther 9:173–176

Da Silva G, Dijkshoorn L, van der Reijden T, van Strijen B, Duarte A (2007) Identification of widespread, closely related *Acinetobacter baumannii* isolates in Portugal as a subgroup of European clone II. Clin Microbiol Infect 13:190–195

Daikos GL, Karabinis A, Paramythiotou E et al (2007) VIM-1-producing *Klebsiella pneumoniae* bloodstream infections: analysis of 28 cases. Int J Antimicrob Agents 29:471–473

Datta N (1969) Drug resistance and R factors in the bowel bacteria of London patients before and after admission to hospital. Br Med J 2:407–411

Diekma DJ, Bollet C, Carlioz A et al (2002) Age-related trends in pathogen frequency and antimicrobial susceptibility of bloodstream isolates in North America SENTRY Antimicrobial Surveillance Program, 1997–2000. Int J Antimicrob Agents 20:412–418

Dozzo P, Moser HE (2008) New aminoglycoside antibiotics. Expert Opin Ther Pat 20:1321–1341

Drlica K, Malik M (2003) Fluoroquinolones: action and resistance. Curr Top Med Chem 3:249–282

Emrich NC, Heisig A, Stubbings W, Labischinski H, Heisig P (2010) Antibacterial activity of finafloxacin under different pH conditions against isogenic strains of *Escherichia coli* expressing combinations of defined mechanisms of fluoroquinolone resistance. J Antimicrob Chemother 65:2530–2533

Eschenburg S, Priestman M, Schonbrunn E (2005) Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamineenolpyruvyltransferase (MurA) is essential for product release. J Biol Chem 280:3757–3763

European Centre for Disease Prevention and Control (ECDC) (2008) Annual epidemiological report on communicable diseases in Europe 2008. Stockholm, Sweden.http://ecdc.europa.eu/en/publications/Publications/0812_SUR_Annual_Epidemiological_Report_2008.pdf. Accessed March 2012

European Centre for Disease Prevention and Control/European Medicines Agency Joint Working Group (ECDC/EMEA) (2009) The bacterial challenge: time to react. www.ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf. Accessed March 2012

Falagas ME, Bliziotis IA, Kasiakou SK et al (2005) Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria. BMC Infect Dis 5:24

Falagas ME, Kopterides P (2006) Risk factors for the isolation of multi-drugresistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: a systematic review of the literature. J Hosp Infect 64:7–15

Falagas ME, Rafailidis PI, Kofteridis D, Virtzili D, Chelvatzoglou FC et al (2007) Risk factors of carbapenem-resistant *Klebsiella pneumoniae* infections: a matched case control study. J Antimicrob Chemother 60:1124–1130

Falagas ME, Rafailidis PI, Matthaiou DK (2010) Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Update 13:132–138

Falagas ME, Rafailidis PI, Matthaiou DK, Virtzili S, Nikita D, Michalopoulos A (2008) Pandrug-resistant *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumanni*i infections: characteristics and outcome in a series of 28 patients. Int J Antimicrob Agents 32:450–454

Farrel DJ, Morrissey I, De Rubeis D et al (2003) A UK study of the antimicrobial susceptibility of bacterial pathogen causing urinary tract infection. J Infect 46:94–100

Fluit AC, Verhoef J, Schmitz FJ (2001) European SENTRY participants. frequency of isolation and antimicrobial resistance of Gram-negative and gram-positive bacteria from patients in intensive care units of 25 European university hospitals participating in the European arm of the SENTRY Antimicrobial Surveillance Program 1997–1998. Eur J Clin Microbiol Infect Dis 20:617–625

Gales AC, Jones RN, Sader HS (2006) Global assessment of the antimicrobial activity of polymyxin B against 54,731 clinical isolates of Gram-negative bacilli: report from the SENTRY antimicrobial surveillance programme (2001–2004). Clin Microbiol Infect 12:315–321

Garcia-Penuela E, Aznar E, Alarcon T, Lopez-Brea M (2006) Susceptibility pattern of *Acinetobacter baumannii* clinical isolates in Madrid vs Hong Kong. Rev Esp Quimioter 19:45–50

Gaynes R, Edwards JR (2005) National nosocomial infections surveillance system. Overview of nococomial infections caused by Gram-negative bacilli. Clin Infect Dis 41:848–854

Georges B, Conil JM, Dubouix A, Archambaud M, Bonnet E, Saivin S et al (2006) Risk of emergence of *Pseudomonas aeruginosa* resistance to beta-lactam antibiotics in intensive care units. Crit Care Med 34:1636–1641

Giamarellou H, Kanellakopoulou K (2008) Current Therapies for *Pseudomonas aeruginosa*. Crit Care Clin 24:261–278

Giske CG, Monnet DL, Cars O, Carmeli Y (2008) Clinical and economic impact of common multidrug-resistant Gram-negative bacilli. Antimicrob Agents Chemother 52:813–821

Go ES, Urban C, Burns J, Kreiswirth B et al (1994) Clinical and molecular epidemiology of *Acinetobacter* infections sensitive only to polymyxin B and sulbactam. Lancet 344:1329–1332

Goa KL, Noble S (2003) Panipenem/betamipron. Drugs 63(9):913–926

Gomi K, Fujimura S, Fuse K et al (2011) Antibacterial activity of carbapenems against clinical isolates of respiratory bacterial pathogens in the northeastern region of Japan in 2007. J Infect Chemother 17:200–206

Gordon KA, Jones RN; SENTRY participant groups (Europe, Latin America, North America) (2003) Susceptibility patterns of orally administered antimicrobials among urinary tract infection pathogens from hospitalized patients in North America: comparison report to Europe and Latin America. results from the SENTRY Antimicrobial Surveillance Program. Diagn Microbiol Infect Dis 45:295–301

Govan JR, Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 60:539–574

Grossman TH, Starosta AL, Fyfe C et al (2012) Target-and resistance-based mechanistic studies with TP-434, a novel fluorocycline antibiotic. Antimicrob Agents Chemother. Feb 21, 2012 (Epub ahead of print)

Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24:1551–1557

Henrichfreise B, Wiegand I, Pfister W et al (2007) Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrob Agents Chemother 51:4062–4070

Hirsch EB, Ledesma KR, Chang KT, Motyl MR, Tam VH (2010*)* In vitro activity of MK-7655 in combination with imipenem (IPM) against carbapenem resistant Gram-negative bacteria. Paper presented at 50th interscience conference on antimicrobial agents and chemotherapy, Boston, MA, USA, 12–15 September 2010

Hornsey M, Ellington MJ, Doumith M et al (2010) AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. J Antimicrob Chemother 65:1589–1593

Hou J, Huang X, Deng Y, He L et al (2012) Dissemination of fosfomycin resistance gene *fosA3* with CTX-M β-lactamase genes and *rmtB* carried on IncFII plasmids among *Escherichia coli* isolates from pets in China. Antimicrob Agents Chemother Jan 9 (Epub ahead of print)

Iredell J, Thomas L, Power D, Mendes E (2007) Tigecycline resistance in Australian antibiotic-resistant Gram-negative bacteria. J Antimicrob Chemother 59:816–818

Ishii Y, Eto M, Mano Y, Tateda K, Yamaguchi K (2010) In vitro potentiation of carbapenems with ME1071, a novel metallo-beta-lactamase inhibitor, against metallo-beta-lactamase-producing *Pseudomonas aeruginosa* clinical isolates. Antimicrob Agents Chemother 54:3625–3629

Jacoby G, Medeiros A (1991) More extended-spectrum β-lactamases. Antimicrob Agents Chemother 35:1697–1704

Jamieson CE, Lambert PA, Simpson IN (2003) In vitro activities of novel oxapenems, alone and in combination with ceftazidime, against Gram-positive and Gram-negative organisms. Antimicrob Agents Chemother 47:2615–2618

Jia B, Lu P, Huang W et al (2010) A multicenter, randomized controlled clinical study on biapenem and imipenem/cilastatin injection in the treatment of respiratory and urinary tract infections. Chemotherapy 56:285–290

Jones RN, Deshpande L, Fritsche TR, Sader HS (2004) Determination of epidemic clonality among multidrug-resistant strains of *Acinetobacter* spp. and *Pseudomonas aeruginosa* in the MYSTIC Programme (USA, 1999–2003). Diagn Microbiol Infect Dis 49:211–216

Kallel H, Bahloul M, Hergafi L, Akrout M et al (2006) Colistin as a salvage therapy for nosocomial infections caused by multidrug-resistant bacteria in the ICU. Int J Antimicrob Agents 28:366–369

Karlowsky JA, Jones ME, Thornsberry C et al (2003) Trends in antimicrobial susceptibilities among *Enterobacteriaceae* isolate from hospitalized patients in the United States from 1998 to 2001. Antimicrob Agents Chemother 47:1672–1680

Kaur K, Adediran SA, Lan MJ, Pratt RF (2003) Inhibition of beta-lactamases by monocyclic acyl phosph(on)ates. Biochemistry 42:1529–1536

Keeney D, Ruzin A, Bradford PA (2007) RamA, a transcriptional regulator, and AcrAB, an RND-type efflux pump, are associated with decreased susceptibility to tigecycline in *Enterobacter cloacae*. Microb Drug Resist 13:1–6

Keller MA, Stiehm ER (2000) Passive immunity in prevention and treatment of infectious diseases. Clin Microbiol Rev 13:602–614

Kitchel B, Rasheed JK, Patel JB et al (2009) Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: clonal expansion of multilocus sequence type 258. Antimicrob Agents and Chemother 53:3365–3370

Klevens RM, Edwards JR, Richards CL et al (2007) Estimating healthcare-associated infections in U.S. hospitals. Public Health Rep 122:160–166

Koga T, Masuda N, Kakuta M, Namba E, Sugihara C, Fukuoka T (2008) Potent In vitro activity of tomopenem (CS-023) against methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 52:2849–2854

Kumarasamy KK, Toleman MA, Walsh TR et al (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10:597–602

Lagacé-Wiens PR, Tailor F, Simner P et al (2011) Activity of NXL104 in combination with β-lactams against genetically characterized *Escherichia coli* and *Klebsiella pneumoniae* isolates producing class A extended-spectrum β-lactamases and class C β-lactamases. Antimicrob Agents Chemother 55:2434–2437

Landman D, Kelly P, Bäcker M et al (2011) Antimicrobial activity of a novel aminoglycoside, ACHN-490, against *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from New York City. J Antimicrob Chemother 66:332–334

Leavitt A, Navon-Venezia S, Chmelnitsky I et al (2007) Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* strains in an Israeli hospital. Antimicrob Agents Chemother 51:3026–3029

Lee H, Yong D, Yum JH et al (2006) Dissemination of 16S rRNA methylase-mediated highly amikacin-resistant isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Korea. Diagn Microbiol Infect Dis 56:305–312

Lee HH, Molla MN, Cantor CR, Collins JJ (2010) Bacterial charity work leads to population-wide resistance. Nature 467:82–85

Lee J, Patel G, Huprikar S, Calfee DP, Jenkins SG (2009) Decreased susceptibility to polymyxin B during treatment for carbapenem-resistant *Klebsiella pneumoniae* infection. J Clin Microbiol 47:1611–1612

Livermore DM (2002) Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis 34:634–640

Livermore DM (2009) Has the era of untreatable infections arrived? J Antimicrob Chemother 64(Suppl 1):i29–i36

Livermore DM, Hope R, Brick G, et al (2008) Non-susceptibility trends among *Enterobacteriaceae* from bacteraemias in the UK and Ireland,2001–06. J Antimicrob Chemother 62(Suppl 2): ii41–ii54

Livermore DM, Mushtaq S, Warner M (2009) Activity of the anti-MRSA carbapenem razupenem (PTZ601) against *Enterobacteriaceae* with defined resistance mechanisms. J Antimicrob Chemother 64:330–335

Livermore DM, Mushtaq S, Warner M (2010) Activity of BAL30376 (monobactam BAL19764 + BAL29880 + clavulanate) versus Gram-negative bacteria with characterized resistance mechanisms. J Antimicrob Chemother 65:2382–2395

Livermore DM, Mushtaq S, Warner M, Zhang JC et al (2011) Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant *Enterobacteriaceae* isolates. J Antimicrob Chemother 66:48–53

Livermore DM, Woodford N (2006) The β-lactamase threat in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter*. Trends Microbiol 14:413–420

Lolans KT, Rice W, Munoz-Price LS, Quinn JP (2006) Multicity outbreak of carbapenem-resistant *Acinetobacter baumannii* isolates producing the carbapenemase OXA-40. Antimicrob Agents Chemother 50:2941–2945

MacNeil IA, Tiong CL, Minor C et al (2001) Expression and isolation of antimicrobial small molecules from soil DNA libraries. J Mol Microbiol Biotechnol 3:301–308

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y et al (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18:268–281

Magnet S, Courvalin P, Lambert T (2001) Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrob Agents Chemother 45:3375–3380

Mahgoub S, Ahmed J, Glatt AE (2002) Completely resistant *Acinetobacter baumannii* strains. Infect Control Hosp Epidemiol 23:477–479

Mansoor UF, Vitharana D, Reddy PA et al (2011) Design and synthesis of potent Gram-negative specific LpxC inhibitors. Bioorg Med Chem Lett 21:1155–1161

Maragakis LL (2010) Recognition and prevention of multidrug-resistant Gram-negative bacteria in the intensive care unit. Crit Care Med 38(Suppl. 8):S345–S351

Maragakis LL, Perl TM (2008) *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. Clin Infect Dis 46:1254–1263

Medina J, Formento C, Pontet J et al (2007) Prospective study of risk factors for ventilator-associated pneumonia caused by *Acinetobacter* species. J Crit Care 22:18–27

Moland ES, Hanson ND, Black JA et al (2009) Prevalence of newer β-lactamases in Gram-negative clinical isolates collected in the United States from 2001 to 2002. J Clin Microbiol 44:3318–3324

Mortensen NP, Fowlkes JD, Sullivan CJ et al (2009) Effects of colistin on surface ultrastructure and nanomechanics of *Pseudomonas aeruginosa* cells. Langmuir 25:3728–3733

Moya B, Zamorano L, Juan C, Pérez JL, Ge Y, Oliver A (2010) Activity of a new cephalosporin, CXA-101 (FR264205), against β-lactam-resistant *Pseudomonas aeruginosa* mutants selected in vitro and after antipseudomonal treatment of intensive care unit patients. Antimicrob Agents Chemother 54:1213–1217

Mushtaq S, Warner M, Livermore D (2010) Activity of the siderophore monobactam BAL30072 against multiresistant non-fermenters. J Antimicrob Chemother 65:266–270

Naas TS, Kernbaum S, Allali S, Nordmann P (2007) Multidrug-resistant *Acinetobacter baumannii*, Russia. Emerg Infect Dis 13:669–671

National Institute of Allergy and Infectious Diseases (NIAID) (2006) The problem of antimicrobial resistance. http://www.idph.state.ia.us/adper/common/pdf/abx/tab9_niaid_resistance.pdf. Accessed March 2012

National Nosocomial Infections Surveillance (NNIS) (2004) System report, data summary from January 1992 through June 2004. Am J Infect Control 32:470–485

Navon-Venezia S, Ben-Ami R, Carmeli Y (2005) Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. Curr Opin Infect Dis 18:306–313

Nemec A, Dolzani L, Brisse S et al (2004) Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones. J Med Microbiol 53:1233–1240

Obara M, Nakae T (1991) Mechanisms of resistance to beta-lactam antibiotics in *Acinetobacter calcoaceticus*. J Antimicrob Chemother 28:791–800

Ohmagari N, Hanna H, Graviss L et al (2005) Risk factors for infections with multidrug-resistant *Pseudomonas aeruginosa* in patients with cancer. Cancer 104:205–212

Oliver A, Canton R, Campo P et al (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science 288:1251–1254

Page MG, Dantier C, Desarbre E (2010) In vitro properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant gram-negative bacilli. Antimicrob Agents Chemother 54:2291–2302

Page MG, Dantier C, Desarbre E, Gaucher B et al (2011) In vitro and in vivo properties of BAL30376, a β-lactam and dual beta-lactamase inhibitor combination with enhanced activity against Gram-negative Bacilli that express multiple β-lactamases. Antimicrob Agents Chemother 55:1510–1519

Paramythiotou E, Lucet JC, Timsit JF et al (2004) Acquisition of multidrug-resistant *Pseudomonas aeruginosa* in patients in intensive care units: role of antibiotics with antipseudomonal activity. Clin Infect Dis 38:670–677

Patel G, Huprikar S, Factor SH et al (2008) Outcomes of carbapenem-resistant *Klebsiella pneumoniae* infection and the impact of antimicrobial and adjunctive therapies. Infect Control Hosp Epidemiol 29:1099–1106

Peleg AY, Bell JM, Hofmeyr A, Wiese P (2006) Inter-country transfer of Gram-negative organisms carrying the VIM-4 and OXA-58 carbapenem-hydrolysing enzymes. J Antimicrob Chemother 57:794–795

Peleg AY, Paterson DL (2006) Multidrug-resistant *Acinetobacter*: a threat to the antibiotic era. Intern Med J 36:479–482

Peleg AY, Seifert H, Paterson DL (2008) *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev 21:538–582

Peters BM, Shirtliff ME, Jabra-Rizk MA (2010) Antimicrobial peptides: primeval molecules or future drugs? PLoS Pathog 6(10):e1001067

Peterson LR (2009) Bad bugs, no drugs: no ESCAPE revisited. Clin Infect Dis 49:992–993

Poirel L, Nordmann P (2006) Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin Microbiol Infect 12:826–836

Quale J, Shah N, Kelly P et al (2011) Activity of polymyxin B and the novel polymyxin analogue CB-182, 804 against contemporary Gram-negative pathogens in New York City. Microb Drug Resist, Dec 23, 2011 (Epub ahead of print)

Ribera A, Ruiz J, Vila J (2003) Presence of the Tet M determinant in a clinical isolate of *Acinetobacter baumannii*. Antimicrob Agents Chemother 47:2310–2312

Rice LB (2006) Challenges in identifying new antimicrobial agents effective for treating infections with *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Clin Infect Dis 43(Suppl 2):S100–S105

Rice LB (2008) Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis 197:1079–1081

Robicsek A, Jacoby GA, Hooper DC (2006) The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis 6:629–640

Schneider P, Hawser S, Islam K (2003) Iclaprim, a novel diaminopyrimidine with potent activity on trimethoprim sensitive and resistant bacteria. Bioorg Med Chem Lett 13:4217–4221

Schwaber MJ, Carmeli Y (2007) Mortality and delay in effective therapy associated with extended-spectrum β-lactamase production in *Enterobacteriaceae* bacteraemia: a systematic review and metaanalysis. J Antimicrob Chemother 60:913–920

Schwaber MJ, Klarfeld-Lidji S, Navon-Venezia S et al (2008) Predictors of carbapenem-resistant *Klebsiella pneumoniae* acquisition among hospitalized adults and effect of acquisition on mortality. Antimicrob Agents Chemother 52:102810–102833

Scott P, Deye G, Srinivasan A, Murray C et al (2007) An outbreak of multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. Clin Infect Dis 44:1577–1584

Seifert H, Stefanik D, Wisplinghoff H (2006) Comparative in vitro activities of tigecycline and 11 other antimicrobial agents against 215 epidemiologically defined multidrug-resistant *Acinetobacter baumannii* isolates. J Antimicrob Chemother 58:1099–1100

Shahid M, Sobia F, Singh A et al (2009) Beta-lactams and beta-lactamase inhibitors in current or potential-clinical practice: a comprehensive update. Crit Rev Microbiol 35:81–108

Shakil S, Khan R, Zarrilli R, Khan AU (2008) Aminoglycosides versus bacteria-a description of the action, resistance mechanism, and nosocomial battleground. J Biomed Sci 15:5–14

Siroy A, Molle V, Lemaitre-Guillier C et al (2005) Channel formation by CarO, the carbapenem resistance-associated outer membrane protein of *Acinetobacter baumannii*. Antimicrob Agents Chemother 49:4876–4883

Speert DP, Campbell ME, Davidson AG, Wong LT (1993) *Pseudomonas aeruginosa* colonization of the gastrointestinal tract in patients with cystic fibrosis. J Infect Dis 167:226–229

Spratt BG (1994) Resistance to antibiotics mediated by target alterations. Science 264:388–393

Sutcliffe JA (2011) Antibiotics in development targeting protein synthesis. Ann N Y Acad Sci 1241:122–152

Takeda S, Nakai T, Wakai Y, Ikeda F, Hatano K (2007) *In vitro* and in vivo activities of a new cephalosporin, FR264205, against *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 51:826–830

Talbot GH (2010) The antibiotic development pipeline for multidrug-resistant Gram-negative bacilli: current and future landscapes. Infect Control Hosp Epidemiol 31(Suppl. 1):S55–S58

Tam VH, Kai-Tai Chang KT, Abdelraouf K et al (2010) Prevalence, resistance mechanisms, and susceptibility of multidrug-resistant bloodstream isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 54:1160–1164

Thomas KD, Adhikari AV, Chowdhury IH et al (2011) Design, synthesis and docking studies of quinoline-oxazolidinone hybrid molecules and their antitubercular properties. Eur J Med Chem 46:4834–4845

Titelman E, Karlsson IM, Ge Y, Giske CG (2011) In vitro activity of CXA-101 plus tazobactam (CXA-201) against CTX-M-14- and CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae*. Diagn Microbiol Infect Dis 70:137–141

Török ME, Chapman AL, Lessing MP, Sanderson F, Seaton RA (2010) Outpatient parenteral antimicrobial therapy: recent developments and future prospects. Curr Opin Investig Drugs 11:929–939

Tzouvelekis LS, Gazouli M, Prinarakis EE, Tzelepi E, Legakis NJ (1997) Comparative evaluation of the inhibitory activities of the novel penicillanic acid sulfone ro 48–1220 against beta-lactamases that belong to groups 1, 2b, and 2be. Antimicrob Agents Chemother 41:475–477

Unal S, Garcia-Rodriguez JA (2005) Activity of meropenem and comparators against *Pseudomonas aeruginosa* and *Acinetobacter* spp. isolated in the MYSTIC Program, 2002–2004. Diagn Microbiol Infect Dis 53:265–271

Urbanowski ML, Lykken GL, Yahr TL (2005) A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system. Proc Natl Acad Sci USA 102:9930–9935

Vaara M, Fox J, Loidl G et al (2008) Novel polymyxin derivatives carrying only three positive charges are effective antibacterial agents. Antimicrob Agents Chemother 52:3229–3236

Vila J, Ruiz J, Goni P, Jimenez de Anta T (1997) Quinolone-resistance mutations in the topoisomerase IV *parC* gene of *Acinetobacter baumannii*. J Antimicrob Chemother 39:757–762

Vila-Farres X, Garcia de la Maria C, López-Rojas R et al (2012) In vitro activity of several antimicrobial peptides against colistin-susceptible and colistin-resistant *Acinetobacter baumannii*. Clin Microbiol Infect 18:383–387

Wachino J, Yamane K, Suzuki S, Kimura K, Yl Arakawa (2010) Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. Antimicrob Agents Chemother 54:3061–3064

Walther-Rasmussen J, Hoiby N (2007) Class A carbapenemases. J Antimicrob Chemother 60:470–482

Xiao XY, Hunt DK, Zhou J et al (2012) Fluorocyclines. 1. 7-fluoro-9-pyrrolidinoacetamido-6-demethyl-6-deoxytetracycline: a potent, broad spectrum antibacterial agent. J Med Chem 55:597–605

Yigit H, Queenan AM, Anderson GJ et al (2001) Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 45:1151–1161

Yong D, Toleman MA, Giske CG et al (2009) Characterization of a new metallo-β-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 53:5046–5054

Zavascki AP, Barth AL, Fernandes JF et al (2006) Reappraisal of *Pseudomonas aeruginosa* hospital-acquired pneumonia mortality in the era of metallo-beta-lactamase-mediated multidrug resistance: a prospective observational study. Crit Care 10:R114

Part II
Families of Novel Candidates and Conventional Antibiotics

Chapter 4
The β-Lactam Antibiotics: Their Future in the Face of Resistance

Erika Leemans, Jed F. Fisher and Shahriar Mobashery

Abstract The search for new β-lactam antibacterial agents is a major challenge in medicinal and pharmaceutical chemistry. Methicillin-resistant *Staphylococcus aureus* (MRSA), pan-resistant *Enterobacteriaceae,* and pan-resistant nonfermenter bacteria are present day clinical scourges. New cephalosporins and carbapenems may solve this problem, but new monosulfactams and monocarbams also show promise. High-molecular-mass PBPs are envisioned as β-lactam targets. Further research keeps revealing interesting aspects about β-lactam resistance by β-lactamases, the existence of sentinel proteins, and the complexity of the cell envelope.

4.1 Introduction

A quintessential component of modern medicine is its ability to treat injury—whether scratch or severe trauma—with confidence that the treatment will not be compromised by infection. The basis for this confidence is chemistry: the availability to the physician of an array of chemotherapeutic agents, each capable of acting in concert with the immune response so as to extinguish bacterial and fungal growth. This confidence is now threatened. While the biochemical mechanisms of resistance are ancient, the profligate use of antibiotics has resurrected and refined these resistance mechanisms. New bacterial strains with an assembly of incremental and complementary resistance mechanisms now can evade the therapeutic concentrations attainable by antibiotics. Moreover, the increasing financial costs for antibiotic discovery and clinical development, and the meager reward once regulatory approval is secured, have diminished commercial interest in new

E. Leemans · J. F. Fisher · S. Mobashery (✉)
Department of Chemistry and Biochemistry, Nieuwland Science Hall,
University of Notre Dame, Notre Dame, IN 46556-5670, USA
e-mail: mobashery@nd.edu

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_4,

antibiotics. This circumstance has been correctly described (Piddock 2012; Wright 2012) as paradoxical: at a time when the collective powers of chemistry, enzymology, and cell biology are unraveling the remarkable interplay among the pathways used by bacteria to detect and perfect a response to the presence of antibiotics, we are at risk of entering a period where this understanding will not translate to improved clinical therapy (Bush et al. 2011).

A response to this paradox is the creation of new value for time-tested, and time-proven, antibiotics. The β-lactam antibacterials are preeminent in terms of efficacy and safety (Kardos and Demain 2011), notwithstanding progressive erosion of their value by the *Scylla* of diminished access to and resistance mutation within their molecular targets (Llarrull et al. 2010); and the *Charybdis* of bacterial enzymes (the β-lactamases) devoted to their molecular destruction (Bush and Fisher 2011). Here we survey the most recent developments in the chemistry, enzymology, and cell biology of the β-lactams, from the perspective of their relevance to the circumvention of bacterial resistance mechanisms and the preservation of the preeminence of the β-lactam antibiotics for future generations.

4.2 Recent Advances in the Chemistry of the β-Lactams

The β-lactam antibiotic sub-classes include the penicillins, cephalosporins, cephamycins, carbapenems, and monobactams (Testero et al. 2010). The antibiotic activity of each sub-class derives from mechanism-based inhibition of bacterial cell-wall biosynthesis. Depending on the structure, the β-lactams are efficacious against both Gram-positive and/or Gram-negative pathogens, and thus possess exceptional clinical value (see Rossolini et al.; Paitan and Ron, this volume). In addition, the weakly antibiotic clavulanate and penicillin sulfone β-lactam sub-classes (and now joined by new bicyclic lactam sub-classes) act as mechanism-based inhibitors of the serine-dependent β-lactamase enzymes, which synergize with the antibiotic β-lactams. The recent developments in the chemistry of these lactams include new cephalosporins, carbapenems, and monobactams (Bush and Pucci 2011; Butler and Cooper 2011), and new strategies for β-lactamase inactivation (Shahid et al. 2009; Drawz and Bonomo 2010; Biondi et al. 2011).

4.2.1 New Cephalosporins

4.2.1.1 Latest Generation Cephalosporins

The newest cephalosporins, each at different stages of clinical development, are ceftaroline, ceftobiprole, and CXA-101 (ceftolozane). The criteria used during the structural optimization of these (and other β-lactam) antibiotics against Gram-negative bacterial infection include the efficient inactivation of the

high-molecular-mass (biosynthetic) penicillin-binding protein (PBP) enzymes (Moya et al. 2010); minimal induction β-lactamase expression, as will result from inactivation of the low-molecular-mass penicillin-binding protein enzymes (Mark et al. 2011); intrinsic resistance to β-lactamase-catalyzed hydrolysis; and unfettered access to the enzyme targets as can be lost as a result of porin deletion (a contributory bacterial-resistance mechanism). The structural features of CXA-101 exemplify this optimization (Fig. 4.1). The aminothiazolyl-oxyimino side chain (left-hand structural segment of this CXA-101 depiction) is found in recent generations of the cephalosporins, and is used for its ability to preserve PBP affinity, while imparting stability toward many β-lactamases. A hydrophilic extension terminating in a cationic amine (seen as the right-hand structural segment of CXA-101) is also a multi-generational structural feature of the cephalosporins, and is used for its ability to enhance PBP affinity. CXA-101 is a parenteral cephalosporin with particular efficacy against *Pseudomonas aeruginosa*. The activity of CXA-101 is further potentiated against Gram-negative *Enterobacteriaceae* (such as *Escherichia coli* and *Klebsiella pneumoniae*) that express extended-spectrum β-lactamases, by co-formulation with tazobactam, a classic β-lactamase inhibitor (Livermore et al. 2010a). The CXA-101–tazobactam combination is not effective, however, against Gram-negative bacteria with more capable β-lactamases, such as the KPC carbapenemases. Indeed, the increasingly limited efficacy of all of the newest generation cephalosporins (and as well of the newest carbapenems) coincides with the increasing concern that an era of untreatable infection is near (Livermore 2012).

4.2.1.2 β-Lactam Hybrid Structures

One solution to the diminishing efficacy of the single agent β-lactam is the pairing of the β-lactam with a second therapeutic having a synergistic mechanism of action (Cottarel and Wierzbowski 2007; Ejim et al. 2011). Methods to identify synergistic pairings are further discussed in Sect. 4.4. This strategy is well exemplified—as just mentioned—by the pairing of clinically important β-lactam antibiotics with a β-lactamase inhibitor. The new pairings may correspond to co-formulation of the β-lactam with the β-lactamase inhibitor and the structural fusion of complementary antibiotics. Two examples of this latter approach are the exploratory cephalosporin-vancomycin hybrid TD-1792, and the design of antibiotic-functionalized cephalosporins for β-lactamase-catalyzed activation (Fig. 4.1).

4.2.1.3 TD-1792, A Covalent Cephalosporin-Vancomycin Hybrid

The chemical criteria for the fusion of two biologically active structures so as to attain mutual synergy are rigorous. Both the selection of the atoms for the interconnection, and the chemistry for the linker, must be perfect. Vancomycin is a

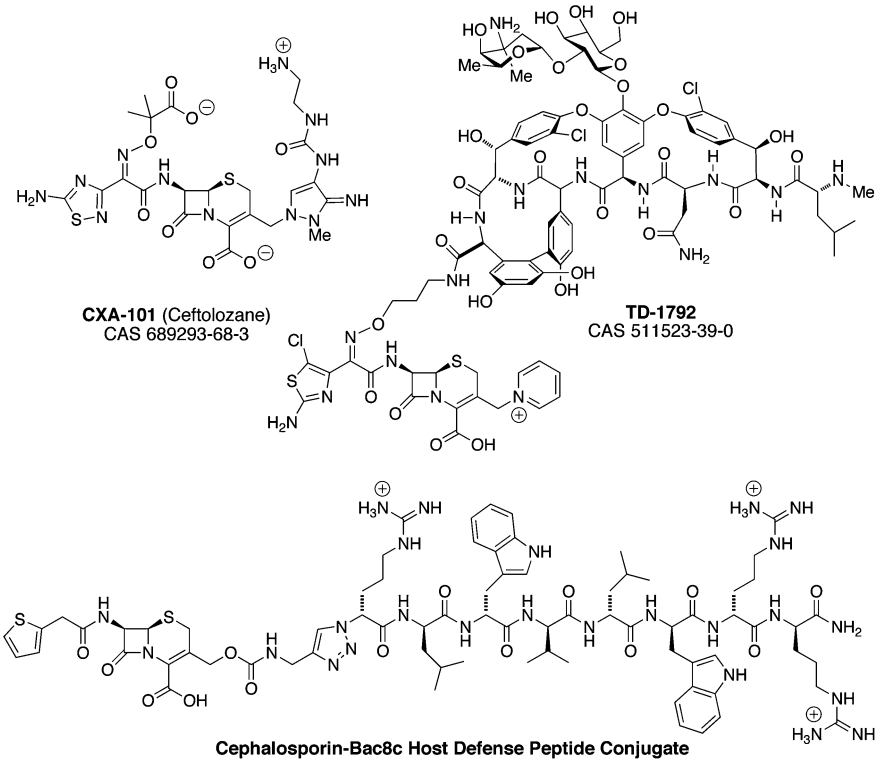


Fig. 4.1 Structures of new cephalosporins

Gram-positive antibiotic that interferes with bacterial cell-wall biosynthesis by a different, but complementary, mechanism compared to the PBP-inactivation mechanism of the β-lactams (Fisher and Mobashery 2010). Synergistic interconnection between the oxyimino segment of the cephalosporin and vancomycin yields the multivalent hybrid antibiotic TD-1792 (Long et al. 2008) having exquisite efficacy (greater than each antibiotic alone) against *Staphylococcus aureus*, including methicillin-susceptible, methicillin-resistant, and vancomycin intermediate-susceptible strains (Blais et al. 2012; Hegde et al. 2012), and as well other Gram-positive bacteria (Tyrrell et al. 2012). Given that the vancomycin structure is itself amenable to structural optimization (Allen 2011), hybrid antibiotics represent a viable strategy for the sustained efficacy of existing β-lactams against emerging pathogens. Although the gargantuan structure of TD-1792 (Fig. 4.1) may ultimately preclude this compound from progressing beyond that of an exploratory structure, it is nonetheless an innovative exemplification of the concept of multi-target synergism.

4.2.1.4 β-Lactamase-Catalyzed Antibiotic Release from Cephalosporins

The antibiotic mechanism of the β-lactams involves functionally irreversible acylation of an active-site serine residue of the PBP enzymes. Bacteria express serine-dependent, and/or metal-dependent, β-lactamase enzymes as a resistance mechanism. β-Lactamases hydrolytically open the β-lactam ring and hence abolish the antibiotic ability of the β-lactam. Cephalosporins have long been known to release their right-hand substituent—appended to C_3; this substituent is not present in the other β-lactam sub-classes—following hydrolytic opening of their β-lactam. While this ability was conceptualized as a means for secondary antibiotic release (Mobashery et al. 1986; Mobashery and Johnston 1987) to β-lactamase-expressing bacteria, its value has now expanded to include imaging of intracellular proteins (Mizukami et al. 2012). A compelling recent example which exemplifies the original conception is the cephalosporin-Bac8c peptide conjugate shown in Fig. 4.1 (Desgranges et al. 2012). The structure of the D-amino acid-derived Bac8c peptide segment used in this conjugate derives from the bovine host defense peptide, bactenecin. The antibacterial activity of the conjugate is distinct from the antibacterial activity of the separate segments, and is consistent with intrinsic activity for the conjugate itself that is abetted by β-lactamase release of the host defense peptide. Given the increasing need for antibiotics selective against emerging pathogens, the potential of this strategy to enhance the therapeutic index of synergistic antibiotic pairs is evident.

4.2.2 New Generation Carbapenems

The first carbapenem to enter into clinical practice in 1986 was imipenem. Subsequent carbapenem approvals include meropenem (1996 U.S.), ertapenem (2001 U.S.), doripenem (2007 U.S.), panipenem/betamipron (1993 Japan), and biapenem (2001 Japan). These carbapenems are categorized by their antibacterial spectrum into three classes. While the clinical impact of this class has been transformative—carbapenems are called the antibiotics of the last resort—during the past decade several experimental carbapenems have failed to progress to clinical approval. These failures suggest the possibility of structure-activity maturity within this class.

4.2.2.1 Anti-Gram-Positive Carbapenems

The continuing diffusion from hospitals to the community of methicillin-resistant *S. aureus* (MRSA) has stimulated efforts toward new anti-MRSA cephalosporin and carbapenem structures (see Rossolini et al. this volume). Carbapenems are the most potent β-lactam antibiotics, stable to hydrolysis by many β-lactamases, and

consequently effective against many cephalosporin-resistant microorganisms. Carbapenems with MRSA activity are termed as Group 3. Regrettably, several new Group 3 carbapenems have failed recently at late-stage clinical evaluation. Razupenem (Fig. 4.2) exemplifies a standard carbapenem structure, with a 1β-methyl substitution (to impart metabolic stability) and a hydrophilic, positively charged C-2 substituent found in various guises in all new carbapenems (Bassetti et al. 2009; El-Gamal and Oh 2010). This positively charged substituent is structurally similar to the substituents found at the equivalent position in ceftobiprole and ceftaroline, and embodies a structural requirement for effective inhibition of their PBP targets. Razupenem has additional antimicrobial activity against vancomycin intermediate *S. aureus* (VISA) and enterococci-like vancomycin-resistant *E. faecium* (VREF), as well as against some Gram-negative bacteria (but not *P. aeruginosa* and *A. baumannii*). Development of razupenem is now discontinued due to unacceptable levels of adverse events. The parenteral carbapenem ME1036 (Fig. 4.2) is structurally similar to razupenem, and is active against multi-drug resistant Gram-positive and Gram-negative bacteria including MRSA, VISA, and extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL). It has strong activity toward *H. influenza* and *Enterococcus faecalis*, and is more active than ceftriaxone and other broad-spectrum cephalosporins (including ceftaroline). ME1036 has potent activity against genotypic penicillin-intermediate *S. pneumonia* (gPISP) strains and genotypic penicillin-resistant *S. pneumonia* (gPRSP) strains that contain more than one mutation in their PBPs. This most recent publication on ME1036 establishes a direct correlation between its bactericidal potency against *S. pneumonia* and its PBP affinity (Hirai et al. 2011). There is no evidence that ME1036 is progressing in clinical evaluation.

The guanidine-pyrrolidine side chain found in tomopenem (Fig. 4.2), a third parenteral 1β-methyl carbapenem, provides high affinity for PBP1, PBP2, and PBP4 of *S. aureus* (Koga et al. 2009). In contrast to older carbapenems, tomopenem demonstrates activity against both MRSA and against methicillin-susceptible *S. aureus* (MSSA), as well as ESBL-producing *E. coli*, *Klebsiella* spp.,

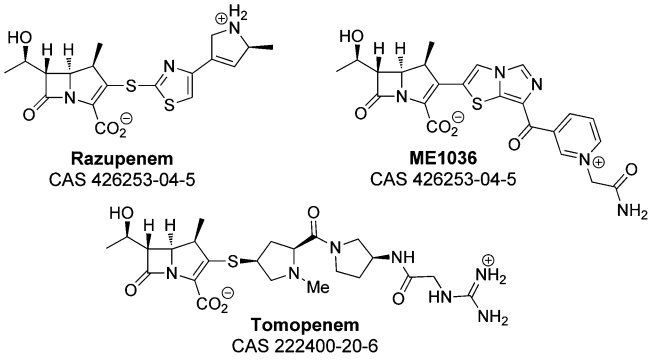


Fig. 4.2 Structures of anti Gram-positive carbapenems

imipenem-resistant and ceftazidime-resistant *P. aeruginosa*, expanded-spectrum cephalosporin-resistant *Enterobacteriaceae* and other Gram-positive and -negative bacteria (Koga et al. 2008). Tomopenem has a superior antibacterial effect against MRSA compared with vancomycin, and a longer half-life compared with other carbapenems except for ertapenem (vide infra). The development of tomopenem for countries outside of Japan is uncertain, as its development rights were returned to the innovator company.

4.2.2.2 Anti-Gram-Negative Carbapenems

A growing concern is future treatment options against pan-resistant *Enterobacteriaceae* and nonfermenter Gram-negative bacteria (see Paitan and Ron, this volume). Gram-negative bacteria acquire resistance to β-lactams by a combination of reduced permeability, active efflux, and expression of plasmid-encoded extended-spectrum β-lactamases (ESBLs, such as the CTX-M-14 and CTX-M-15 enzymes) and serine- and metal-dependent carbapenemases (Lascols et al. 2012). Often multiple β-lactamases appear in a single organism.

Imipenem (Fig. 4.3), a derivative of the natural product thienamycin, is used in combination with cilastatin (to inhibit its hydrolysis by the renal enzyme dehydropeptidase-I). It is a broad-spectrum Group 2 (nonfermenter) carbapenem. However, imipenem induces expression of the AmpC β-lactamase, a key resistance mechanism of many Gram-negatives. The more recent Group 2 meropenem (Fig. 4.3) has improved metabolic stability and reduced β-lactamase induction, and is used extensively for complicated urinary and respiratory infection. Meropenem is not as potent as imipenem or doripenem (vide infra) against *P. aeruginosa*. Biapenem (Fig. 4.3) is a broad-spectrum (active against many Gram-negative and Gram-positive aerobic and anaerobic bacteria, including β-lactamase producers) zwitterionic carbapenem, used in Japan since 2002 and more active than imipenem against ESBL-expressing *Enterobacteriaceae.*

Ertapenem (Fig. 4.3) is a Group 1 (limited non-fermenter activity) carbapenem that is structurally related to meropenem, and approved in the U.S. in 2001 for its activity against ESBL-producing *Enterobacteriaceae* (but inactive against *P. aeruginosa* and *A. baumannii*). Doripenem (Fig. 4.3), a new 1β-methyl carbapenem that was approved in Japan in 2005, has excellent Gram-positive, Gram-negative, and anaerobic coverage, including difficult to treat pathogens such as *E. coli* and *Klebsiella spp.* that produce extended-spectrum β-lactamases, penicillin-resistant *S. pneumonia*, *Pseudomonas* spp., *Citrobacter* spp., and *A. baumannii* with MIC values at least 16-fold lower than those for imipenem against the same isolates. *P. aeruginosa* isolates resistant to imipenem and meropenem may retain susceptibility to doripenem. Hydrolysis of doripenem is 2- to 150-fold slower than that of imipenem. Tebipenem-pivoxil (Fig. 4.3) is an oral carbapenem whose active metabolite shows broad-spectrum activity against Gram-positive bacteria and *Enterobacteriaceae*. Tebipenem-pivoxil is not active against MRSA, and unlike meropenem it has no activity against β-lactamase-producing *P. aeruginosa*.

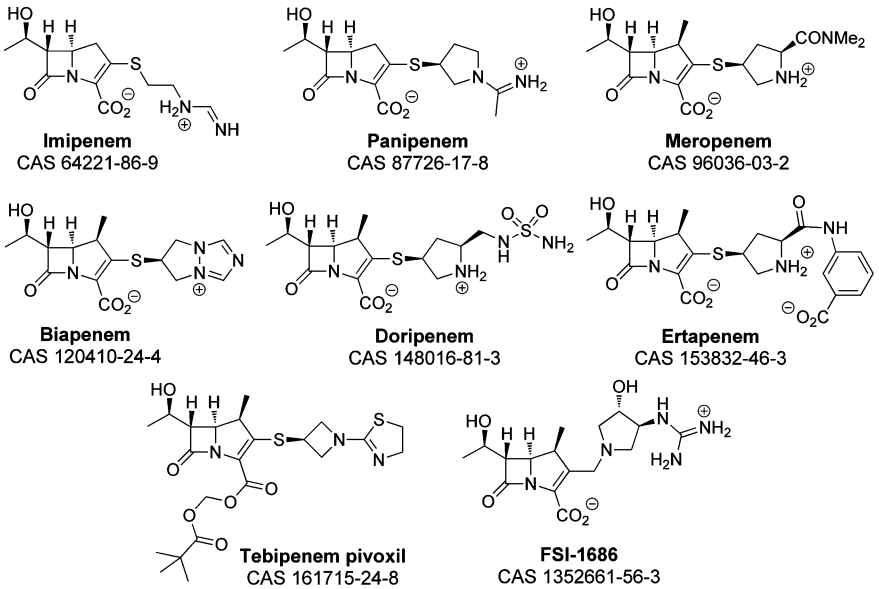


Fig. 4.3 Structures of anti Gram-negative carbapenems

It displays excellent activity against *S. pneumonia* strains, including penicillin-resistant strains. The most recent carbapenem entry is FSI-1686 (Fig. 4.3), representing a new carbapenem class that is very effective against multi-drug resistant Gram-negative bacteria including carbapenem-resistant *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*.

4.2.2.3 The Future of the Carbapenems

As is evident from inspection of these carbapenem structures, the structural evolution of the carbapenems since imipenem coincides with the incorporation of the 1β-methyl to attain renal metabolic stability, and the iterative exploration at C-2 of hydrophilic, positively charged substitution. It may be argued that the guanidinopyrrolidine found in FSI-1686 (Fig. 4.3) represents culmination of this strategy: functionality that attains new breadth of antibacterial activity, while simultaneously presenting challenges with respect to drug synthesis, drug formulation, and patient tolerability. Whether chemical space remains for further C-2 exploration is uncertain. The newest development with the carbapenems is the co-formulation of existing carbapenems with β-lactamase inhibitors, in response to the aggressive expansion of β-lactamase catalytic ability resulting in the dramatic erosion of the β-lactamase stability of the carbapenems. For example, combination of imipenem with MK-7655 (see 4.3.2.1), a new Class C and Class A β-lactamase

inhibitor, restored the antibacterial activity of imipenem against Class A (serine) carbapenemase-producing *Enterobacteriaceae* and multi-drug-resistant *P. aeruginosa* isolates. MK-7655 is currently undergoing Phase 1 safety evaluation, in anticipation of therapeutic combination with a carbapenem (Bush and Pucci 2011). The appearance of activity against *Mycobacterium tuberculosis* by combination of meropenem with clavulanic acid suggests the possibility of a new therapeutic approach against these Gram-positive bacteria (Hugonnet et al. 2009) and previously regarded as fully β-lactam refractory due to its constitutive expression of a powerful β-lactamase (Tremblay et al. 2010).

Further structural optimization of the carbapenems will likely follow two paths. The correlation of carbapenemase structure to kinetic analyses may provide structure-based design opportunity against specific pathogens (Frase et al. 2009; Ke et al. 2012). As a further example, change to the heretofore invariant 6-(hydroxyethyl) substituent of the carbapenems may provide opportunity for optimization against specific emerging carbapenemases, as suggested by initial studies (using a penicillin core) with a Class D enzyme (Testero et al. 2009; Verma et al. 2011).

4.2.3 New Generation Monocyclic β-Lactams

The absence of antibacterial activity in the monocyclic β-lactam obtained from the reductive desulfurization of penicillin was a key observation in the structural and mechanistic elucidation of the penicillin structure. This seeming correlation—between the absence of antibacterial activity and a monocyclic β-lactam structure—was confounded by the discovery some 30 years ago of the Gram-negative monobactam antibiotics, exemplified by the monobactam aztreonam (Fig. 4.4). While the eventual clinical value of aztreonam was limited as a result of its solely Gram-negative spectrum, against the habits of antibiotic use and prevalence of Gram-positive pathogens, interest in monocylic β-lactams has increased sharply with the emergence of multi-drug resistant Gram-negative bacteria (Canton and Lumb 2011; Kollef et al. 2011; Walsh and Toleman 2012). Aztreonam, and its more recent congener structures, have intrinsic stability toward many β-lactamases (including metallo-β-lactamases) and thus potential clinical value against newer pan-resistant *Pseudomonas*, *Acinetobacter*, *Escherichia*, and *Klebsiella* species. Their β-lactamase stability is further improved by combination with β-lactamase inhibitors, as prominently exemplified by the triple combination antibiotic (coded as BAL-30376, from Basilea Pharmaceutica) comprising the monobactam BAL-19764 (Fig. 4.4) with two β-lactam-based β-lactamase inhibitors, BAL-29880 and clavulanic acid (Page et al. 2011). Perspectives on new strategies for β-lactamase inhibition are given in Sect. 4.3.2.1. Comparison of the aztreonam and BAL-19764 structures calls attention to the (1,4-dihydro-1,5-dihydroxy-4-oxo-2-pyridinyl)methoxy substitution to the imine of the monobactam side chain in the latter, and which is absent in aztreonam. This substituent is iron-chelating and acts as a bacterial siderophore mimetic. Its use was conceptualized as a means of evading

porin deletion as a resistance mechanism, a common resistance event in Gram-negative bacteria. The several-decade history of the siderophore conception is summarized concisely elsewhere (Flanagan et al. 2011). Regardless of the absence to date of experimental data demonstrating their uptake by iron transporters, the "siderophore" monocyclic β-lactams clearly demonstrate expanded antibacterial potency and a low frequency of spontaneous resistance development. Accordingly, this same siderophore substitution is retained in the newest monocyclic β-lactams, those of the monosulfactam and monocarbam sub-classes.

4.2.3.1 The Monosulfactams

Neither the monosulfactam sub-class nor the monocarbam sub-class (Sect. 4.2.3.2) is as yet fully exemplified, in terms of structure–activity study, outside of the patent literature. For this reason, and because only in vitro activity data are available for those few structures that are disclosed, it is not possible to assess the long-term clinical potential of either sub-class. Nonetheless, the initial data for both are quite promising. Examination of the structures within the three monocyclic β-lactam sub-classes suggests that a diversity of acyl activating groups on the nitrogen of the β-lactam may be used. Comparison of the monosulfactam BAL-30072 (Fig. 4.4) with the monocarbam MC-1 (Fig. 4.4) further suggests tolerance for the structural placement of the siderophore segment. If these generalizations are sustained, significant future potential for the structure–activity development of both monocyclic β-lactams sub-classes is anticipated.

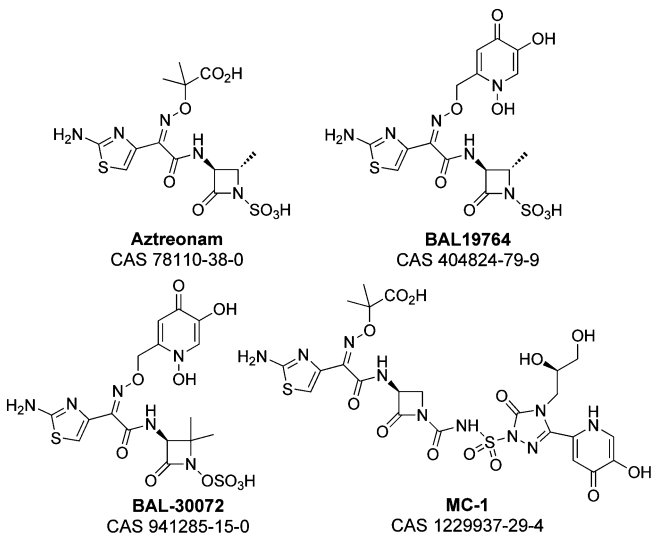


Fig. 4.4 Structures of new generation monocyclic β-lactams

The monocyclic sulfactam sub-class is exemplified thus far by BAL-30072 (Basilea) (Fig. 4.4). In vitro evaluation of BAL-30072 showed broad-spectrum Gram-negative activity, including activity against many carbapenem-resistant strains as a result of expression of Class C (AmpC) and Class D (Oxa) β-lactamases (Mushtaq et al. 2010; Page et al. 2010; Mima et al. 2011; Russo et al. 2011). For example, BAL-30072 was more active against meropenem-non-susceptible *A. baumannii* (65 % of the isolates, MIC $\leq$ 8 mg/L) when compared to other β-lactams (including imipenem, cefepime, aztreonam) and as well other antibacterials (including levofloxacin, amikacin, rifampicin) representing different antibacterial classes (Higgins et al. 2012). No correlation was found between the MIC values for those isolates with elevated BAL-30072 MIC values, with elevated MIC values for the other antibacterials.

4.2.3.2 The Monocarbams

Renewed interest in the monocarbams is evidenced by recent disclosures from Pfizer. An initial structure-activity study (Flanagan et al. 2011) has identified a prototype structure, MC-1 (Fig. 4.4). Its structure was evaluated as a point of reference for structure-based drug design, as described in the context of its crystal structure (as the acyl-enzyme) with the penicillin-binding protein targets encountered in the opportunistic Gram-negative pathogens *A. baumanii* and *P. aeruginosa* (Han et al. 2010, 2011). While numerous points for structural optimization were identified, the value of PBP acyl-enzyme structure for structure-based design toward improved kinetics for serine acylation from the Michaelis complex is uncertain (Nicola et al. 2010). These acyl-enzyme structures may provide, however, the basis for initiating computational evaluation of the key transition-state structures leading to the acyl-enzyme complex. The first QM/MM studies on PBP catalysis have been described (My et al. 2011; Shi et al. 2011).

4.3 Recent Advances in the Enzymology of the β-Lactams

The historical foci for the enzymology of the β-lactams have been their target enzymes, the penicillin-binding proteins, and the β-lactamase enzymes, as a preeminent β-lactam resistance mechanism. While the importance of these two enzyme classes is undiminished, new proteins and enzymes—both within these classes and related to them through resistance pathways—are emerging with direct relevance to the preservation of the clinical value of the β-lactams.

4.3.1 Penicillin-Binding Proteins

Cell-wall biosynthesis is required for every bacterium. Even among the "cell wall-less" bacteria a portion of the cell-wall biosynthetic pathway (exactly what portion(s) remains uncertain) is required to complete cell division (Henrichfreise et al. 2009; Gaballah et al. 2011). For those bacteria with a fully functional cell-wall biosynthetic pathway, the cell wall is assembled using several high-molecular-mass (HMM) PBP enzymes dedicated to the processes of cell-wall growth and septation. Many HMM PBPs are bifunctional, with separate transglycosylase and transpeptidase domains. The transpeptidase activity is the molecular target of the β-lactam antibiotics. Cell-wall assembly is refined using low-molecular-mass (LMM) PBP enzymes, which possess a single active site nearly identical to the transpeptidase active site of the HMM PBPs, but here with endopeptidase and/or carboxypeptidase activities. Although these enzymes are also molecular targets of the β-lactams, LMM PBP inactivation is now more clearly understood to directly connect to β-lactam resistance induction, rather than to the antibiotic activity. New revelations, within each of these two PBP sub-classes, are now influencing future thought with respect to β-lactam design.

4.3.1.1 The High-Molecular-Mass PBPs as ß-Lactam Targets

Study of the high-molecular-mass PBPs has been limited by their membrane-associated character, and by the extraordinary difficulty in accessing their substrates so as to enable meaningful evaluation of their in vitro catalytic activity. Both limitations are being addressed. Laborious, but reliable, syntheses of analogs of Lipid II—substrates for the transglycosylase activity—are now in place (Gampe et al. 2011). As membrane-associated enzymes, structural analysis of these enzymes using crystallography heretofore has used as constructs that lack the membrane-binding domain of these enzymes. This limitation is diminishing. For example, synthetic and crystallographic studies are converging with respect to understanding the interaction of the membrane-proximal transglycosylase domain of these PBPs with Lipid II, and understanding inhibitor occupancy of this same transglycosylase active site (Fuse et al. 2010; Huang et al. 2012). While the difficulty in the assay of their transpeptidase activity remains, robust screening methods for evaluating binding to the transpeptidase/β-lactamase active sites are becoming available (Bobba et al. 2011; Inglis et al. 2012).

The opportunity for structure-based optimization of β-lactam structure against Gram-negative bacteria against their specific HMM PBP target was discussed previously (Sect. 4.2.3.2). In the Gram-positive pathogen methicillin-resistant *S. aureus* (MRSA), β-lactam resistance is achieved through the acquisition of a gene encoding a new transpeptidase-specific PBP having intrinsic resistance against inactivation as a result of β-lactam antibiotic acylation (Fuda et al. 2004; Llarrull et al. 2009). While the endogenous PBP transpeptidase activity is lost as the result of β-lactam acylation of its active site serine, MRSA completes its cell

wall using the acquired PBP (termed PBP 2a). The structural basis for the intrinsic β-lactam resistance of PBP 2a is believed to be a pH-dependent steric interaction between a peptide loop guarding the active site, that is preferentially exerted against β-lactam inhibitors as opposed to the peptidoglycan substrate (Lemaire et al. 2008, 2009). Gratifyingly, experimental evidence supports allosteric modulation of this loop by the peptidoglycan itself to ameliorating the loop steric interaction, allow peptidoglycan entry (Fuda et al. 2005, 2006, 2007), and a positive correlation between β-lactam efficacy against MRSA and the ability of the β-lactam to evade this loop interaction (Villegas-Estrada et al. 2008). Future β-lactam design will be guided by the molecular structure of its PBP target (Livermore 2006). Indeed, this approach has led to unusual macrocyclic β-lactams as exploratory structures for PBP inhibition (Sliwa et al. 2012a, b).

4.3.1.2 The Connection Between the Low-Molecular-Mass PBPs and β-Lactam Resistance

The functional role of the LMM PBPs in bacteria is enigmatic (Ghosh et al. 2008). Nonetheless, key progress has been made with respect to the central roles of these enzymes in the separate events of Gram-negative bacterial growth and resistance expression. With respect to growth, select LMM PBPs "condition" the peptidoglycan polymer so as to determine cell shape, and to coordinate with the assembly of cytoskeletal proteins to enable cell division (Potluri et al. 2012). With respect to resistance development, a connection has been made recently between β-lactam structure and the differential ability of the β-lactam to induce a resistance response. While the existence of this connection is long known to the medicinal chemist, the molecular mechanisms determining this ability now are coming into focus. The key process connecting β-lactam structure to resistance is the recycling of the cell-wall peptidoglycan. During growth, new peptidoglycan is incorporated into the existing peptidoglycan. This incorporation occurs with substantial liberation of peptidoglycan components (called muropeptides). These muropeptides are liberated in the periplasmic space of the Gram-negative bacteria, proximal to the cell wall, and following enzymatic processing are internalized to the cytoplasm through dedicated permeases for further enzymatic recycling. The presumed purposes of this recycling are nutrient recovery, minimization of the innate immune response by the host—which is also muropeptide-structure-dependent (Boudreau et al. 2012)—and also control of the β-lactamase resistance response. This last purpose occurs through the involvement of LMM PBPs. Certain of these LMM PBPs alter the precise molecular composition of these muropeptides, resulting in a different "effector" pool of structures when the LMM PBP is catalytically active, compared to when it is not (as occurs upon its inactivation by β-lactams). The altered molecular composition of this effector pool is then sensed by one particular—AmpR—transcription factor (Balcewich et al. 2010). While derepression of β-lactamase expression is the notable event governed by AmpR, in some pathogenic Gram-negative bacteria the breadth of resistance and virulence

factors controlled through AmpR is extensive (Balasubramanian et al. 2012; Hennequin et al. 2012). With increasing awareness of the relationship between the muropeptide recycling pathway and resistance, the particular roles of the individual recycling enzymes in determining the effector pool are being identified: genetic deletion of one results in high-level β-lactamase expression, while co-administration of an inhibitor of another represses β-lactamase expression (Mark et al. 2011; Yamaguchi et al. 2012). The potential of synergistic drug pairings with β-lactams to forestall AmpR-dependent resistance is evident. A critical absence thus far for this understanding is the specific transformation(s) exerted on the effector pool by the LMM PBP. Notwithstanding considerable effort to identify the endogenous LMM PBP catalytic transformations through in vitro experimentation, the particular identity of the reactions they accomplish in vivo remains uncertain (Nemmara et al. 2011).

This same opportunity for synergistic combinations with the β-lactams may exist for Gram-positive pathogens. The presumption that Gram-positive bacteria cannot recycle muropeptides, since they have a peptidoglycan exoskeleton and a less-defined periplasmic space, is refuted for at least (thus far) some Gram-positives (Reith and Mayer 2011). Moreover, the BlaI transcription factor (which controls β-lactamase expression and can control that of PBP2a—a homolog of the MecI transcription factor that directly controls PBP2a expression in MRSA) is also responsive to a muropeptide effector pool (Amoroso et al. 2012). As discussed for AmpR, processes molecular strategies that alter the Gram-positive muropeptide effector pool may also forestall β-lactam-induced expression of resistance responses in these Gram-positives.

4.3.2 β-Lactamases

The separate but coincident discoveries of clavulanate and the penam sulfones (exemplified by tazobactam) as mechanism-based inhibitors of the serine β-lactamases inaugurated a three decade era of successful antibacterial therapy, that continues to this day (Shahid et al. 2009). Both inhibitors are used clinically in combination with penicillins (Drawz and Bonomo 2010). The value of these combinations is such that recent arguments favoring their use instead of carbapenems (as a carbapenem-resistance-sparing strategy) in certain infections (such as ESBL-*E. coli* bloodstream infections) have been advanced (Perez and Bonomo 2012; Rodriguez-Bano et al. 2012). Indeed, the possibility of advantageous combinations of clavulanate (or penam sulfones) with other β-lactam classes (cephalosporins, carbapenems, monobactam-type) dominates current β-lactam clinical development. The driving force behind these initiatives is the recent emergence of β-lactamases—both serine and metallo—with enhanced hydrolytic ability against the best cephalosporins and carbapenems, and impervious to the inactivation mechanisms of clavulanate and penam sulfones. This emergence, arguably more than any other event, is the basis for the concern that we are now entering an era of untreatable Gram-negative infections (Livermore 2009; Walsh and Toleman 2012).

4.3.2.1 Responding to β-Lactamase Evolution with Inhibitors

The responses against this possibility are currently the evaluation of β-lactamase nuance (the new β-lactamases are not omnipotent enzymes: as they gain one β-lactam as a substrate, they may lose this capability against another); the exploration of new β-lactamase inhibitor combinations (vide supra); and the discovery of new β-lactam-β-lactamase inhibitor combinations. The first response will have value only when clinical diagnostic methodologies are greatly advanced from where they are currently. The evaluation of new pairings of known β-lactams, the second response, is eminently sensible. The third response arguably represents the future. In this regard, the future is best described as not without hope, notwithstanding the bifurcated—metallo and serine—threat posed by these new β-lactamases.

The sudden appearance of an entirely new, and entirely capable, metallo-β-lactamase variant (the NDMs) (Nordmann et al. 2012), has underscored forcefully the absence of a useful inhibitor against these (and other) metallo-β-lactamases. Numerous structures have been premised as inhibitor leads, on the basis of millimolar-level inhibition in vitro as a result of chelation of the active site metal(s) (for example: (Faridoon et al. 2012; Schlesinger et al. 2013)). Nonetheless, none have progressed, and as a consequence the possibility of therapeutic intervention with as the simple metal chelator, EDTA, has been explored in pharmacological models (Aoki et al. 2010). A recent discovery of notable interest is the synergism (by an as yet unknown mechanism) of 2-imidazolamines with β-lactams in suppressing the resistance of certain metallo-β-lactamase-expressing strains (Worthington et al. 2012). This same 2-imidazolamine functional group class also exerts interesting anti-biofilm activity through quorum-sensing modulation (Frei et al. 2012). New β-lactam discovery in light of these emerging β-lactamases focuses on the monobactam, monosulfactam, and monocarbam β-lactam classes, in recognition of the intrinsic stability of these β-lactams to especially metallo-β-lactamase hydrolysis (Page et al. 2011).

Not surprisingly, the newest inhibitors of the serine β-lactamases incorporate similar chemical substructure as have the monobactam-type structures: the bicyclic β-lactam-sulfamate as exemplified by BAL-29880 and by MK-8712 (a congener of MK-7655), and the bicyclic imidazolidinone-*N*-sulfate exemplified by Avibactam (NXL104) (Fig. 4.5). BAL-29880 inhibits the serine Class C β-lactamases via formation of an acyl enzyme that progresses to a hydrolytically stable acyl enzyme by further reaction (Endimiani et al. 2010). BAL-29880 is formulated as one component of the triple β-lactam combination BAL-30376 (with clavulanate as a second β-lactamase inactivator and the siderophore monobactam BAL-19764), currently in clinical evaluation against β-lactam-resistant *Enterobacteriaceae* due to expression of the metallo-, AmpC, ESBL, or carbapenemase β-lactamases (Livermore et al. 2010b; Page et al. 2011). The combination of MK-7655 (Chen et al. 2011) with imipenem dramatically improved the in vitro antibacterial efficacy of imipenem against KPC-2 β-lactamase-expressing *K. pneumoniae* and *P. aeruginosa* (Hirsch et al. 2012). Avibactam (NXL-104) is a structurally

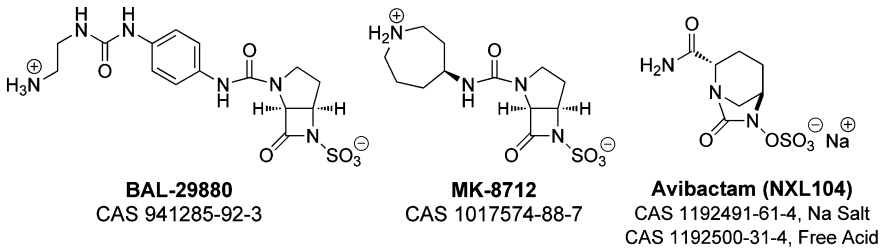


Fig. 4.5 Structures of new β-lactamase inhibitors

unprecedented β-lactamase inhibitor, active against numerous Class A (including KPC), Class C, and Class D β-lactamases, and is currently in two separate mid-stage clinical trials with two different cephalosporins, ceftazidime, and ceftaroline. Together, these two combinations could potentially encompass an impressive breadth of Gram-negative and Gram-positive bacterial pathogens, respectively (Bush and Pucci 2011). Mechanistic study shows surprising efficacy for avibactam as an irreversible acylating agent of the serine β-lactamases (Stachyra et al. 2010; Xu et al. 2012).

4.3.2.2 The Future of β-Lactamase Inhibition

The newest serine β-lactamase inhibitors represent new lactam chemotypes. While the BAL-29880/MK-7655/8712 class has been extensively explored through empirical medicinal chemistry (Chen et al. 2011), neither has yet been fully vetted by the structure-based design opportunities presented by analysis of their irreversible acylation of susceptible β-lactamases, and of the kinetic and mutational analysis of non-susceptible β-lactamases. While these structures present extraordinary challenge with respect to process-scale total chemical synthesis (Mangion et al. 2011), as was once also true for the carbapenems, chemical synthesis provides its own opportunities for structural diversification. It is entirely reasonable to contemplate, for example, appropriate modification of avibactam such that it is not just β-lactamases, but penicillin-binding proteins, that are also irreversibly acylated.

4.4 Recent Advances in the Cell Biology of the β-Lactams

For these first decades of their clinical use, the context of the molecular mechanism of the antibiotic action of the β-lactams was their mechanism-based inactivation of the PBPs. While the shortsightedness of this context was long recognized—for example, why are some β-lactams bacteriostatic, and others bactericidal?—the biological framework to more deeply understand the events

ensuing from the loss of particular PBPs was not in place. These frameworks are now emerging, and their emergence will profoundly affect the future of the β-lactams. Two emerging frameworks may be cited: the discovery that sentinel proteins are used to detect the presence of β-lactams, and secondly the imperative that peptidoglycan biosynthesis is coordinated with that of the other structural components of the cell envelope.

4.4.1 The Existence of Sentinel Proteins

While many bacterial resistance enzymes are expressed constitutively, others are not. A preeminent example of the latter is the PBP2a transpeptidase that is expressed by MRSA bacteria following the loss of the catalytic activity of the transpeptidase domain of their bifunctional PBP, as a consequence of β-lactam acylation. The reason for control of PBP2a expression is the fitness cost of this resistance mechanism. A second example is the mechanism used in many Gram-negative bacteria for β-lactamase expression. In both examples, bacteria use β-lactam-initiated signal transduction to control the resistance response. While in both examples the molecular event for β-lactam signaling is known, in neither are the details of the signaling pathways fully understood. Nevertheless, the prospect is before us that concurrent interference with the signal transduction may resensitize resistant bacteria to β-lactams.

The molecular events of these two examples are summarized. MRSA bacteria express a receptor protein for detection of β-lactams (a cognate receptor protein is used to control the expression of their β-lactamase, and is the better studied of the two). This receptor protein has cell-surface, transmembrane, and cytosolic domains (Llarrull et al. 2011). In the presence of β-lactams, a serine in the antibiotic-binding site of the cell-surface domain is irreversibly acylated (Kumarasiri et al. 2012). The occurrence of this acylation on the membrane surface propagates to the cytoplasmic domain through transduction of information entailing conformational changes, which ultimately result in derepression of the gene for PBP2a or for β-lactamase. The gene derepression takes place via the activated cytoplasmic metalloproteinase domain of the sensor protein, which degrades the gene suppressor protein. Evasion of the signaling of this resistance mechanism may be envisioned as occurring through β-lactam structure optimization to minimize receptor protein acylation, through interference with the proteolysis, or through manipulation of the muropeptide structures (Amoroso et al. 2012) that control the affinity of the repressor for its DNA. A remarkably similar pathway is used to control expression of the AmpC β-lactamase in certain Gram-negatives. Here, a low-molecular-mass PBPs acts as a sentinel enzyme for the detection of the presence of β-lactams. As a result of β-lactam-dependent loss of its catalytic activity, the composition of the muropeptide pool entering from the periplasm to the cytoplasm for recycling is altered (Boudreau et al. 2012). Structural alteration within the muropeptide pool is sensed directly by the gene regulator controlling

the β-lactamase gene, and results in transcription of the DNA. Here, inhibition of a key enzyme (nagZ) in the recycling pathway diverts the muropeptide pool so as to maintain repression of AmpC expression, and preservation of β-lactam sensitivity (Mark et al. 2011).

4.4.2 Cell-Wall Components

The second framework relates to the structural complexity of the bacterial cell envelope (Silhavy et al. 2010). During bacterial growth, a functional peptidoglycan is necessary but insufficient: the growing peptidoglycan must structurally integrate with simultaneous growth elsewhere, including the other cell-wall components, the membrane(s), and the cytoskeleton (Hanson and Neely 2012). The intuitive surmise that concurrent interference with peptidoglycan biosynthesis and a key companion event of cell growth might give profound mutual synergy is now proven (Ejim et al. 2011). In Gram-positives, coordination is required between wall teichoic acid and peptidoglycan biosynthesis (Atilano et al. 2010; Campbell et al. 2011). Inhibitors of wall teichoic acid biosynthesis that synergize β-lactam activity as a "synthetically lethal" combination have been identified (Campbell et al. 2012). Moreover, this nexus is not unique. Genomic-based technologies such as whole-genome sequencing, genotyping, and gene-expression profiling have the potential to identify similarly efficacious pairings (Roemer et al. 2012). Restoration of MRSA susceptibility to β-lactams has been demonstrated through synergy with inhibitors of other events in peptidoglycan biosynthesis (Huber et al. 2009), with other seemingly unrelated enzymes (such as glutamine synthase: Roemer et al. 2012). These synergies define the β-lactam genetic potentiation network of MRSA *S. aureus*. Remarkably, the genetic potentiation network for one β-lactam structure need not be the same as for a different β-lactam structure (Roemer et al. 2012). These same approaches can be used to define the β-lactam genetic potentiation network for specific β-lactam structures against specific bacterial pathogens.

4.4.3 Bacterial Cell Death

Further opportunity for β-lactam genetic potentiation may likewise be found through understanding of the ultimate mechanism resulting in bacterial cell death. This mechanism is just now unfolding. Regardless of their primary targets, cell death from exposure to different classes of bactericidal antibiotics results from stress-induced reactive oxygen (ROS) oxidative damage (Belenky and Collins 2011; Lee and Collins 2012; Foti et al. 2012). Accordingly, bacteria use strategies to combat the ROS produced as a result of antibiotic exposure, including exploration of mutation-conferring resistance (Kohanski et al. 2010a). The adaptive

stress responses used to detoxify the ROS may provide a framework for the development of new antibiotics (Kohanski et al. 2010b). Likewise, the use of extracellular metabolites that provoke different energetic pathways may enable the use of other antibiotics to selectively kill persister bacteria (Lewis 2010) while not affecting normal antibiotic-sensitive cells, as exemplified by even single-chemical supplementation (Kim et al. 2011). For example, Shatalin et al. found that both Gram-negative and Gram-positive bacteria were sensitized to a wide array of antibiotics by deleting (or inhibiting) enzymes that produce H_2S, implicating a direct involvement of H_2S in antibiotic tolerance (Shatalin et al. 2011).

4.5 Conclusions

Notwithstanding an interlude of 30 years since the discovery of entirely new classes of β-lactams, new opportunities will emerge within the identity of the β-lactams as natural products. These opportunities will originate from creative screening methods to empirically identify β-lactam synergy within other classes of antibacterials (Huber et al. 2009; Worthington et al. 2012), from innovative technologies that facilitate the identification of antibiotic overproducers (Charusanti et al. 2012), from epigenetic manipulation of antibiotic-producing bacteria (Wang et al. 2010), and from the ultimate dissection of the β-lactam-biosynthetic pathways (Bodner et al. 2011) coupled to robust and sensitive assays for the identification of new β-lactam structures (Phelan et al. 2012). β-Lactams are preeminent examples of natural products harnessed for the benefit of human health, and emerging discoveries and technologies within the cell networks that define β-lactam biosynthesis, resistance mechanism expression, stress response, and potentiation network will preserve this preeminence.

Antibiotic resistance is inexorable. The continuous emergence of new and powerful resistance mechanisms against the β-lactams reflects their enormous clinical value. We cannot suppress resistance development. Rather, its impact must be limited through improved clinical practice, and countered by exploiting the advantages disclosed through basic research. The challenge—and this is not a small challenge—is bringing these advantages into clinical practice in a timely, practical, and affordable manner. For a seemingly aged antibiotic class, an astonishing breadth of new discovery concerning the β-lactams is now being revealed: with respect to new β-lactam structures, new understanding of their protein targets, new understanding of their resistance pathways, new understanding of their cytotoxicity, and a better appreciation as to how all of these interrelate in terms of bacterial cellular biology. Each of these discoveries has the potential to contribute to the future of the β-lactams as an ageless antibiotic class.

References

Allen NE (2011) From vancomycin to oritavancin: the discovery and development of a novel lipoglycopeptide antibiotic. Anti-Infect Agents Med Chem 9:23–47. doi:1871-5214/10

Amoroso A, Boudet J, Berzigotti S, Duval S, Teller N, Mengin-Lecreulx D, Luxen A, Simorre J-P, Joris B (2012) A peptidoglycan fragment triggers β-lactam resistance in *Bacillus licheniformis*. PLoS Pathog 8:e1002571. doi:10.1371/journal.ppat.1002571

Aoki N, Ishii Y, Tayeda K et al (2010) Efficacy of calcium-EDTA as an inhibitor for metallo-β-lactamase in a mouse model of *Pseudomonas aeruginosa* pneumonia. Antimicrob Agents Chemother 54:4582–4588. doi:10.1128/AAC.00511-10

Atilano ML, Pereira PM, Yates J, Reed P, Veiga H, Pinho MG, Filipe SR (2010) Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*. Proc Natl Acad Sci USA 107:18991–18996. doi:10.1073/pnas.1004304107

Balasubramanian D, Schneper L, Merighi M, Smith R, Narasimhan G, Lory S, Mathee K (2012) The regulatory repertoire of *Pseudomonas aeruginosa* AmpC β-lactamase regulator AmpR includes virulence genes. PLoS ONE 7:e34067. doi:10.1371/journal.pone.0034067

Balcewich MD, Reeve TM, Orlikow EA, Donald LJ, Vocadlo DJ, Mark BL (2010) Crystal structure of the AmpR effector binding domain provides insight into the molecular regulation of inducible AmpC β-lactamase. J Mol Biol 400:998–1010. doi:10.1016/j.jmb.2010.05.040

Bassetti M, Nicolini L, Esposito S, Righi E, Viscoli C (2009) Current status of newer carbapenems. Curr Med Chem 16:564–575. doi:0929-8673/09

Belenky P, Collins JJ (2011) Microbiology. Antioxidant strategies to tolerate antibiotics. Science 334:915–916. doi:10.1126/science.1214823

Biondi S, Long S, Panunzio M, Qin WL (2011) Current trends in β-lactam based β-lactamases inhibitors. Curr Med Chem 18:4223–4236

Blais J, Lewis SR, Krause KM, Benton BM (2012) Antistaphylococcal activity of TD-1792, a multivalent glycopeptide-cephalosporin antibiotic. Antimicrob Agents Chemother 56:1584–1587. doi:10.1128/AAC.05532-11

Bobba S, Ponnaluri VK, Mukherji M, Gutheil WG (2011) Microtiter plate-based assay for inhibitors of penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 55:2783–2787. doi:10.1128/AAC.01327-10

Bodner MJ, Li R, Phelan RM, Freeman MF, Moshos KA, Lloyd EP, Townsend CA (2011) Definition of the common and divergent steps in carbapenem β-lactam antibiotic biosynthesis. ChemBioChem 12:2159–2165. doi:10.1002/cbic.201100366

Boudreau MA, Fisher JF, Mobashery S (2012) Messenger functions of the bacterial cell wall-derived muropeptides. Biochemistry 51:2974–2990. doi:10.1021/bi300174x

Bush K, Courvalin P, Dantas G et al (2011) Tackling antibiotic resistance. Nat Rev Microbiol 9:894–896. doi:10.1038/nrmicro2693

Bush K, Fisher JF (2011) Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from Gram-negative bacteria. Annu Rev Microbiol 65:455–478. doi:10.1146/annurev-micro-090110-102911

Bush K, Pucci MJ (2011) New antimicrobial agents on the horizon. Biochem Pharmacol 82:1528–1539. doi:10.1016/j.bcp.2011.07.077

Butler MS, Cooper MA (2011) Antibiotics in the clinical pipeline in 2011. J Antibiot 64:413–425. doi:10.1038/ja.2011.44

Campbell J, Singh AK, Santa Maria JPJ, Kim Y, Brown S, Swoboda JG, Mylonakis E, Wilkinson BJ, Walker S (2011) Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in *Staphylococcus aureus*. ACS Chem Biol 6:106–116. doi:10.1021/cb100269f

Campbell J, Singh AK, Swoboda JG, Gilmore MS, Wilkinson BJ, Walker S (2012) An antibiotic that inhibits a late step in wall teichoic acid biosynthesis induces the cell wall stress stimulon in *S. aureus*. Antimicrob Agents Chemother 56:1810–1820. doi:10.1128/AAC.05938-11

Canton R, Lumb J (2011) Emerging resistance in Gram-negative pathogens and implications for clinical practice. Future Microbiol 6:19–22. doi:10.2217/fmb.10.150

Charusanti P, Fong NL, Nagarajan H, Pereira AR, Li HJ, Abate EA, Su Y, Gerwick WH, Palsson BO (2012) Exploiting adaptive laboratory evolution of *Streptomyces clavuligerus* for antibiotic discovery and overproduction. PLoS ONE 7:e33727. doi:10.1371/journal.pone.0033727

Chen H, Blizzard TA, Kim S et al (2011) Side chain SAR of bicyclic β-lactamase inhibitors (BLIs). 2. *N*-Alkylated and open chain analogs of MK-8712. Bioorg Med Chem Lett 21:4267–4270. doi:10.1016/j.bmcl.2011.05.065

Cottarel G, Wierzbowski J (2007) Combination drugs, an emerging option for antibacterial therapy. Trends Biotechnol 25:547–555. doi:10.1016/j.tibtech.2007.09.004

Desgranges S, Ruddle CC, Burke LP, McFadden TM, O'Brien JE, Fitzgerald-Hughes D, Humphreys H, Smyth TP, Devocelle M (2012) β-Lactam-host defence peptide conjugates as antibiotic prodrug candidates targeting resistant bacteria. RSC Adv 2:2480–2492. doi:10.1039/C2RA01351G

Drawz SM, Bonomo RA (2010) Three decades of β-lactamase inhibitors. Clin Microbiol Rev 23:160–201. doi:10.1128/CMR.00037-09

Ejim L, Farha MA, Falconer SB, Wildenhain J, Coombes BK, Tyers M, Brown ED, Wright GD (2011) Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. Nat Chem Biol 7:348–350. doi:10.1038/nchembio.559

El-Gamal MI, Oh CH (2010) Current status of carbapenem antibiotics. Curr Top Med Chem 10:1882–1897

Endimiani A, Doi Y, Bethel CR et al (2010) Enhancing resistance to cephalosporins in class C β-lactamases: impact of Gly214Glu in CMY-2. Biochemistry 49:1014–1023. doi:10.1021/bi9015549

Faridoon Hussein WM, Vella P, Islam NU, Ollis DL, Schenk G, McGeary RP (2012) 3-Mercapto-1,2,4-triazoles and *N*-acylated thiosemicarbazides as metallo-β-lactamase inhibitors. Bioorg Med Chem Lett 22:380–386. doi:10.1016/j.bmcl.2011.10.116

Fisher JF, Mobashery S (2010) Host-guest chemistry of the peptidoglycan. J Med Chem 53:4813–4829. doi:10.1021/jm100086u

Flanagan ME, Brickner SJ, Lall M et al (2011) Preparation, Gram-negative antibacterial activity, and hydrolytic stability of novel siderophore-conjugated monocarbam diols. ACS Med Chem Lett 2:385–390. doi:10.1021/ml200012f

Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC (2012) Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. Science 336:315–319. doi:10.1126/science.1219192

Frase H, Shi Q, Testero SA, Mobashery S, Vakulenko SB (2009) Mechanistic basis for the emergence of catalytic competence against carbapenem antibiotics by the GES family of β-lactamases. J Biol Chem 284:29509–29513. doi:10.1074/jbc.M109.011262

Frei R, Breitbach AS, Blackwell HE (2012) 2-Aminobenzimidazole derivatives strongly inhibit and disperse *Pseudomonas aeruginosa* biofilms. Angew Chem Int Ed 51:5226–5229. doi:10.1002/anie.201109258

Fuda C, Suvorov M, Vakulenko SB, Mobashery S (2004) The basis for resistance to β-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. J Biol Chem 279:40802–40806. doi:10.1074/jbc.M403589200

Fuda C, Hesek D, Lee M, Morio K, Nowak T, Mobashery S (2005) Activation for catalysis of penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus* by bacterial cell wall. J Am Chem Soc 127:2056–2057. doi:10.1021/ja0434376

Fuda C, Hesek D, Lee M, Heilmayer W, Novak R, Vakulenko SB, Mobashery S (2006) Mechanistic basis for the action of new cephalosporin antibiotics effective against methicillin- and vancomycin-resistant *Staphylococcus aureus*. J Biol Chem 281:10035–10041. doi:10.1074/jbc.M508846200

Fuda C, Suvorov M, Shi Q, Hesek D, Lee M, Mobashery S (2007) Shared functional attributes between the mecA gene product of *Staphylococcus sciuri* and penicillin-binding protein 2a of

methicillin-resistant *Staphylococcus aureus*. Biochemistry 46:8050–8057. doi:10.1021/bi7004587

Fuse S, Tsukamoto H, Yuan Y, Wang TS, Zhang Y, Bolla M, Walker S, Sliz P, Kahne D (2010) Functional and structural analysis of a key region of the cell wall inhibitor moenomycin. ACS Chem Biol 5:701–711. doi:10.1021/cb100048q

Gaballah A, Kloeckner A, Otten C, Sahl H-G, Henrichfreise B (2011) Functional analysis of the cytoskeleton protein MreB from *Chlamydophila pneumoniae*. PLoS ONE 6:e25129. doi:10.1371/journal.pone.0025129

Gampe CM, Tsukamoto H, Wang T-SA, Walker S, Kahne D (2011) Modular synthesis of diphospholipid oligosaccharide fragments of the bacterial cell wall and their use to study the mechanism of moenomycin and other antibiotics. Tetrahedron 67:9771–9778. doi:10.1016/j.tet.2011.09.114

Ghosh AS, Chowdhury C, Nelson DE (2008) Physiological functions of D-alanine carboxypeptidases in *E. coli*. Trends Microbiol 16:309–317. doi:10.1016/j.tim.2008.04.006

Han S, Zaniewski RP, Marr ES, Lacey BM, Tomaras AP, Evdokimov A, Miller JR, Shanmugasundaram V (2010) Structural basis for effectiveness of siderophore-conjugated monocarbams against clinically relevant strains of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 107:22002–22007. doi:10.1073/pnas.1013092107

Han S, Caspers N, Zaniewski RP, Lacey BM, Tomaras AP, Feng X, Geoghegan KF, Shanmugasundaram V (2011) Distinctive attributes of β-lactam target proteins in *Acinetobacter baumannii* relevant to development of new antibiotics. J Am Chem Soc 133:20536–20545. doi:10.1021/ja208835z

Hanson BR, Neely MN (2012) Coordinate regulation of Gram-positive cell surface components. Curr Opin Microbiol 15:204–210. doi:10.1016/j.mib.2011.12.011

Hegde SS, Okusanya OO, Skinner R, Shaw J-P, Obedencio G, Ambrose PG, Blais J, Bhavnani SM (2012) Pharmacodynamics of TD-1792, a novel glycopeptide-cephalosporin heterodimer antibiotic used against Gram-positive bacteria, in a neutropenic murine thigh model. Antimicrob Agents Chemother 56:1578–1583. doi:10.1128/AAC.05382-11

Hennequin C, Robin F, Cabrolier N, Bonnet R, Forestier C (2012) Characterization of a DHA-1-producing *Klebsiella pneumoniae* strain involved in an outbreak and role of the AmpR regulator in virulence. Antimicrob Agents Chemother 56:288–294. doi:10.1128/AAC.00164-11

Henrichfreise B, Schiefer A, Schneider T et al (2009) Functional conservation of the lipid II biosynthesis pathway in the cell wall-less bacteria *Chlamydia* and *Wolbachia*: why is lipid II needed? Mol Microbiol 73:913–923. doi:10.1111/j.1365-2958.2009.06815.x

Higgins PG, Stefanik D, Page MG, Hackel M, Seifert H (2012) In vitro activity of the siderophore monosulfactam BAL30072 against meropenem-non-susceptible *Acinetobacter baumannii*. J Antimicrob Chemother 67:1167–1169. doi:10.1093/jac/dks009

Hirai Y, Takahata S, Yamada K, Ida T, Maebashi K (2011) Correlation of the antimicrobial activity of ME1036 with its binding affinities to the penicillin-binding proteins from *Streptococcus pneumoniae* strains. J Antibiot 64:741–746. doi:10.1038/ja.2011.76

Hirsch EB, Ledesma KR, Chang KT, Schwartz MS, Motyl MR, Tam VH (2012) In vitro activity of MK-7655, a novel β-lactamase inhibitor, in combination with imipenem against carbapenem resistant Gram-negative bacteria. Antimicrob Agents Chemother, doi:10.1128/AAC.05927-11

Huang CY, Shih HW, Lin LY, Tien YW, Cheng TJ, Cheng WC, Wong CH, Ma C (2012) Crystal structure of S*taphylococcus aureus* transglycosylase in complex with a Lipid II analog and elucidation of peptidoglycan synthesis mechanism. Proc Natl Acad Sci USA 109:6496–6501. doi:10.1073/pnas.1203900109

Huber J, Donald RG, Lee SH et al (2009) Chemical genetic identification of peptidoglycan inhibitors potentiating carbapenem activity against methicillin-resistant *Staphylococcus aureus*. Chem Biol 16:837–848. doi:10.1016/j.chembiol.2009.05.012

Hugonnet JE, Tremblay LW, Boshoff HI, Barry CE, Blanchard JS (2009) Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. Science 323:1215–1218. doi:10.1126/science.1167498

Inglis SR, Strieker M, Rydzik AM, Dessen A, Schofield CJ (2012) A boronic-acid-based probe for fluorescence polarization assays with penicillin binding proteins and β-lactamases. Anal Biochem 420:41–47. doi:10.1016/j.ab.2011.08.036

Kardos N, Demain AL (2011) Penicillin: the medicine with the greatest impact on therapeutic outcomes. Appl Microbiol Biotechnol 92:677–687. doi:10.1007/s00253-011-3587-6

Ke W, Bethel CR, Papp-Wallace KM, Pagadala SR, Nottingham M, Fernandez D, Buynak JD, Bonomo RA, van den Akker F (2012) Crystal structures of KPC-2 β-lactamase in complex with 3-NPBA and PSR-3-226. Antimicrob Agents Chemother 56:2713–2718. doi:10.1128/AAC.06099-11

Kim JS, Heo P, Yang TJ et al (2011) Selective killing of bacterial persisters by a single chemical compound without affecting normal antibiotic-sensitive cells. Antimicrob Agents Chemother 55:5380–5383. doi:10.1128/AAC.00708-11

Koga T, Masuda N, Kakuta M, Namba E, Sugihara C, Fukuoka T (2008) Potent in vitro activity of tomopenem (CS-023) against methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 52:2849–2854. doi:10.1128/AAC.00413-08

Koga T, Sugihara C, Kakuta M, Masuda N, Namba E, Fukuoka T (2009) Affinity of tomopenem (CS-023) for penicillin-binding proteins in *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 53:1238–1241. doi:10.1128/AAC.01433-08

Kohanski MA, Depristo MA, Collins JJ (2010a) Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 37:311–320. doi:10.1016/j.molcel.2010.01.003

Kohanski MA, Dwyer DJ, Collins JJ (2010b) How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 8:423–435. doi:10.1038/nrmicro2333

Kollef MH, Golan Y, Micek ST, Shorr AF, Restrepo MI (2011) Appraising contemporary strategies to combat multidrug resistant Gram-negative bacterial infections–proceedings and data from the Gram-negative resistance summit. Clin Infect Dis 53(Suppl. 2):S33–S55. doi:10.1093/cid/cir475

Kumarasiri M, Llarrull LI, Borbulevych O, Fishovitz J, Lastochkin E, Baker BM, Mobashery S (2012) An amino-acid position at the crossroads of evolution of protein function: antibiotic-sensor domain of the BlaR1 protein from *Staphylococcus aureus* vs. class D β-lactamases. J Biol Chem 287:8232–8241. doi:10.1074/jbc.M111.333179

Lascols C, Hackel M, Hujer AM, Marshall SH, Bouchillon SK, Hoban DJ, Hawser SP, Badal RE, Bonomo RA (2012) Using nucleic acid microarrays to perform molecular epidemiology and detect novel β-lactamases: a snapshot of ESBLs throughout the world. J Clin Microbiol 50:1167–1169. doi:10.1128/JCM.06115-11

Lee HH, Collins JJ (2012) Microbial environments confound antibiotic efficacy. Nat Chem Biol 8:6–9. doi:10.1038/nchembio.740

Lemaire S, Fuda C, Van Bambeke F, Tulkens PM, Mobashery S (2008) Restoration of susceptibility of methicillin-resistant *Staphylococcus aureus* to β-lactam antibiotics by acidic pH: Role of PBP 2a. J Biol Chem 283:12769–12776. doi:10.1074/jbc.M800079200

Lemaire S, Glupczynski Y, Duval V, Joris B, Tulkens PM, Van Bambeke F (2009) Activity of ceftobiprole and other cephalosporins against extracellular and intracellular (THP-1 macrophages, keratinocytes) forms of methicillin-sensitive (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA). Antimicrob Agents Chemother 53:2289–2297. doi:10.1128/AAC.01135-08

Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372. doi:10.1146/annurev.micro.112408.134306

Livermore DM (2006) Can β-lactams be re-engineered to beat MRSA? Clin Microbiol Infect 12(Suppl. 2):11–16. doi:10.1111/j.1469-0691.2006.01403.x

Livermore DM (2009) Has the era of untreatable infections arrived? J Antimicrob Chemother 64(Suppl. 1):i29–i36. doi:10.1093/jac/dkp255

Livermore DM (2012) Fourteen years in resistance. Int J Antimicrob Agents 39:283–294. doi:10.1016/j.ijantimicag.2011.12.012

Livermore DM, Mushtaq S, Ge Y (2010a) Chequerboard titration of cephalosporin CXA-101 (FR264205) and tazobactam versus β-lactamase-producing *Enterobacteriaceae*. J Antimicrob Chemother 65:1972–1974. doi:10.1093/jac/dkq248

Livermore DM, Mushtaq S, Warner M (2010b) Activity of BAL30376 (monobactam BAL19764 + BAL29880 + clavulanate) versus Gram-negative bacteria with characterized resistance mechanisms. J Antimicrob Chemother 65:2382–2395. doi:10.1093/jac/dkq310

Llarrull LI, Fisher JF, Mobashery S (2009) Molecular basis and phenotype of methicillin resistance in *Staphylococcus aureus* and insights into new β-lactams that meet the challenge. Antimicrob Agents Chemother 53:4051–4063. doi:10.1128/AAC.00084-09

Llarrull LI, Testero SA, Fisher JF, Mobashery S (2010) The future of the β-lactams. Curr Opin Microbiol 13:551–557. doi:10.1016/j.mib.2010.09.008

Llarrull LI, Toth M, Champion MM, Mobashery S (2011) Activation of BlaR1 protein of methicillin-resistant *Staphylococcus aureus*, its proteolytic processing, and recovery from induction of resistance. J Biol Chem 286:38148–38158. doi:10.1074/jbc.M111.288985

Long DD, Aggen JB, Chinn J et al (2008) Exploring the positional attachment of glycopeptide/β-lactam heterodimers. J Antibiot 61:603–614. doi:10.1038/ja.2008.80

Mangion IK, Ruck RT, Rivera N, Huffman MA, Shevlin M (2011) A concise synthesis of a β-lactamase inhibitor. Org Lett 13:5480–5483. doi:10.1021/ol202195n

Mark BL, Vocadlo DJ, Oliver A (2011) Providing β-lactams a helping hand: targeting the AmpC β-lactamase induction pathway. Future Microbiol 6:1415–1427. doi:10.2217/fmb.11.128

Mima T, Kvitko BH, Rholl DA, Page MGP, Desarbre E, Schweizer HP (2011) In vitro activity of BAL30072 against *Burkholderia pseudomallei*. Int J Antimicrob Agents 38:157–159. doi:10.1016/j.ijantimicag.2011.03.019

Mizukami S, Watanabe S, Akimoto Y, Kikuchi K (2012) No-wash protein labeling with designed fluorogenic probes and application to real-time pulse-chase analysis. J Am Chem Soc 134:1623–1629. doi:10.1021/ja208290f

Mobashery S, Johnston M (1987) Inactivation of alanine racemase by β-chloro-L-alanine released enzymatically from amino acid and peptide C10-esters of deacetylcephalothin. Biochemistry 26:5878–5884

Mobashery S, Lerner SA, Johnston M (1986) Conscripting β-lactamase for use in drug delivery. Synthesis and biological activity of a cephalosporin C10-ester of an antibiotic dipeptide. J Am Chem Soc 108:1685–1686

Moya B, Zamorano L, Juan C, Ge Y, Oliver A (2010) Affinity of the new cephalosporin CXA-101 to penicillin-binding proteins of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 54:3933–3937. doi:10.1128/AAC.00296-10

Mushtaq S, Warner M, Livermore D (2010) Activity of the siderophore monobactam BAL30072 against multiresistant non-fermenters. J Antimicrob Chemother 65:266–270. doi:10.1093/jac/dkp425

My NH, Hirao H, Van DU, Morokuma K (2011) Computational studies of bacterial resistance to β-lactam antibiotics: mechanism of covalent inhibition of the penicillin-binding protein 2a (PBP2a). J Chem Inf Model 51:3226–3234. doi:10.1021/ci2004175

Nemmara VV, Dzhekieva L, Sarkar KS, Adediran SA, Duez C, Nicholas RA, Pratt RF (2011) Substrate specificity of low-molecular mass bacterial DD-peptidases. Biochemistry 50:10091–10101. doi:10.1021/bi201326a

Nicola G, Tomberg J, Pratt RF, Nicholas RA, Davies C (2010) Crystal structures of covalent complexes of β-lactam antibiotics with *Escherichia coli* penicillin-binding protein 5: toward an understanding of antibiotic specificity. Biochemistry 49:8094–8104. doi:10.1021/bi100879m

Nordmann P, Boulanger AE, Poirel L (2012) NDM-4 metallo-β-lactamase with increased carbapenemase activity from *Escherichia col*i. Antimicrob Agents Chemother 56:2184–2186. doi:10.1128/AAC.05961-11

Page MGP, Dantier C, Desarbre E (2010) In vitro properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant Gram-negative bacilli. Antimicrob Agents Chemother 54:2291–2302. doi:10.1128/AAC.01525-09

Page MGP, Dantier C, Desarbre E, Gaucher B, Gebhardt K, Schmitt-Hoffmann A (2011) *In vitro* and in vivo properties of BAL30376, a β-lactam/dual β-lactamase inhibitor combination with enhanced activity against Gram-negative Bacilli that express multiple β-lactamases. Antimicrob Agents Chemother 55:1510–1519. doi:10.1128/AAC.01370-10

Perez F, Bonomo RA (2012) Can we really use β-lactam/β-lactam inhibitor combinations for the treatment of infections caused by extended-spectrum β-lactamase-producing bacteria? Clin Infect Dis 54:175–177. doi:10.1093/cid/cir793

Phelan RM, Dipardo BJ, Townsend CA (2012) A high-throughput screen for the engineered production of β-lactam antibiotics. ACS Chem Biol 7:835–840. doi:10.1021/cb200504g

Piddock LJV (2012) The crisis of no new antibiotics-what is the way forward? Lancet Infect Dis 12:249–253. doi:10.1016/S1473-3099(11)70316-4

Potluri LP, de Pedro MA, Young KD (2012) *Escherichia coli* low-molecular-weight penicillin-binding proteins help orient septal FtsZ, and their absence leads to asymmetric cell division and branching. Mol Microbiol 84:203–224. doi:10.1111/j.1365-2958.2012.08023.x

Reith J, Mayer C (2011) Peptidoglycan turnover and recycling in Gram-positive bacteria. Appl Microbiol Biotechnol 92:1–11. doi:10.1007/s00253-011-3486-x

Rodriguez-Bano J, Navarro MD, Retamar P, Picon E, Pascual A (2012) β-Lactam/β-lactam inhibitor combinations for the treatment of bacteremia due to extended-spectrum β-lactamase-producing *Escherichia c*oli: a post hoc analysis of prospective cohorts. Clin Infect Dis 54:167–174. doi:10.1093/cid/cir790

Roemer T, Davies J, Giaever G, Nislow C (2012) Bugs, drugs and chemical genomics. Nat Chem Biol 8:46–56. doi:10.1038/nchembio.744

Russo TA et al (2011) In vivo and in vitro activity of the siderophore monosulfactam BAL30072 against *Acinetobacter baumannii*. J Antimicrob Chemother 66:867–873. doi:10.1093/jac/dkr013

Schlesinger SR, Bruner B, Farmer PJ, Kim SK (2013) Kinetic characterization of a slow-binding inhibitor of Bla2: thiomaltol. J Enzyme Inhib Med Chem 28:137–142. doi:10.3109/14756366.2011.640632

Shahid M, Sobia F, Singh A, Malik A, Khan HM, Jonas D, Hawkey PM (2009) β-Lactams and β-lactamase-inhibitors in current- or potential-clinical practice: a comprehensive update. Crit Rev Microbiol 35:81–108. doi:10.1080/10408410902733979

Shatalin K, Shatalina E, Mironov A, Nudler E (2011) H_2S: a universal defense against antibiotics in bacteria. Science 334:986–990. doi:10.1126/science.1209855

Shi Q, Meroueh SO, Fisher JF, Mobashery S (2011) A computational evaluation of the mechanism of penicillin-binding protein-catalyzed cross-linking of the bacterial cell wall. J Am Chem Soc 133:5274–5283. doi:10.1021/ja1074739

Silhavy TJ, Kahne D, Walker S (2010) The bacterial cell envelope. Cold Spring Harb Perspect Biol 2:a000414. doi:10.1101/cshperspect.a000414

Sliwa A, Dive G, Marchand-Brynaert J (2012a) 12- to 22-Membered bridged β-lactams as potential penicillin-binding protein inhibitors. Chem Asian J 7:425–434. doi:10.1002/asia.201100732

Sliwa A, Dive G, Zervosen A, Verlaine O, Sauvage E, Marchand-Brynaert J (2012b) Unprecedented inhibition of resistant penicillin binding proteins by bis-2-oxoazetidinyl macrocyles. Med Chem Commun 3:344–351. doi:10.1039/C2MD00251E

Stachyra T, Pechereau MC, Bruneau JM, Claudon M, Frere JM, Miossec C, Coleman K, Black MT (2010) Mechanistic studies of the inactivation of TEM-1 and P99 by NXL104, a novel non-β-lactam β-lactamase inhibitor. Antimicrob Agents Chemother 54:5132–5138. doi:10.1128/AAC.00568-10

Testero SA, O'Daniel PI, Shi Q, Lee M, Hesek D, Ishiwata A, Noll BC, Mobashery S (2009) Regiospecific syntheses of 6α-(1*R*-hydroxyoctyl) penicillanic acid and 6β-(1*R*-hydroxyoctyl) penicillanic acid as mechanistic probes of class D β-lactamases. Org Lett 11:2515–2518. doi:10.1021/ol900668k

Testero SA, Fisher JF, Mobashery S (2010) β-Lactam antibiotics. Burger's Medicinal Chemistry, Drug Discovery and Development, 7th edn 7:259–404. doi: 10.1002/0471266949.bmc226

Tremblay LW, Fan F, Blanchard JS (2010) Biochemical and structural characterization of *Mycobacterium tuberculosis* β-lactamase with the carbapenems ertapenem and doripenem. Biochemistry 49:3766–3773. doi:10.1021/bi100232q

Tyrrell KL, Citron DM, Warren YA, Goldstein EJ (2012) In vitro activity of TD-1792, a multivalent glycopeptide-cephalosporin antibiotic, against 377 strains of anaerobic bacteria and 34 strains of *Corynebacterium* species. Antimicrob Agents Chemother 56:2194–2197. doi:10.1128/AAC.06274-11

Verma V, Testero SA, Amini K et al (2011) Hydrolytic mechanism of OXA-58 enzyme, a carbapenem-hydrolyzing class D β-lactamase from *Acinetobacter baumannii*. J Biol Chem 286:37292–37303. doi:10.1074/jbc.M111.280115

Villegas-Estrada A, Lee M, Hesek D, Vakulenko SB, Mobashery S (2008) Co-opting the cell wall in fighting methicillin-resistant *Staphylococcus aureus*: potent inhibition of PBP 2a by two anti-MRSA β-lactam antibiotics. J Am Chem Soc 130:9212–9213. doi:10.1021/ja8029448

Walsh TR, Toleman MA (2012) The emergence of pan-resistant Gram-negative pathogens merits a rapid global political response. J Antimicrob Chemother 67:1–3. doi:10.1093/jac/dkr378

Wang X, Sena Filho JG, Hoover AR, King JB, Ellis TK, Powell DR, Cichewicz RH (2010) Chemical epigenetics alters the secondary metabolite composition of guttate excreted by an atlantic-forest-soil-derived *Penicillium citreonigrum*. J Nat Prod 73:942–948. doi:10.1021/np100142h

Worthington RJ, Bunders CA, Reed CS, Melander C (2012) Small molecule suppression of carbapenem resistance in NDM-1 producing Klebsiella pheumoniae. ACS Med Chem Lett 3: 357–361. doi: dx.doi.org/ 10.1021/ml200290p

Wright GD (2012) Antibiotics: a new hope. Chem Biol 19:3–10. doi:10.1016/j.chembiol.2011.10.019

Xu H, Hazra S, Blanchard JS (2012) NXL104 irreversibly inhibits the β-lactamase from *Mycobacterium tuberculosis*. Biochemistry 51:4551–4557. doi:10.1021/bi300508r

Yamaguchi T, Blázquez B, Hesek D, Lee MK, Llarrull LI, Boggess B, Oliver AG, Fisher JF, Mobashery S (2012) Inhibitors for bacterial cell-wall recycling. ACS Med Chem Lett 3:238–242. doi:org/10.1021/ml2002746

Chapter 5
Glycopeptides: An Old But Up-to-Date Successful Antibiotic Class

Giorgia Letizia Marcone and Flavia Marinelli

Abstract Glycopeptides are one of the oldest but still critically important antibiotic class that target Gram-positive pathogens. They are used as essential drugs for the treatment of life-threatening infections of relevant pathogens such as *Staphylococcus aureus, Enterococcus* spp., and *Clostridium difficile*. Antibacterial glycopeptides arrest bacterial cell wall biosynthesis by binding to the acyl-D-alanyl-D-alanine terminus of the nascent peptidoglycan, blocking its extracellular polymerization, and subsequently inhibiting cell growth and division. Chemically, these agents consist of a heptapeptide core structure that is transformed in the mature active antibiotic by intramolecular cyclizations among aromatic amino acid residues and by addition of glycosidic moieties, chlorine atoms, and occasionally lipid chains. First-generation glycopeptides (vancomycin and teicoplanin) are natural products from soil actinomycetes. Second-generation molecules (dalbavancin, oritavancin, telavancin) are produced by chemical modification of natural products. Glycopeptide resistance required nearly 30 years to appear following clinical introduction of vancomycin. High-level resistance is due to a collection of genes (named *van*) that reorder cell wall biosynthesis enabling bacteria to bypass the critical steps susceptible to inhibition by vancomycin and other glycopeptide antibiotics. There have been various efforts, with mixed success, to identify novel glycopeptide structures able to circumvent high-level glycopeptide resistance. This chapter is an overview on the features, mode of action, mechanism of resistance, biosynthesis of this old but up-to-date successful antibiotic class.

G. L. Marcone · F. Marinelli (✉)
Department of Biotechnology and Life Sciences, University of Insubria,
via J. H. Dunant 3, 21100 Varese, Italy
e-mail: flavia.marinelli@uninsubria.it

G. L. Marcone · F. Marinelli
The Protein Factory Research Center, Politecnico of Milano,
ICRM CNR Milano and University of Insubria, Varese, Italy

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_5,

5.1 Introduction

Glycopeptide antibiotics are an important class of natural products composed of glycosylated non-ribosomal peptides produced by a diverse group of actinomycetes. The most known glycopeptide antibiotics are vancomycin and teicoplanin (Fig. 5.1) that are currently considered drugs of last resort for life-threatening infections caused by multi-drug resistant Gram-positive pathogens. Vancomycin (from the word "vanquish") was first discovered in 1953 and was the first approved glycopeptide antibiotic; it is considered the prototypic example of this class. It was discovered by Eli Lilly scientists during the fermentation of *Streptomyces orientalis* (then reclassified as *Amycolatopsis orientalis*) isolated from a soil sample from Borneo. Vancomycin has been in clinical use following its rapid approval in 1958 till today to treat serious infections due to *Staphylococcus aureus* and other Gram-positive bacteria (Ehrenkranz 1958; McCormick et al. 1955). Shortly after being introduced, vancomycin was eclipsed by antibiotics (cephalosporins) that were considered less toxic and equally or more efficacious. Beginning in the early 1980s, a significant vancomycin use occurred, with 1100-fold increase in clinical settings occurring over the next two decades. Two events were primarily responsible for this resurgence. The first was the advent of pseudomembranous enterocolitis, an uncommon but potentially serious disease, resulting in acute diarrhea and characterized by colonic pseudomembranes. Pseudomembranous colitis may develop in debilitated patients after chemotherapy with antibiotics like lincomycin and clindamycin, but the condition has also been reported in children after treatment with other broad-spectrum antibiotics like ampicillin and penicillin (Neu et al. 1977; Prince and Neu 1979). The response to vancomycin was excellent, and the presence of this disease became an indication for the use of vancomycin (Geraci and Hermans 1983). Although *Clostridium difficile* is the primary agent of pseudomembranous enterocolitis, *S. aureus* is an

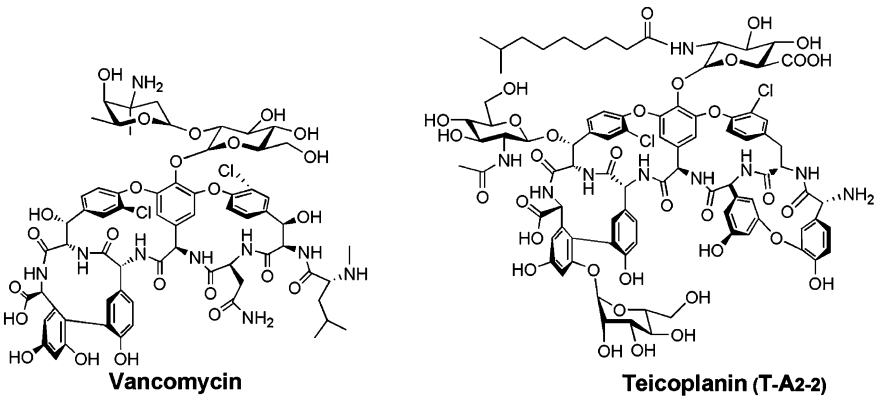


Fig. 5.1 Structures of vancomycin and teicoplanin factor T-$A_{2\text{-}2}$

occasional cause of the disorder (Gravet et al. 1999). Because vancomycin is active against both pathogens and is poorly absorbed from the intestinal tract, it became the drug of choice for treating pseudomembranous enterocolitis. However, this widespread vancomycin use, especially via the oral route, is considered today as one of the causes for the development of vancomycin-resistant enterococci (VRE) (see Sect. 5.4.1, see Rossolini et al., this volume). The second event leading to increased vancomycin use was the widespread appearance of β-lactam-resistant pathogens: first, methicillin-resistant *S. aureus* (MRSA), and then, penicillin-resistant *Streptococcus pneumoniae*. Known since 1978 as a cause for nosocomial infection (Peacock et al. 1980), MRSA appeared suddenly in 1980 in community isolates in Detroit and, later, diffused throughout the world (Saravolatz et al. 1982a, b). With its predictable activity against MRSA, vancomycin was selected as the drug of choice for MRSA infections, thus beginning a new era in the history of this antibiotic (Levine 2006).

Lipoglycopeptides represent a subgroup of the glycopeptide antibiotic class. Natural product lipoglycopeptides are identified by presence of a lipophilic, often long chain fatty acid, attached to an amino sugar on a central aromatic amino acid. Teicoplanin was the first discovered natural product lipoglycopeptide (Fig. 5.1) during a screening conducted on ca. 20,000 newly isolated *Actinoplanes* spp. in Lepetit labs (Lancini and Demain 2006). Teicoplanin was first reported in 1978 (Parenti et al. 1978) and then introduced in clinics for treatment of Gram-positive infections in Europe and in Japan in 1988 and 1998, respectively. The antibacterial activity of teicoplanin against Gram-positive bacteria, including those expressing resistance to unrelated compounds, is similar to that of vancomycin but with increased potency, particularly against *Streptococcus* spp. and *Enterococcus* spp.. Some strains of coagulase-negative *Staphylococcus* spp., particularly *S. haemolyticus* are less susceptible to teicoplanin than to vancomycin (Parenti et al. 2000). Teicoplanin is more active against some vancomycin-resistant enterococci (VanB and VanC), but is not active on VanA-resistant enterococci and staphylococci (see Sect. 5.4.1, see Rossolini et al., this volume). Benefits of teicoplanin include more favorable pharmacokinetics (longer half-life) and reduced occurrence of nephrotoxicity compared to vancomycin (Peetermans et al. 1990; Rybak et al. 1992). Notwithstanding these positive characteristics, teicoplanin has never been approved for clinical use in the US.

5.2 Structures of Glycopeptides

Glycopeptides are heptapeptides produced by non-ribosomal peptide synthesis. The common structural motif occurring in all type I–IV glycopeptides is a core heptapeptide scaffold containing aromatic amino acids that have undergone extensive oxidative cross-linking and decoration with different moieties, such as sugar residues, chlorine atoms, and lipid chains. They are subdivided into five types of which four (types I–IV) display antibacterial properties (Nicolaou et al. 1999). Of the

varying structural subtypes, type I structures, such as vancomycin (Fig. 5.1), contain five aromatic amino acids and two aliphatic ones (Leu_1 and Asn_3), whereas in types II, III, and IV all the seven amino acids are aromatic. The electron-rich side chains of aromatic amino acid residues facilitate the oxidative cross-linking that sets the polycyclic architecture of the heptapeptide scaffolds of these drugs. Types I and II contain three ring systems among aromatic residues, whereas III and IV contain an extra ring system. Type IV compounds, such as teicoplanin (Fig. 5.1), have an additional long fatty-acid chain attached to the sugar appended to the central 4-hydroxyphenylglycine (Hpg_4). Type V, such as complestatin and kistamicin A and B, that have antiviral properties (Matsuzaki et al. 1994), contain a characteristic tryptophan moiety linked to the central amino acid.

Nicolaou and colleagues (Nicolaou et al. 1999) listed all the functional groups attached to the different positions in the various glycopeptides. These modifications of the core heptapeptide include primary modifications, whereas the amino acid components of the heptapeptide scaffold are directly modified—such as halogenation, sulfation, methylation, and glycosylation—and secondary modifications, referring to tailoring of primary modifications such as acylation of the amino sugar linked to Hpg_4 in teicoplanin (Thaker and Wright 2012). In the case of antibacterial glycopeptides, the heptapeptide core offers the antibiotic binding pocked that sequesters the dipeptide terminus D-alanine-D-alanine (D-Ala-D-Ala) of the peptidoglycan (PG) precursors, blocking transpeptidation and transglycosylation reactions in the late extracellular stages of PG cross-linking (see Sects. 5.3.1 and 5.3.2). Primary and secondary modifications were reported to increase solubility, impart stability, affect dimerization, determine membrane attachment, limit conformational flexibility, avoid degradation, and evade resistance (Chan et al. 2011; Grdadolnik et al. 1998). Chlorination is the most common modification on glycopeptides, ranging from one to four chlorine atoms addition in different glycopeptides (Nicolaou et al. 1999; Thaker and Wright 2012). Chlorination is implicated in intramolecular dimerization and in conferring a lipophilic nature that might contribute to the affinity for the membrane compartment (Nicolaou et al. 1999). Sulfation is relatively uncommon but it is reported to play a role in evading the induction of resistance (Kalan et al. 2013). Glycosylation of glycopeptides was studied in details: the spectrum ranges from the aglycon A47934 to ristocetin (see Sect. 5.2.3) bearing six sugar residues (Kahne et al. 2005; Thaker and Wright 2012). The sugar groups of the glycopeptides play important roles in delivering the antibiotics to their target by modulating solubility and determining variation in antibiotic activity (Grdadolnik et al. 1998; Mackay et al. 1994b). Acylation of teicoplanin and teicoplanin-like A40926 (see Sects. 5.2.1 and 5.2.2) confers a lipophilic nature to the antibiotics that was suggested to impart them superior antimicrobial activity and pharmacokinetics in comparison to vancomycin (Ashford and Bew 2012; Kahne et al. 2005; Mackay et al. 1994b). As a consequence, most of the second-generation semi-synthetic glycopeptides were prepared introducing hydrophobic moieties in the heptapeptide scaffold in order to confer increased membrane anchoring ability, leading to improved drugs (James et al. 2012; Zhanel et al. 2010).

5.2.1 First Generation: Vancomycin and Teicoplanin

Vancomycin and teicoplanin are closely related structures (Fig. 5.1). Their heptapeptide scaffold includes two unusual aromatic amino acids, 4-hydroxyphenylglycine (Hpg) and 3,5-dihydroxyphenylglycine (Dpg), and the β-hydroxy version of tyrosine (Bht). Five of the seven residues in vancomycin are aromatic and two are aliphatic (Leu_1-Bht_2-Asn_3-Hpg_4-Hpg_5-Bht_6-Dpg_7), while all seven are aromatic in teicoplanin (Hpg_1-Tyr_2-Dpg_3-Hpg_4-Hpg_5-Bht_6-Dpg_7). There are five non-proteinogenic phenylglycines in teicoplanin and three in vancomycin. In vancomycin, the remaining two aromatic residues are modified tyrosines, with chlorine at the meta position of the aromatic ring and an OH substituent at the benzylic carbon of the side chain. In teicoplanin, Tyr_2 and Bht_6 are also chlorinated. In vancomycin, three cross-links join the aromatic rings at positions 2–4 and 4–6 in aryl ether linkages and 5–7 in a direct C–C coupling. In teicoplanin, a fourth cross-link between aromatic rings at positions 1–3 is added. These aglycone scaffolds are glycosylated, by a disaccharide chain on residue 4 in vancomycin or by three monosaccharides at residues 4, 6, and 7 in teicoplanin. The disaccharide in vancomycin is a D-glucosyl-2, 1-D-vancosamine, linked through C_1 of glucose to the phenolic oxygen of Hpg_4 of the cross-linked peptide. The vancosamine unit is a 2,3,6-trideoxy-L-hexose, with methyl and amino substituents at C_3. In teicoplanin, there is a glucosamine attached to Hpg_4 residue, a N-acetyl glucosamine (GlcNAc) at Bht_6, and a mannose on Dpg_7. The glucosamine moiety in teicoplanin is acylated by a fatty-acid chain. Clinically used teicoplanin is a mixture of five molecules slightly differing in the length (C10–C11) and branching of the fatty-acid tails, whose main component is the one bearing the 8-methylnonanoic (iso-C10:0) acid and named T-$A_{2\text{-}2}$ (Borghi et al. 1991; Taurino et al. 2011).

5.2.2 Second Generation: Oritavancin, Telavancin, Dalbavancin

The continued spread of MRSA and the appearance of high-level resistance to vancomycin have renewed interest in this antibacterial class. Semi-synthetic analogs based on traditional glycopeptide scaffolds were synthesized with modified side chains to improve the activity against vancomycin-resistant strains (Ashford and Bew 2012; Jovetic et al. 2010; Malabarba et al. 1997; Pace and Yang 2006). Oritavancin, telavancin, and dalbavancin (Fig. 5.2) were developed and telavancin recently achieved approval for clinical use, while oritavancin and dalbavancin are in late clinical development programs (Guskey and Tsuji 2010).

Originally discovered and developed by Eli Lilly, oritavancin (LY333328) was obtained by reductive alkylation of the natural glycopeptide chloroeremomycin, which differs from vancomycin in having a 4-epi-vancosamine sugar substituted for vancosamine in the disaccharide attached at residue 4 and an additional

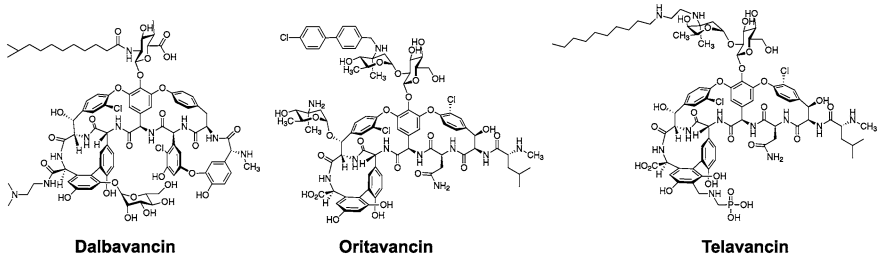


Fig. 5.2 Structures of semi-synthetic second-generation glycopeptides

4-epi-vancosamine attached at residue 6 of the linear heptapeptide. Oritavancin (Fig. 5.2) is the *N*-alkyl-*p*-chlorophenyl-benzyl derivative of chloroeremomycin. Although oritavancin presents a general spectrum of activity comparable to that of vancomycin, it offers considerable advantages in terms of intrinsic activity (especially against streptococci), and remains insensitive to the resistance mechanisms developed by staphylococci and enterococci; it is also active against *Clostridium difficile* (Bouza and Burillo 2010). According to recent investigations (Belley et al. 2010), the biaryl group is involved in cell membrane depolarization and in increase of membrane permeability. The superior activity against Gram-positive pathogens including those resistant to vancomycin is due to this dual-action mechanism either inhibiting cell wall biosynthesis or affecting membrane integrity. Additional favorable characteristics of oritavancin include its rapid bactericidal action, long-lasting post-antibiotic effects, and a prolonged half-life of 2 weeks. Its pharmacodynamic properties make it an ideal antibiotic for a once-a-day or even single dose regimen (Bouza and Burillo 2010; Van Bambeke 2006; Zhanel et al. 2010). Acquired by The Medicine Co. in 2009, oritavancin is currently running in phase III of clinical trials for the treatment of acute bacterial skin and skin structure infections.

On 11 September 2009, the FDA approved the drug telavancin (trade name Vibativ) for complicated skin and skin structure infections. Telavancin (TD-6424) is a semi-synthetic derivative of vancomycin, possessing a hydrophobic side chain on the vancosamine sugar (decylaminoethyl) and a methyl aminophosphonate group in the biaryl region of vancomycin (Fig. 5.2). The length of the hydrophobic side chain was chosen to reach a compromise between optimized activity against MRSA (8–10 C) and VRE (12–16 C). The hydrophilic properties of the phosphonate group improve adsorption, distribution, metabolism, and excretion profile (ADME) of the compound (Leadbetter et al. 2004). Pharmacological studies suggest that the enhanced activity of telavancin versus vancomycin on *S. pneumoniae*, *S. aureus* (to a lesser extent), and staphylococci or enterococci (including those harboring the *vanA* gene cluster, see Sect. 5.4.1) results from a complex mechanism of action which involves a perturbation of lipid synthesis and possibly membrane disruption (Higgins et al. 2005). The global antimicrobial activity is comparable to that of oritavancin (Van Bambeke 2006). Advantageous properties include a prolonged

post-antibiotic effect and a concentration-dependent bactericidal activity. The polar group introduced on the molecule improves the distribution of the molecule in the body and counterbalances the prolonging effect of the lipophilic side chain on the half-life, which is approximately 7 h and still compatible with a once-daily administration (Van Bambeke 2006; Van Hal and Paterson 2011; Zhanel et al. 2010).

Dalbavancin (previously named BI 397, Fig. 5.2) is a semi-synthetic derivative of the natural glycopeptide A40926 (Fig. 5.3), a teicoplanin analog. A40926 was isolated in Lepetit laboratories from a strain of *Nonomuraea* sp. collected in the mid-1980s from an Indian sample of soil during a screening for new glycopeptide antibiotics (Goldstein et al. 1987). In comparison with teicoplanin, A40926 lacks the GlcNAc at Bht_6. It also differs from teicoplanin due to the presence of an acylaminoglucuronic acid on amino acid 4 instead of the acylglucosamine. Other structural differences between A40926 and teicoplanin include the terminal methylamino group, the position of one chlorine atom, and the length of the fatty-acid chain (Malabarba and Ciabatti 2001). A40926 was used by Lepetit chemists as a scaffold for chemical derivatization producing several clinical leads (Malabarba and Ciabatti 2001; Malabarba et al. 1997). Dalbavancin was selected to proceed in clinical development. Dalbavancin is a dimethylaminopropyl amide derivative of the microbial lipoglycopeptide A40926 (Figs. 5.2, 5.3) (Malabarba and Goldstein 2005). Dalbavancin underwent a phase III clinical trial for adults with complicated skin infections, but in December 2007 the FDA said more data was needed before approval. Durata Therapeutics acquired the rights to dalbavancin in December 2009 from Pfizer and initiated two new Phase III clinical trials for treatment of acute bacterial skin and skin structure infections. Dalbavancin (trade name Zeven) is extremely active and bactericidal against staphylococci and streptococci but loses activity against VanA enterococci and staphylococci in comparison to oritavancin and telavancin. Dalbavancin shows an unusually prolonged half-life that its plasma concentration exceeds the minimal bactericidal concentration of target organisms even at 1 week after administration of a single 1,000 mg dose. Dalbavancin has a half-life of ca. 7 days, due to the strong binding to serum proteins and cell compartmentalization, whereas vancomycin has an elimination half-life of 3.4 h. Accordingly, the drug is currently being evaluated in clinical studies using once-a-week scheme of administration. Pilot phase II trials show an excellent clinical success (>90 %) in patients receiving 1,000 mg dalbavancin at day 1 and 500 mg at day 8 for the treatment of skin and soft tissue infections or catheter-related bloodstream infections by Gram-positive organisms (Van Bambeke 2006; Zhanel et al. 2010).

The success of these three second-generation glycopeptides as drug candidates and their potentiated antibacterial activities in comparison to vancomycin, has stimulated further efforts in developing glycopeptides derivatives. A recent extensive review (Ashford and Bew 2012) reports the current chemical approaches followed to modify existing scaffolds by introducing hydrophobic side chains, by facilitating dimerization and by changing amino acids inside the binding pocket. Due to the progresses achieved by total synthesis of glycopeptides by the group of

Boger since the 1999, single atom changes in vancomycin are possible today and pave the way to rationally redesign glycopeptides exhibiting potent antibacterial activity against VRE and vancomycin-resistant *Staphylococcus aureus* (VRSA) (James et al. 2012). Another innovative approach based on structural-activity relationship (SAR) is the synthesis by click chemistry of hybrid antibiotics that consisted of vancomycin and lantibiotic (nisin) fragments (see Cortes, this volume) which targets different portions of Lipid II involved in cell wall biosynthesis (see Sect. 5.3.1). These resulted hybrid antibiotics are significantly more active than the separate fragments against VRE (Arnusch et al. 2008).

5.2.3 Other Natural Glycopeptides

After the clinical success of vancomycin and teicoplanin, many other glycopeptides were isolated by screening actinomycetes. A complete list of them is reported in (Nicolaou et al. 1999). To our knowledge, none of them have been developed for clinical use.

Chloroeremomycin (the precursor of the semi-synthetic oritavancin, Fig. 5.2) and balhimycin (Fig. 5.3) have the identical heptapeptide scaffold of vancomycin, but differ in the pattern of glycosylation. A47934 (Fig. 5.3) is an aglycone antibiotic belonging to the teicoplanin family. It is produced by *Streptomyces toyocaensis* NRRL 15009. It is peculiar among reported glycopeptides in that it contains a sulfate ester on the N-terminal *p*-hydroxyphenylglycine residue, and is not glycosylated (Boeck and Mertz 1986). Studies on the genes involved in the biosynthesis of balhimycin, chloroeremomycin, and A47934 were pivotal to understand the biosynthetic pathway of glycopeptides (Pelzer et al. 1999; Pootoolal et al. 2002; Van Wageningen et al. 1998, see Sect. 5.5). A40926 (Fig. 5.3), the precursor of the second-generation dalbavancin (Fig. 5.2), is another teicoplanin molecule isolated by a soil actinomycete (Goldstein et al. 1987), described in the section above.

Differently important even if not in use, avoparcin (Fig. 5.3) is a glycopeptide antimicrobial agent related to vancomycin, isolated from *Streptomyces candidus* (then reclassified as *Amycolatopsis coloradensis* sp.) (Labeda 1995; Pensack et al. 1982). It was extensively used as a growth promoter in animal feeds for more than two decades from the early 1970s, until a link was established between its use and the increased prevalence of VRE in the food chain (Lu et al. 2004; Marshall et al. 1998). In April 1997, avoparcin was banned in the EU. More evidence of the role of avoparcin in the emergence of VRE in food animals was then provided. Bager showed a statistically significant decline of VRE isolated from broilers after the ban of avoparcin in Denmark (Bager et al. 1999). Kruse also demonstrated a strong and statistically significant association between the use of avoparcin in Norwegian poultry production and the occurrence of VRE (Kruse et al. 1999). Pantosti demonstrated, 18 months after the banning of avoparcin, a decline in the percentage of poultry meat samples containing *vanA* gene-positive VRE (see Sect. 5.4.1) from

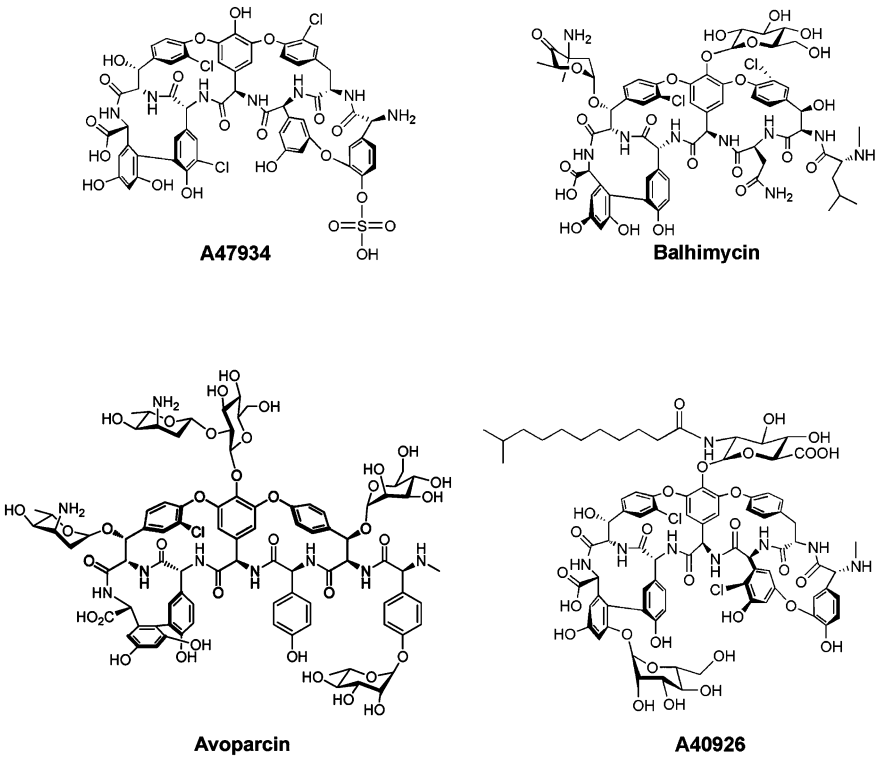


Fig. 5.3 Structures of natural glycopeptides

14.6 to 8 % (Pantosti et al. 1999). Thus, avoparcin animal feeding data correlate well with the prevalence of *vanA* VRE in animals.

5.3 Mode of Action of Glycopeptides

5.3.1 Peptidoglycan Synthesis

Glycopeptide antibiotics block the synthesis of bacterial cell wall through inhibition of extracellular steps in PG biosynthesis. The main component of the PG is a polysaccharidic matrix of unbranched chains containing alternating subunits of GlcNAc and N-acetylmuramic acid (MurNAc) linked by peptide bridges. For detailed description of bacterial cell wall composition and synthesis, readers can refer to reviews (Kahne et al. 2005; Vollmer et al. 2008). The starting pathways for preparing PG precursors occur in the cytoplasm. The pyrimidine (UDP) is linked to glucosamine-1-phosphate to form the first subunit of PG precursors, the UDP-N-acetylglucosamine (UDP-GlcNAc). The UDP-GlcNAc combines with phosphoenol

pyruvate to form the second glycan subunit, the UDP-N-acetyl-muramic acid (UDP-MurNAc) to which five amino acids are subsequently linked to form the UDP-MurNAc pentapeptide also known as Park nucleotide. The Park nucleotide has different amino acid composition within different bacteria (Vollmer et al. 2008). Usually, an L-Ala is the first amino acid of the peptide stem added by the MurC ligase to the UDP-MurNAc residue followed frequently by a D-glutamate added by MurD. The addition of the third amino acid is catalyzed by MurE ligase. This amino acid is generally a diamino acid either L-lysine (most Gram-positives) or *meso*-diaminopimelic acid (most Gram-negatives). Amino acids at positions 4 and 5 are added as a dipeptide, in most cases D-Ala-D-Ala. In most Gram-positive species, synthesis of the dipeptide is carried out by a ligase (Ddl) enzyme and its incorporation into peptide stem by MurF to form the UDP-MurNAc pentapeptide (UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala). The Park nucleotide is then bound to C55-undecaprenylphosphate to form Lipid intermediate I, which is the cytoplasmic membrane PG transporter. The UDP-GlcNAc subunit is added to Lipid I, forming Lipid intermediate II. Lipid II is then transferred across the membrane, and on the outer membrane surface transglycosylase and transpeptidase enzymes catalyze the formation of the mature PG. Transglycosylases link the sugar moieties GlcNAc-MurNAc to form long polysaccharide chains. C55-undecaprenylphosphate becomes free and thus available for accepting other cytoplasmic Park nucleotides. Transpeptidases recognize the sequence D-Ala-D-Ala at the end of pentapeptide chain, cleave off the terminal alanine and join the remainder to the third amino acid from an adjacent polysaccharide chains. Theoretically, all D-Ala-D-Ala motifs should be consumed in the cross-bridging reaction: however, still 20–30 % of subunits retain D-Ala-D-Ala termini. The direct crosslink, described above between pentapeptide side chains, is present in some bacteria as *Escherichia coli* and *Bacillus subtilis*, while in *Staphylococcus aureus* and in *Streptomyces* spp. There is a cross-bridge of pentaglycine and monoglycine, respectively. The peptide cross-linking provides the structural rigidity of mature PG required for maintenance of cell shape and prevention of cell lysis (Vollmer et al. 2008).

5.3.2 Mode of Action

Glycopeptide antibiotics interact with the D-Ala-D-Ala terminus of the uncross-linked PG precursor pentapeptide. Previous NMR and X-ray studies (Mackay et al. 1994a; Schäfer et al. 1996) demonstrated that the interaction between acyl-D-Ala-D-Ala and vancomycin consisted of a network of five hydrogen bonds to form a non-covalent complex that effectively sequesters the D-Ala-D-Ala dipeptide, thereby physically inhibiting the action of transpeptidases/transglycosylases that generate the cross-linked PG needed for the appropriate tensile strength of cell wall. The requirement for direct access of the large antibiotic to the target PG explains the selective inhibition of Gram-positive bacteria. Gram-positive expose PG on the external surface of cytoplasmic membrane, whereas in Gram-negative

bacteria PG is protected by the presence of an outer lipopolysaccharide membrane impermeable to large biomolecules (Kahne et al. 2005).

Many glycopeptides of vancomycin-type have the capacity to form dimers. X-ray crystallography investigations (Schäfer et al. 1996) and NMR studies (Williams et al. 1993) revealed the dimerization activity of vancomycin, but recent X-ray crystal structures (Nitanai et al. 2009) provided a more detailed picture of "back-to-back," "face-to-face," and "side-to-side" arrangements observed in vancomycin binding to analogs of the PG terminus. These dimeric structures, held together by four hydrogen bonds, contain two binding sites for acyl-D-Ala-D-Ala units. The tendency of vancomycin-type glycopeptide antibiotics to dimerize has been correlated with their antimicrobial potency (Mackay et al. 1994a, b). In contrast to vancomycin, teicoplanin, A40926, and similar lipoglycopeptides exhibit high antibiotic activity despite their apparent inability to dimerize in solution (Mackay et al. 1994a). An explanation for the enhanced potency of teicoplanin is provided by another secondary effect exhibited by lipoglycopeptides, that of anchoring into the cells phospholipid bilayer through a long hydrocarbon chain. Thus, the lipophilic chain of teicoplanin attached to one of its carbohydrate units is supposed to localize the antibiotic to its site of action (Jovetic et al. 2010; Kahne et al. 2005; Mackay et al. 1994a). A scheme illustrating the double type of interaction is reported in Fig. 5.4.

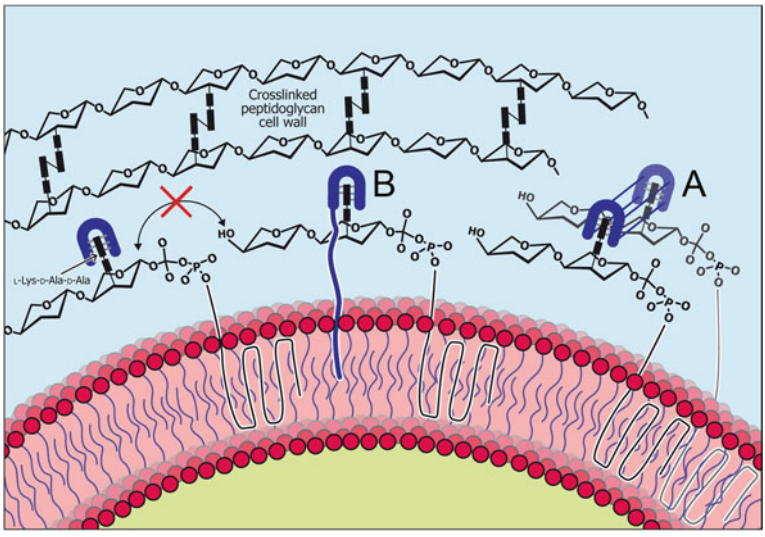


Fig. 5.4 Peptidoglycan structure and mechanism of action of glycopeptide antibiotics. Glycopeptides inhibit transglycosylation and transpeptidation by the antibiotic binding to the C-terminal D-Ala-D-Ala of late peptidoglycan precursor. A Mechanism of action of glycopeptides vancomycin-like is based on the dimerization that enhances the binding with the target peptide through both cooperative and allosteric effects. B Lipoglycopeptides (e.g., teicoplanin and its derivatives) own a fatty acyl chain anchored in the phospholipid bilayer thus enhancing the binding affinity (from Jovetic et al. 2010)

5.4 Resistance to Glycopeptides

5.4.1 Resistance in Enterococci and Staphylococci

The onset of vancomycin resistance was long-delayed in comparison to all other antibiotics. Even after its first three decades of use, there was no notable resistance to vancomycin reported. Glycopeptide resistance in enterococci was originally reported in the late 1980s (Uttley et al. 1989) and has undergone a global diffusion during the past two decades, especially in *E. faecium* (see Rossolini, this volume). Following the spread of resistance in enterococci, concern arose over the emergence of vancomycin-resistant *S. aureus*. *S. aureus* strains showing reduced susceptibility to vancomycin (vancomycin-intermediate *S. aureus* [VISA], vancomycin MIC = 4–8 mg/L) have been encountered with increasing frequency worldwide (Howden et al. 2010, see Rossolini, this volume), thereby compromising the therapeutic efficiency of this drug, which is the one to which MRSA remain uniformly susceptible. Originally isolated in 1996 in Japan, similar VISA phenotypes have been isolated in many countries, particularly from patients having received prolonged vancomycin therapy. The first case of a fully vancomycin-resistant *S. aureus* strain (VRSA, vancomycin MIC > 16 mg/L) strain was reported in 2002, and it was isolated from a dialysis patient in Michigan co-infected by a vancomycin-resistant *E. faecalis*, implicating horizontal gene transfer mediated by a mobile genetic element known as *Tn1546* (Weigel et al. 2003). Since that time, a few true vancomycin-resistant *S. aureus* (VRSA) isolates have been reported mainly in USA hospitals. VSRA has also been described in Iran and India, although fortunately it remains rare worldwide. It seems that, due to several biological constraints such as a potent restriction system of *S. aureus* that limits the transfer of mobile genetic elements from other species and a fitness defect associated with the modified cell wall structure, dissemination of VRSA has so far been limited, although it remains an extremely serious concern for patients infected by such bacteria (Périchon and Courvalin 2009).

In the two most common phenotypes of VRE, VanA and VanB, and in the VRSA the PG precursor terminates with a D-alanine-D-lactate (D-Ala-D-Lac), incorporating an ester linkage in place of the amide of the D-Ala-D-Ala. The replacement of a dipeptide with a depsipeptide removes one of the hydrogen bonding interactions and leads to lone pair–lone pair repulsion, reducing of 1000-fold the affinity of the glycopeptides to their molecular target, and resulting in a corresponding 1000-fold loss in antimicrobial activity (Bugg et al. 1991; Crowley and Boger 2006; Lessard and Walsh 1999). Vancomycin-resistant bacteria sense the antibiotic challenge and subsequently remodel their precursor peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-Lac. Normal synthesis of Lipid intermediate I and II continues, but a late stage remodeling to D-Ala-D-Lac of PG termini ensues to avoid the action of vancomycin. One key indication that emerges from this characterization of vancomycin-resistant bacteria is that the most direct efforts to

redesign vancomycin for their treatment should target compounds that bind D-Ala-D-Lac and maintain binding to D-Ala-D-Ala (James et al. 2012). The mechanism by which the vancomycin resistance is induced in VRE and VRSA is well understood (Arthur et al. 1996; Evers et al. 1996; Healy et al. 2000), entailing a two-component signaling cascade (VanS/VanR). Initiated by a ligand-induced dimerization of a cell surface His-kinase (VanS), it activates the signaling transcription factor VanR by phosphorylation and dimerization. In turn, the activated VanR binds to DNA and induces the expression of three genes encoding three enzymes (VanH, VanA, and VanX) that remodel the peptidoglycan precursor from D-Ala-D-Ala to D-Ala-D-Lac. VanH converts pyruvate into D-lactate, Van A is a D-Ala-D-Lac ligase, and Van X is D-Ala-D-Ala dipeptidase that cleaves any residual D-Ala-D-Ala dipeptide, ensuring that peptidoglycan precursors terminate mostly in D-Ala-D-Lac (Arthur et al. 1996; Jovetic et al. 2010; Kahne et al. 2005). Vancomycin and teicoplanin induce resistance in VanA enterococci, whereas VanB strains sense vancomycin but are resistant to teicoplanin, opening not yet completed answered questions on the real inducer of VanS/R (James et al. 2012; Reynolds and Courvalin 2005). Several other variants of such gene clusters have been discovered (e.g., *vanC, vanD, vanE, vanG, vanL, vanM, vanN*) that can be associated with variable glycopeptide-resistance phenotypes and are often carried on transposable elements such as *Tn1546* (Reynolds and Courvalin 2005; Lebreton et al. 2011; Xu et al. 2010; Sujatha and Praharaj 2012). It seems that these diverse sets of *van* genes and of sensing mechanisms were most likely co-opted from the glycopeptide-producing organisms, which use them to avoid the antibiotic effects, rather than being orchestrated by sensitive bacteria upon continued treatment (Marshall et al. 1998). As such, it is probable that pathogenic bacteria have not yet independently evolved a unique and effective resistance mechanism to glycopeptide challenges (Hong et al. 2008).

5.4.2 Self-Resistance in the Producer Bacteria

An interesting hypothesis is that bacterial pathogens have actually acquired antibiotic resistance mechanisms from antibiotic producers, although there is no conclusive evidence which proves gene transfer. *van*-like genes with a high level of homology and an organization similar to those in pathogenic enterococci were identified in many glycopeptide-producing actinomycetes. *van*-like genes are present in *Amycolatopsis* spp., that produce vancomycin, and vancomycin-type molecules chloroeremomycin, ristocetin, and avoparcin (Marshall et al. 1998); in *Streptomyces toyocaensis* NRRL15009, which is the producer of the teicoplanin-like A47934 (Pootoolal et al. 2002); and in the teicoplanin producer *Actinoplanes teichomyceticus* ATCC 31121 (Beltrametti et al. 2007). In these last two actinomycetes *van*-like genes are located within the clusters of genes dedicated to the biosynthesis of A47934 (*sta* biosynthetic cluster) and teicoplanin (*tcp* biosynthetic cluster), suggesting a close correlation between the expression of resistance and

antibiotic synthesis. The amino acid sequences of VanH, VanA, and VanX of *S. toyocaensis* show a high sequence homology with those found in VRE (Pootoolal et al. 2002). Their gene expression is induced by A47934. Co-regulation of resistance genes and biosynthetic genes involved in A47934 production was confirmed by the inactivation of *vanA*. The resulting mutant was more susceptible to the glycopeptide and its production was delayed of 16 h, until the cells were completely grown and not more sensitive to the glycopeptide action (Pootoolal et al. 2002). In *A. teichomyceticus* sp. ATCC 31121, the teicoplanin producer, the genes involved in the resistance mechanism are constitutively expressed to produce PG precursors terminating in the depsipeptide D-Ala-D-Lac terminus. Consequently *A. teichomyceticus* is highly resistant to vancomycin and teicoplanin (Beltrametti et al. 2007).

Surprisingly also *S. coelicolor*, which does not produce any glycopeptide, possesses *van* genes, that confers to this microorganism high inducible resistance level to vancomycin, but not to teicoplanin. The genetic cluster consists of seven genes *vanSRJKHAX*: VanR, VanS, and VanHAX have the same role seen in VRE. VanJ is a protein involved in cell wall modification and teicoplanin resistance (Novotna et al. 2012) and VanK is a member of Fem proteins family which add the glycine/s to the stem pentapeptide of PG precursors (Hong et al. 2004). Analysis of PG precursor pool in *S. coelicolor* shows that Lipid II terminates in D-Ala-D-Ala or D-Ala-D-Lac in the absence or in the presence of vancomycin, respectively (Hong et al. 2005). These findings on vancomycin resistance of *S. coelicolor* suggest that this actinomycete might have acquired *van* genes, gaining a selective advantage toward the glycopeptide-producing actinomycetes which inhabit the same ecological niche (Hutchings et al. 2006).

Recently, a novel mechanism of glycopeptide resistance was described in *Nonomuraea* sp. ATCC 39727, the producer of A40926 (Marcone et al. 2010). The *dbv* gene cluster for A40926 production does not contain *vanHAX* genes but only *vanY*, that is considered an ancillary resistance gene in enterococci, encoding an enzyme capable of removing the terminal D-Ala residue of pentapeptide peptidoglycan precursors (Binda et al. 2012). The produced tetrapeptide is a poor substrate for glycopeptide binding, thus reducing the level of susceptibility to glycopeptides. Analysis of UDP-linked peptidoglycan precursors in *Nonomuraea* sp. ATCC 39727 confirmed the predominant presence of the tetrapeptide UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-Ala. Consistent with this, a *vanY*-null mutant of *Nonomuraea* sp. ATCC 39727 demonstrated a reduced level of glycopeptide resistance and heterologous expression of *vanY* in a sensitive *Streptomyces* species resulted in a higher level of glycopeptide resistance (Binda et al. 2013; Schäberle et al. 2011).

The vancomycin-resistance mechanism was recently investigated also in *Amycolatopsis balhimycina*, the producer of balhimycin (Schäberle et al. 2011). The structure of the peptidoglycan precursor terminating in D-Ala-D-Lac is consistent with the presence of *vanHAX* genes, which were identified outside the balhimycin (*bal*) synthesis cluster. A *vanY* gene with high similarity to that from *Nonomuraea* sp. ($\sim$48 %) was found inside the *bal* cluster (Binda et al. 2012).

These studies indicate that a thorough understanding of self-resistance mechanisms in different glycopeptide-producer actinomycetes can contribute not only to a better comprehension of evolution of resistant determinants in pathogens, but also to an early warning system for novel emerging resistance mechanisms following introduction of new glycopeptide antibiotics in the environment and clinics (D'Costa et al. 2011).

5.5 Biosynthesis

Although the total synthesis of vancomycin and teicoplanin family of glycopeptides was accomplished, the complexity of these natural products makes fermentation process the only viable route for their pharmaceutical production. Considerable progress has been made toward understanding the biosynthetic sequence by which the glycopeptide antibiotics are produced within bacteria. Almost all biosynthetic steps were characterized biochemically and/or by combination of genetics and analytical chemistry. Based upon other biosynthetic pathways, formation of these structures was expected to proceed through three steps: first, the construction of the basic building blocks by specific biosynthetic pathways; second, formation of the linear heptapeptide, interconnection of the aromatic side chains, and halogenation; third, modification of the aglycone by glycosyltransferases, a methyltransferase and in the case of type-IV glycopeptides additionally by acyltransferases and eventually by sulfotransferases. First indications were achieved by isotopic labeling studies: feeding with ^{13}C-labeled substrates proved the origin of Bht and Hpg from tyrosine, and Dpg from acetate in vancomycin (Hammond et al. 1982). Sequencing of the chloroeremomycin biosynthesis gene cluster yielded insight into the gene arrangement, and putative gene functions were deduced (Van Wageningen et al. 1998). The sequence of the balhimycin biosynthesis gene cluster (Pelzer et al. 1999) and of other glycopeptide antibiotic genes followed shortly thereafter. The A47934 cluster was the first complete sequence for a teicoplanin class glycopeptide. The cluster includes 34 ORFs encompassing 68 kb and all of the genes predicted to be required to synthesize A47934 and regulate its biosynthesis (Pootoolal et al. 2002). To date, six gene clusters involved in the biosynthesis of balhimycin (*bal*) (Pelzer et al. 1999), chloroeremomycin (*cep*) (Van Wageningen et al. 1998), A47934 (*sta*) (Pootoolal et al. 2002), A40926 (*dbv*) (Sosio et al. 2003), teicoplanin (*tcp/tei*) (Li et al. 2004; Sosio et al. 2004), and sulfo-teicoplanin (*teg*) (Lamb et al. 2006) are available in GenBank, providing the basis for elucidating most of the biosynthesis steps.

All the genes required for the biosynthesis of the non-proteinogenic amino acids (Bht, Hpg, and Dpg) are located in the glycopeptide gene clusters (Wohlleben et al. 2009). As example, the biosynthesis of Bht was understood in the balhimycin-producer *Amycolatopsis balhimycina* by gene inactivation of the genes *bhp*, *bpsD,* and *oxyD* of the *bal* cluster. The in-frame deletion delivered null mutants unable to produce balhimycin, and the biosynthesis was restored by

adding Bht to the medium (Puk et al. 2002, 2004). The biosynthesis of Hpg was investigated by heterologous expression in *E. coli* of the putative genes from the *cep* cluster of *Amycolatopsis orientalis* (Choroba et al. 2000) or by inactivation of the corresponding *pgat/hmaS/hmO* genes in *bal* cluster (Stegmann et al. 2010; Süssmuth and Wohlleben 2004). Dpg is synthesized by a polyketide synthase mechanism and inactivation of the gene encoding for the predicted polyketide synthase *dpgA* resulted in the loss of balhimycin production (Stegmann et al. 2010; Süssmuth and Wohlleben 2004). The biosynthesis of unusual carbohydrates falls equally into this part of monomer built-up. The biosynthetic pathways of many carbohydrate residues attached to glycopeptide aglycones are often found in other antibiotics or may even come from primary metabolism (like mannose and galactose). The carbohydrates vancosamine, *epi*-vancosamine, and *oxo*-vancosamine are characteristic representatives of the type I-glycopeptides. Their biosynthetic genes are present in vancomycin, chloroeremomycin, and balhimycin producers and the structures at the same time represent the differentiation criterion for these glycopeptides (Wohlleben et al. 2009).

Regarding the second step, the assembly of the component amino acids of the glycopeptide antibiotics into the heptapeptide backbone occurs through a non-ribosomal peptide synthesis mechanism involving a multi-enzyme thio-template. According to this mechanism, each amino acid is recognized and activated by the appropriate module of the non-ribosomal peptide synthetase (NRPS). The glycopeptide NRPS comprises seven modules and is formed by three enzymes in vancomycin-type molecule and four in teicoplanin-type glycopeptides. Each module is responsible for the activation and thiolation of a single amino acid. Condensation domains, epimerization domains, and one thioesterase domain complete the typical domain organization of NRPS (Stegmann et al. 2010; Süssmuth and Wohlleben 2004).

The final steps leading to the aglycon from the linear heptapeptide require oxygenases whose sequences is similar to P450 monooxygenases. These include hydroxylation of amino acids at position 2 and 6, ring closures to form the two diarylethers and the biaryl system in vancomycin-type; in teicoplanin one additional ring closure occurs between amino acids 1 and 3. Chlorination and side chain cyclization probably occur on the NRPS complex. N-methylation is indeed a late step in glycopeptide biosynthesis (Stegmann et al. 2010; Süssmuth and Wohlleben 2004). Glycosylation is catalyzed by three glycosyl-transferases in chloroeremomycin and balhimycin biosynthetic cluster (Pelzer et al. 1999; Van Wageningen et al. 1998) whereas in the vancomycin biosynthetic gene cluster are present only two (Choroba et al. 2000).

Additional genes present in the glycopeptide clusters are those devoted to regulation, self-resistance (see Sect. 5.4.2), and export of the antibiotics outside the cell. Sequence analysis of all glycopeptide clusters revealed a gene for StrR-ike regulator that was first described in the streptomycin gene cluster (Retzlaff and Distler 1995). There are two genes in all clusters that encode putative transporters playing a role in antibiotic secretion.

5.6 Chemo-Enzyme Synthesis

The current knowledge of the biosynthetic clusters for different glycopeptides offer new enzymatic tools to modify existing scaffold creating novel combinations by chemo-enzyme synthesis. Genes for methyltransferases, halogenases, sulfotransferases, deacetylase/acyltransferase, and glycosyltransferases were identified in the biosynthetic clusters, overexpressed in surrogate hosts and the purified proteins used to generate library of novel glycopeptides. These results have been covered by recent extensive reviews (Li et al. 2012; Thaker and Wright 2012). Using a glycorandomization approach where nucleotide-activated sugars were generated in *Escherichia coli,* the vancomycin aglycone was glycosylated biochemically (Fu et al. 2003). Novel glycopeptides were prepared by an innovative way reported by Liu et al. (2011). During the recent structure determination studies of the flavin-containing hexose oxidase (Ddv29), these authors serendipitously discovered a solvent-exposed reaction intermediate that they cleverly used in generating a series of teicoplanin analogs with amidated and aminated lipid chains. Some of these derivatives showed an excellent activity against vancomycin and teicoplanin *E. faecalis* strains. Metagenomics approach was also used to identify groups of glycopeptide-related gene clusters that encode the biosynthesis of new structural variants. Banik et al. (2008) screened a soil DNA cosmid library for clones containing genes associated with the biosynthesis of teicoplanin- and vancomycin-like glycopeptide antibiotics, and identified two new biosynthetic gene clusters. One of these glycopeptide clusters contains unique genes encoding three sulfotransferases, a class of tailoring enzymes that has rarely been associated with glycopeptide biosynthesis. Using the teicoplanin aglycone as a substrate, seven new anionic glycopeptide congeners were identified in vitro using the environmental DNA-derived sulfotransferase (Banik et al. 2010). Such novel approaches for enzymatic modification of glycopeptide scaffolds may result in a third generation of glycopeptides.

5.7 Conclusions

In the past three decades, the clinical use of antibacterial glycopeptides has constantly increased because of global diffusion of infections caused by Gram-positive microorganisms such as MRSA and enterococcal isolates resistant to ampicillin and aminoglycosides, and due to the increasing incidence of nosocomial infection from *Clostridium difficile*. Despite the alarm arisen following the isolation of the first clinical vancomycin highly resistant isolates, resistance to glycopeptides has remained fortunately very uncommon among MRSA strains, whereas from the clinical perspective, the most important issue is represented by acquired diffused glycopeptide-resistance in enterococcal infections caused by ampicillin-resistant strains (see Rossolini, this volume). The continued spread of MRSA and the

appearance of high-level resistance to vancomycin has anyhow renewed a multidisciplinary interest in this antibiotic class stimulating research to wide chemical diversity by preparing semi-synthetic analogs based on structural-activity relationship (SAR) (Ashford and Bew 2012; James et al. 2012) and by using advanced molecular tools of chemo-enzyme synthesis to prepare novel hybrid molecules (Li et al. 2012; Thaker and Wright 2012). NMR and crystallography studies, that have elucidated the mode of action and resistance of old and novel glycopeptides, indicate the way to redesign future improvement (James et al. 2012). Study of the biosynthetic clusters in the metagenome (Banik and Brady 2008) and investigation on biosynthesis and mechanism of self-resistance (Jovetic et al. 2010; Thaker and Wright 2012; Wohlleben et al. 2009) in the producing actinomycetes is providing new scaffolds and contributing to the surveillance of resistance evolution.

References

Arnusch CJ, Bonvin AM, Verel AM, Jansen WT, Liskamp RM, de Kruijff B, Pieters RJ, Breukink E (2008) The vancomycin-nisin (1–12) hybrid restores activity against vancomycin resistant enterococci. Biochemistry 47:12661–12663

Arthur M, Reynolds PE, Depardieu F, Evers S, Dutka-Malen S, Quintiliani R, Courvalin P (1996) Mechanisms of glycopeptide resistance in enterococci. J Infect 32:11–16

Ashford PA, Bew SP (2012) Recent advances in the synthesis of new glycopeptide antibiotics. Chem Soc Rev 41:957–978

Bager F, Aarestrup FM, Madsen M, Wegener HC (1999) Glycopeptide resistance in *Enterococcus faecium* from broilers and pigs following discontinued use of avoparcin. Microb Drug Resist 5:53–56

Banik JJ, Brady SF (2008) Cloning and characterization of new glycopeptide gene clusters found in an environmental DNA megalibrary. Proc Natl Acad Sci USA 105:17273–17277

Banik JJ, Craig JW, Calle PY, Brady SF (2010) Tailoring enzyme-rich environmental DNA clones: a source of enzymes for generating libraries of unnatural natural products. J Am Chem Soc 132:15661–15670

Belley A, McKay GA, Arhin FF, Sarmiento I, Beaulieu S, Fadhil I, Parr TR, Moeck G (2010) Oritavancin disrupts membrane integrity of *Staphylococcus aureus* and vancomycin-resistant enterococci to effect rapid bacterial killing. Antimicrob Agents Chemother 54:5369–5371

Beltrametti F, Consolandi A, Carrano L, Bagatin F, Rossi R, Leoni L, Zennaro E, Selva E, Marinelli F (2007) Resistance to glycopeptide antibiotics in the teicoplanin producer is mediated by *van* gene homologue expression directing the synthesis of a modified cell wall peptidoglycan. Antimicrob Agents Chemother 51:1135–1141

Binda E, Marcone GL, Pollegioni L, Marinelli F (2012) Characterization of VanYn, a novel D, D-peptidase/D, D-carboxypeptidase involved in glycopeptide antibiotic resistance in *Nonomuraea* sp. ATCC 39727. FEBS J 279:3203–3213

Binda E, Marcone GL, Berini F, Pollegioni L, Marinelli F (2013) *Streptomyces* spp. as efficient expression system for a D, D-peptidase/D, D-carboxypeptidase involved in glycopeptide antibiotic resistance. BMC Biotechnol 13:24

Boeck LD, Mertz FP (1986) A47934, a novel glycopeptide-aglycone antibiotic produced by a strain of *Streptomyces toyocaensis* taxonomy and fermentation studies. J Antibiot (Tokyo) 39:1533–1540

Borghi A, Edwards D, Zerilli LF, Lancini GC (1991) Factors affecting the normal and branched-chain acyl moieties of teicoplanin components produced by *Actinoplanes teichomyceticus*. J Gen Microbiol 137:587–592

Bouza E, Burillo A (2010) Oritavancin: a novel lipoglycopeptide active against gram-positive pathogens including multiresistant strains. Int J Antimicrob Agents 36:401–407

Bugg TD, Wright GD, Dutka-Malen S, Arthur M, Courvalin P, Walsh CT (1991) Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry 30:10408–10415

Chan HC, Huang YT, Lyu SY, Huang CJ, Li YS, Liu YC, Chou CC, Tsai MD, Li TL (2011) Regioselective deacetylation based on teicoplanin-complexed *orf*2* crystal structures. Mol BioSyst 7:1224–1231

Choroba OW, Williams DH, Spencer JB (2000) Biosynthesis of the vancomycin group of antibiotics: involvement of an unusual dioxygenase in the pathway to (s)-4-hydroxyphenylglycine. J Am Chem Soc 122:5389–5390

Crowley BM, Boger DL (2006) Total synthesis and evaluation of [psi[ch2nh]tpg4]vancomycin aglycon: reengineering vancomycin for dual D-Ala-D-Ala and D-Ala-D-Lac binding. J Am Chem Soc 128:2885–2892

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 (2011) Antibiotic resistance is ancient. Nature 477:457–461

Ehrenkranz NJ (1958) The clinical evaluation of vancomycin in treatment of multi-antibiotic refractory staphylococcal infections. Antibiot Annu 6:587–594

Evers S, Quintiliani R, Courvalin P (1996) Genetics of glycopeptide resistance in enterococci. Microb Drug Resist 2:219–223

Fu X, Albermann C, Jiang J, Liao J, Zhang C, Thorson JS (2003) Antibiotic optimization via in vitro glycorandomization. Nat Biotechnol 21:1467–1469

Geraci JE, Hermans PE (1983) Vancomycin. Mayo Clin Proc 58:88–91

Goldstein BP, Selva E, Gastaldo L, Berti M, Pallanza R, Ripamonti F, Ferrari P, Denaro M, Arioli V, Cassani G (1987) A40926, a new glycopeptide antibiotic with anti-*Neisseria* activity. Antimicrob Agents Chemother 31:1961–1966

Gravet A, Rondeau M, Harf-Monteil C, Grunenberger F, Monteil H, Scheftel JM, Prévost G (1999) Predominant *Staphylococcus aureus* isolated from antibiotic-associated diarrhea is clinically relevant and produces enterotoxin A and the bicomponent toxin lukE-lukD. J Clin Microbiol 37:4012–4019

Grdadolnik SG, Pristovsek P, Mierke DF (1998) Vancomycin: conformational consequences of the sugar substituent. J Med Chem 41:2090–2099

Guskey MT, Tsuji BT (2010) A comparative review of the lipoglycopeptides: oritavancin, dalbavancin, and telavancin. Pharmacotherapy 30:80–94

Hammond SJ, Williamson MP, Williams DH, Boeck LD, Marconi GG (1982) On the biosynthesis of the antibiotic vancomycin. J Chem Soc Chem Commun 344–346

Healy VL, Lessard IA, Roper DI, Knox JR, Walsh CT (2000) Vancomycin resistance in enterococci: reprogramming of the D-Ala-D-Ala ligases in bacterial peptidoglycan biosynthesis. Chem Biol 7:R109–R119

Higgins DL, Chang R, Debabov DV, Leung J, Wu T, Krause KM, Sandvik E, Hubbard JM, Kaniga K, Schmidt DE, Gao Q, Cass RT, Karr DE, Benton BM, Humphrey PP (2005) Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall synthesis and cell membrane integrity in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 49:1127–1134

Hong HJ, Hutchings MI, Neu JM, Wright GD, Paget MS, Buttner MJ (2004) Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. Mol Microbiol 52:1107–1121

Hong HJ, Hutchings MI, Hill LM, Buttner MJ (2005) The role of the novel fem protein VanK in vancomycin resistance in *Streptomyces coelicolor*. J Biol Chem 280:13055–13061

Hong HJ, Hutchings MI, Buttner MJ (2008) Vancomycin resistance *vanS/vanR* two-component systems. Adv Exp Med Biol 631:200–213

Howden BP, Davies JK, Johnson PD, Stinear TP, Grayson ML (2010) Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. Clin Microbiol Rev 23:99–139

Hutchings MI, Hong HJ, Buttner MJ (2006) The vancomycin resistance VanRS two-component signal transduction system of *Streptomyces coelicolor*. Mol Microbiol 59:923–935

James RC, Pierce JG, Okano A, Xie J, Boger DL (2012) Redesign of glycopeptide antibiotics: back to the future. ACS Chem Biol 7:797–804

Jovetic S, Zhu Y, Marcone GL, Marinelli F, Tramper J (2010) β-lactam and glycopeptide antibiotics: first and last line of defense? Trends Biotechnol 28:596–604

Kahne D, Leimkuhler C, Lu W, Walsh C (2005) Glycopeptide and lipoglycopeptide antibiotics. Chem Rev 105:425–448

Kalan L, Perry J, Koteva K, Thaker M, Wright G (2013) Glycopeptide sulfation evades resistance. J Bacteriol 195:167–171

Kruse H, Johansen BK, Rørvik LM, Schaller G (1999) The use of avoparcin as a growth promoter and the occurrence of vancomycin-resistant enterococcus species in norwegian poultry and swine production. Microb Drug Resist 5:135–139

Labeda DP (1995) *Amycolatopsis coloradensis* sp. nov., the avoparcin (ll-av290)-producing strain. Int J Syst Bact 45:124–127

Lamb SS, Patel T, Koteva KP, Wright GD (2006) Biosynthesis of sulfated glycopeptide antibiotics by using the sulfotransferase *stal*. Chem Biol 13:171–181

Lancini G, Demain A (2006) Bacterial pharmaceutical products. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The Prokaryotes. Vol 1: Symbiotic assosiations, biotechnology, applied microbiology. Springer, Heidelberg

Leadbetter MR, Adams SM, Bazzini B, Fatheree PR, Karr DE, Krause KM, Lam BM, Linsell MS, Nodwell MB, Pace JL, Quast K, Shaw JP, Soriano E, Trapp SG, Villena JD, Wu TX, Christensen BG, Judice JK (2004) Hydrophobic vancomycin derivatives with improved ADME properties: discovery of telavancin (td-6424). J Antibiot (Tokyo) 57:326–336

Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V (2011) D-Ala-D-Ser VanN-type transferable vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother 55:4606–4612

Lessard IA, Walsh CT (1999) Mutational analysis of active-site residues of the enterococcal D-Ala-D-Ala dipeptidase VanX and comparison with *Escherichia coli* D-Ala-D-Ala ligase and D-Ala-D-Ala carboxypeptidase VanY. Chem Biol 6:177–187

Levine DP (2006) Vancomycin: a history. Clin Infect Dis 42(Suppl 1):S5–S12

Li TL, Huang F, Haydock SF, Mironenko T, Leadlay PF, Spencer JB (2004) Biosynthetic gene cluster of the glycopeptide antibiotic teicoplanin: characterization of two glycosyltransferases and the key acyltransferase. Chem Biol 11:107–119

Li TL, Liu YC, Lyu SY (2012) Combining biocatalysis and chemoselective chemistries for glycopeptide antibiotics modification. Curr Opin Chem Biol 16:170–178

Liu YC, Li YS, Lyu SY, Hsu LJ, Chen YH, Huang YT, Chan HC, Huang CJ, Chen GH, Chou CC, Tsai MD, Li TL (2011) Interception of teicoplanin oxidation intermediates yields new antimicrobial scaffolds. Nat Chem Biol 7:304–309

Lu K, Asano R, Davies J (2004) Antimicrobial resistance gene delivery in animal feeds. Emerg Infect Dis 10:679–683

Mackay JP, Gerhard U, Beauregard DA, Maplestone RA, Williams DH (1994a) Dissection of the contributions toward dimerization of glycopeptide antibiotics. J Am Chem Soc 116:4573–4580

Mackay JP, Gerhard U, Beauregard DA, Westwell MS, Searle MS, Williams DH (1994b) Glycopeptide antibiotic activity and the possible role of dimerization: a model for biological signaling. J Am Chem Soc 116:4581–4590

Malabarba A, Nicas TI, Thompson RC (1997) Structural modifications of glycopeptide antibiotics. Med Res Rev 17:69–137

Malabarba A, Ciabatti R (2001) Glycopeptide derivatives. Curr Med Chem 8:1759–1773

Malabarba A, Goldstein BP (2005) Origin, structure, and activity in vitro and in vivo of dalbavancin. J Antimicrob Chemother 55(Suppl 2):15–20

Marcone GL, Beltrametti F, Binda E, Carrano L, Foulston L, Hesketh A, Bibb M, Marinelli F (2010) Novel mechanism of glycopeptide resistance in the A40926 producer *Nonomuraea* sp. ATCC 39727. Antimicrob Agents Chemother 54:2465–2472

Marshall CG, Lessard IA, Park I, Wright GD (1998) Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. Antimicrob Agents Chemother 42:2215–2220

Matsuzaki K, Ikeda H, Ogino T, Matsumoto A, Woodruff HB, Tanaka H, Omura S (1994) Chloropeptins I and II, novel inhibitors against gp120-CD4 binding from *Streptomyces* sp. J Antibiot (Tokyo) 47:1173–1174

McCormick MH, McGuire JM, Pittenger GE, Pittenger RC, Stark WM (1955) Vancomycin, a new antibiotic. I. Chemical and biological properties. Antibiot Annu 3:606–611

Neu HC, Prince A, Neu CO, Garvey GJ (1977) Incidence of diarrhea and colitis associated with clindamycin therapy. J Infect Dis 135(Suppl):S120–S125

Nicolaou KC, Boddy CN, Bräse S, Winssinger N (1999) Chemistry, biology, and medicine of the glycopeptide antibiotics. Angew Chem Int Ed Engl 38:2096–2152

Nitanai Y, Kikuchi T, Kakoi K, Hanamaki S, Fujisawa I, Aoki K (2009) Crystal structures of the complexes between vancomycin and cell-wall precursor analogs. J Mol Biol 385:1422–1432

Novotna G, Hill C, Vincent K, Liu C, Hong HJ (2012) A novel membrane protein, VanJ, conferring resistance to teicoplanin. Antimicrob Agents Chemother 56:1784–1796

Pace JL, Yang G (2006) Glycopeptides: update on an old successful antibiotic class. Biochem Pharmacol 71:968–980

Pantosti A, Del Grosso M, Tagliabue S, Macrì A, Caprioli A (1999) Decrease of vancomycin-resistant enterococci in poultry meat after avoparcin ban. Lancet 354:741–742

Parenti F, Beretta G, Berti M, Arioli V (1978) Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. Sp. I. Description of the producer strain, fermentation studies and biological properties. J Antibiot 31:276–283

Parenti F, Schito GC, Courvalin P (2000) Teicoplanin chemistry and microbiology. J Chemother 12:5–14

Peacock JE, Marsik FJ, Wenzel RP (1980) Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. Ann Intern Med 93:526–532

Peetermans WE, Hoogeterp JJ, Hazekamp-van Dokkum AM, van den Broek P, Mattie H (1990) Antistaphylococcal activities of teicoplanin and vancomycin in vitro and in an experimental infection. Antimicrob Agents Chemother 34:1869–1874

Pelzer S, Süssmuth R, Heckmann D, Recktenwald J, Huber P, Jung G, Wohlleben W (1999) Identification and analysis of the balhimycin biosynthetic gene cluster and its use for manipulating glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM 5908. Antimicrob Agents Chemother 43:1565–1573

Pensack JM, Wang GT, Simkins L (1982) Avoparcin—a growth-promoting feed antibiotic for broiler chickens. Poult Sci 61:1009–1012

Périchon B, Courvalin P (2009) VanA-type vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53:4580–4587

Pootoolal J, Thomas MG, Marshall CG, Neu JM, Hubbard BK, Walsh CT, Wright GD (2002) Assembling the glycopeptide antibiotic scaffold: the biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL 15009. Proc Natl Acad Sci USA 99:8962–8967

Prince AS, Neu HC (1979) Antibiotic-associated pseudomembranous colitis in children. Pediatr Clin North Am 26:261–268

Puk O, Huber P, Bischoff D, Recktenwald J, Jung G, Süssmuth RD, van Pée KH, Wohlleben W, Pelzer S (2002) Glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM 5908: function of a halogenase and a haloperoxidase/perhydrolase. Chem Biol 9:225–235

Puk O, Bischoff D, Kittel C, Pelzer S, Weist S, Stegmann E, Süssmuth RD, Wohlleben W (2004) Biosynthesis of chloro-beta-hydroxytyrosine, a nonproteinogenic amino acid of the peptidic backbone of glycopeptide antibiotics. J Bacteriol 186:6093–6100

Retzlaff L, Distler J (1995) The regulator of streptomycin gene expression, StrR of *Streptomyces griseus* is a DNA binding activator protein with multiple recognition sites. Mol Microbiol 18:151–162

Reynolds PE, Courvalin P (2005) Vancomycin resistance in enterococci due to synthesis of precursors terminating in D-alanyl-D-serine. Antimicrob Agents Chemother 49:21–25

Rybak MJ, Bailey EM, Warbasse LH (1992) Absence of "Red man syndrome" in patients being treated with vancomycin or high-dose teicoplanin. Antimicrob Agents Chemother 36:1204–1207

Saravolatz LD, Markowitz N, Arking L, Pohlod D, Fisher E (1982a) Methicillin-resistant *Staphylococcus aureus*. Epidemiologic observations during a community-acquired outbreak. Ann Intern Med 96:11–16

Saravolatz LD, Pohlod DJ, Arking LM (1982b) Community-acquired methicillin-resistant *Staphylococcus aureus* infections: a new source for nosocomial outbreaks. Ann Intern Med 97:325–329

Schäberle TF, Vollmer W, Frasch HJ, Hüttel S, Kulik A, Röttgen M, von Thaler AK, Wohlleben W, Stegmann E (2011) Self-resistance and cell wall composition in the glycopeptide producer *Amycolatopsis balhimycina*. Antimicrob Agents Chemother 55:4283–4289

Schäfer M, Schneider TR, Sheldrick GM (1996) Crystal structure of vancomycin. Structure 4:1509–1515

Sosio M, Stinchi S, Beltrametti F, Lazzarini A, Donadio S (2003) The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by *Nonomuraea* species. Chem Biol 10:541–549

Sosio M, Kloosterman H, Bianchi A, de Vreugd P, Dijkhuizen L, Donadio S (2004) Organization of the teicoplanin gene cluster in *Actinoplanes teichomyceticus*. Microbiology 150:95–102

Stegmann E, Frasch HJ, Wohlleben W (2010) Glycopeptide biosynthesis in the context of basic cellular functions. Curr Opin Microbiol 13:595–602

Sujatha S, Praharaj I (2012) Glycopeptide resistance in Gram-positive cocci: a review. Interdiscip Perspect Infect Dis 2012:781679

Süssmuth RD, Wohlleben W (2004) The biosynthesis of glycopeptide antibiotics -a model for complex, non-ribosomally synthesized, peptidic secondary metabolites. Appl Microbiol Biotechnol 63:344–350

Taurino C, Frattini L, Marcone GL, Gastaldo L, Marinelli F (2011) *Actinoplanes teichomyceticus* ATCC 31121 as a cell factory for producing teicoplanin. Microb Cell Fact 10:82

Thaker MN, Wright GD (2012) Opportunities for synthetic biology in antibiotics: expanding glycopeptide chemical diversity. ACS Synth Biol doi:10.1021/sb300092n

Uttley AH, George RC, Naidoo J, Woodford N, Johnson AP, Collins CH, Morrison D, Gilfillan AJ, Fitch LE, Heptonstall J (1989) High-level vancomycin-resistant enterococci causing hospital infections. Epidemiol Infect 103:173–181

Van Bambeke F (2006) Glycopeptides and glycodepsipeptides in clinical development: a comparative review of their antibacterial spectrum, pharmacokinetics and clinical efficacy. Curr Opin Investig Drugs 7:740–749

Van Hal SJ, Paterson DL (2011) New Gram-positive antibiotics: better than vancomycin? Curr Opin Infect Dis 24:515–520

Van Wageningen AM, Kirkpatrick PN, Williams DH, Harris BR, Kershaw JK, Lennard NJ, Jones M, Jones SJ, Solenberg PJ (1998) Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. Chem Biol 5:155–162

Vollmer W, Blanot D, de Pedro MA (2008) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32:149–167

Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC (2003) Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science 302:1569–1571

Williams DH, Searle MS, Mackay JP, Gerhard U, Maplestone RA (1993) Toward an estimation of binding constants in aqueous solution: studies of associations of vancomycin group antibiotics. Proc Natl Acad Sci USA 90:1172–1178

Wohlleben W, Stegmann E, Süssmuth RD (2009) Chapter 18. Molecular genetic approaches to analyze glycopeptide biosynthesis. Methods Enzymol 458:459–486

Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M (2010) *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob Agents Chemother 54:4643–4647

Zhanel GG, Calic D, Schweizer F, Zelenitsky S, Adam H, Lagacé-Wiens PR, Rubinstein E, Gin AS, Hoban DJ, Karlowsky JA (2010) New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. Drugs 70:859–886

Chapter 6
Daptomycin and Related Lipopeptides Produced by Fermentation, Chemical Modification, and Combinatorial Biosynthesis

Richard H. Baltz

Abstract Among the structurally related ten-membered ring acidic cyclic lipopeptide antibiotics, daptomycin was the first to gain FDA approval in the USA. Daptomycin and related lipopeptides require Ca^{2+} for activity, and Ca^{2+}-bound daptomycin acts as a cationic peptide and interacts with the negatively charged phosphotidylglycerol (PG) in the cytoplasmic membrane to trigger its antibacterial effects. Mutants of *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium,* and *Bacillus subtilis*, which display incremental increases in resistance to daptomycin, have mutations in genes that cause reductions in the negative charge on the membrane or thickening of cell walls. Daptomycin was approved to treat skin and skin structure infections caused by Gram-positive pathogens, and bacteremia including right-sided endocarditis caused by *S. aureus*, but has not been approved to treat *Streptococcus pneumoniae* pneumonia. Many derivatives of A21978C (containing the core peptide of daptomycin) and A54145 have been generated by chemical modification or by combinatorial biosynthesis to identify antibiotics superior to daptomycin for the treatment of community-acquired pneumonia (CAP). Several compounds had antibacterial activity superior to daptomycin, but were not as active as vancomycin in a mouse model for pneumonia. Lipopeptide CB-813,315, however, was more active than daptomycin and vancomycin against *Clostridium difficile* in vitro, and is currently undergoing clinical trials to treat *C. difficile*-associated diarrhea (CDAD).

6.1 Introduction

A number of acidic, cyclic lipopeptide antibiotics containing ten-membered rings and exocyclic amino acid tails coupled to long chain fatty acids have been discovered by screening fermentation broths from actinomycetes (Baltz et al. 2005;

R. H. Baltz (✉)
CognoGen Biotechnology Consulting, 7636 Andora Drive, Sarasota, FL 34238, USA
e-mail: rbaltz923@gmail.com

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_6,

Baltz 2008). Among these are two cyclic depsipeptide complexes, A21978C (Debono et al. 1987) and A54145 (Boeck et al. 1990), discovered by Eli Lilly and Company (Lilly). Other related cyclic peptides are friulimicin and amphomycin. Friulimicin (Schneider et al. 2009; Vértesy et al. 2000; Wecke et al. 2009) and an amphomycin derivative, MX-2401 (Dugourd et al. 2011; Rubinchik et al. 2011), have undergone preclinical studies, but neither has progressed to late stage clinical development. Calcium-dependent antibiotic (CDA), which is produced by *Streptomyces coelicolor* (Hojati et al. 2002) and *Streptomyces lividans* (Penn et al. 2006), is a related cyclic depsipeptide that has undergone extensive biosynthetic studies (Baltz et al. 2005; Baltz 2008, 2010a, b, c; Strieker and Marahiel 2009). Daptomycin is semi-synthetic derivative of A21978C discovered at Lilly and developed by Cubist Pharmaceuticals (Cubist) for treatment of complicated skin and skin structure infections caused by Gram-positive pathogens (Arbeit et al. 2004) and bacteremia including right-sided endocarditis caused by *Staphylococcus aureus* strains, including those resistant to methicillin (MRSA) (Fowler et al. 2006, see Rossolini et al., this volume). In this chapter, I discuss the: (i) discovery and development of daptomycin; (ii) current understanding of the mechanisms of action (MOA), and resistance (MOR) to daptomycin; (iii) current approaches to discover and develop improved lipopeptide antibiotics by medicinal chemistry and combinatorial biosynthesis; (iv) development of a second-generation lipopeptide for treatment of *Clostridium difficile*-associated diarrhea (CDAD); and (v) future prospects for expanding combinatorial biosynthesis and coupling with medicinal chemistry to generate lipopeptide antibiotics with superior properties.

6.2 Discovery of Lipopeptide Antibiotics at Lilly

Lilly discovered the cyclic lipopeptide antibiotic complexes A21978C (Debono et al. 1987) and A54145 (Boeck et al. 1990) by screening fermentation broths for antibiotic activities. Initially, there was little interest in developing these molecules because of the successes achieved with the structurally simpler cephalosporin antibiotics, and the relatively small market for their other peptide antibiotic, vancomycin, in the pre-MRSA era (Baltz 2005; Kirst et al. 1998). However, Lilly's interest in lipopeptides and glycopeptides increased in the late 1980s as MRSA infections became more prevalent (see Rossolini et al., Marcone and Marinelli, this volume).

6.2.1 Fermentation Products of Streptomyces roseosporus and Streptomyces fradiae

A21978C, which contains the tridecapeptide that served as the scaffold to develop daptomycin (Debono et al. 1988), is produced in fermentation by *Streptomyces roseosporus*. A54145, which has a cyclic lipopeptide structure similar to A21978C

(Fig. 6.1), is produced by *Streptomyces fradiae*. A21978C is comprized of a mixture of lipopeptides containing an invariant tridecapeptide coupled to different long chain fatty acids, primarily *anteiso*-undecanoic acid, *iso*-dodecanoic acid, and *anteisio*-tridecanoic acid (Fig. 6.1). The A54145 fermentation is more complex: four different tridecapeptides are coupled to *n*-decanoyl, *iso*-decanoyl, or *anteiso*-undecanoyl side chains. The four peptides contain combinations of $3mGlu_{12}$ or Glu_{12} and Ile_{13} or Val_{13}. The fermentation can be simplified by feeding Ile to enrich for two compounds containing Ile_{13}, $3mGlu_{12}$ or Glu_{12}, and an *anteiso*-undecanoyl side chain (Boeck and Wetzel 1990). Molecular genetic approaches at Cubist further focused the fermentation into a single product by disrupting the *lptI* gene encoding a methyltransferase involved in the formation of $3mGlu_{12}$ (Alexander et al. 2010).

6.2.2 *Chemical Substitutions for the Lipid Side Chains of A21978C and A54145, and Discovery of Daptomycin*

The A21978C (Debono et al. 1987) and A54145 (Boeck and Wetzel 1990; Boeck et al.1990; Counter et al. 1990; Fukuda et al. 1990a, b) lipopeptide factors were isolated and characterized for biological activities. The fatty acids were removed

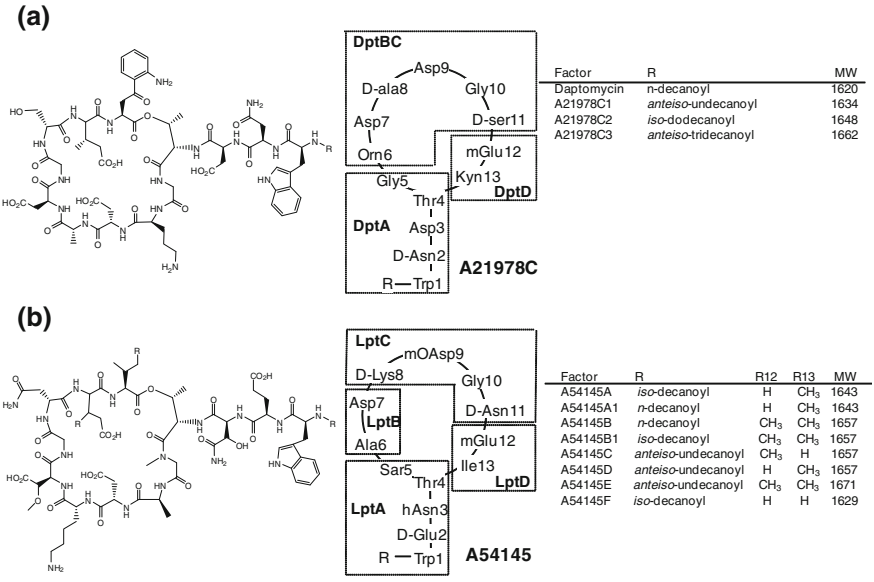


Fig. 6.1 Structures of lipopeptide antibiotics and NRPS subunit content. **a** A21978C major factors and daptomycin produced by *S. roseosporus*. **b** A54145 major factors produced by *S. fradiae*. Note that H or CH_3 indicate the presence of Glu or 3mGlu; H or CH_3 at R13 indicate the presence of Val or Ile (The figure is reproduced from Baltz et al. 2010b)

by incubation with *Actinoplanes utahensis*, which secreted a deacylase enzyme (Boeck et al. 1988; Fukuda et al. 1990b). The deacylase gene was later cloned and expressed in *S. lividans* to produce high levels of the secreted protein (Kreuzman et al. 2000). Removal of the lipid side chains enabled chemical modifications at the *N*-terminal Trp to produce acyl and aryl derivatives of A21978C (Debono et al. 1988) and A54145 (Fukuda et al. 1990b). Limited structure–activity studies indicated that the *N*-decanoyl derivative of the A21978C core peptide (daptomycin) showed good in vivo efficacy against Gram-positive pathogens and low toxicity in mice (Debono et al. 1988). Some of the A54145 derivatives containing $3mGlu_{12}$ were as active as daptomycin in vitro, but were toxic in mice (Counter et al. 1990). Daptomycin was pursued as a clinical candidate, and work on A54145 derivatives was discontinued.

6.3 Clinical Development of Daptomycin

6.3.1 Clinical Studies at Lilly

Daptomycin was pursued in clinical trials sponsored by Lilly to address complicated skin and skin structure infections, and bacteremia including endocarditis caused by Gram-positive pathogens. In dose escalation studies aimed at achieving appropriate tissue concentrations to treat endocarditis, Lilly encountered muscle toxicity in a small number of patients, and discontinued the clinical development of daptomycin in the early 1990s (Baltz 2010a; Eisenstein et al. 2010).

6.3.2 Clinical Studies and Launch of Daptomycin by Cubist

Cubist developed an interest in daptomycin, and licenced it from Lilly in 1997 (Baltz 2010a; Eisenstein et al. 2010). Based upon studies in dogs, Cubist scientists predicted that the muscle toxicity encountered in the Lilly clinical trials might have been due to twice-per-day dosing, and that once-per-day dosing might mitigate the muscle toxicity (Eisenstein et al. 2010; Oleson et al. 2000). Cubist carried out clinical trials with once-per-day dosing in humans, and obtained successful outcomes in the treatment of complicated skin and skin structure infections (Arbeit et al. 2004), and in treatment of bacteremia and right-sided endocarditis caused by *S. aureus* (Fowler et al. 2006). Cubicin (daptomycin for injection) was approved by the FDA for these indications in 2003 and 2006, respectively.

6.4 Mechanism of Resistance to Daptomycin

Resistance mechanisms employed by antibiotic-producing organisms can be similar to those encountered in clinical isolates, and understanding resistance mechanisms can help identify the mechanisms of action (MOA). So far, there is no correlation between a resistance mechanism in *S. roseosporus* and the incremental resistances observed in Gram-positive pathogens. However, the mechanism of resistances (MORs) observed in clinical isolates have shed light on the MOA(s) of daptomycin.

6.4.1 Daptomycin Resistance in S. roseosporus

S. roseosporus produces over 1 g/l of daptomycin during fermentation (Huber et al. 1988). If *S. roseosporus* has an antibacterial target(s) for daptomycin, then it needs to have a mechanism for self-resistance. *Streptomyces ambofaciens* BES2074 (Richardson et al. 1990) is very sensitive to daptomycin (Alexander et al. 2004), and is amenable to genetic manipulation (Baltz 2010c). Alexander et al. (2004) demonstrated that a single gene from the daptomycin gene cluster, *dptP*, was sufficient to confer daptomycin resistance in *S. ambofaciens*. The *dptP* gene has homologs in the A54145 gene cluster (Miao et al. 2006a), the cryptic daptomycin-like cluster in *Saccharomonospora viridis* (Baltz 2010b), and an uncharacterized NRPS cluster in *Streptomyces ghanaensis* (Baltz, unpublished). All four of the DptP-like proteins have four membrane spanning helices (Baltz, unpublished). In all four cases, the *dptP-like* genes are adjacent to *dptM-* and *dptN-like* genes that encode ABC transporters. Thus, DptP might play a role in export of lipopeptides in conjunction with DptM and DptN (Baltz 2008). If so, DptP expressed in *S. ambofaciens* might interact with another ABC transporter to export or block entry of daptomycin. Importantly, BLASTP searches have revealed no DptP homologs encoded by *S. aureus*, *S. pneumoniae*, *E. faecalis*, *E. faecium*, or *C. difficile*, so it is unlikely that this mechanism of resistance will be encountered in low G+C Gram-positive pathogens.

6.4.2 Resistance in Other Actinomycetes

Nearly 100 % of 480 randomly isolated soil streptomycetes were resistant to 20 μg/ml daptomycin (D'Costa et al. 2006). 80 % of the 80 strains tested inactivated daptomycin during 48 h of growth. More recently, D'Costa et al. (2012) studied 60 of the daptomycin-inactivating strains in more detail, and demonstrated that all have daptomycin MICs > 256 μg/ml. 44 % of the strains inactivated daptomycin by cleavage of the ester bond between Kyn_{13} and Thr_4, and 29 %

deacylated the lipid tail. 79 % of the strains displayed secondary degradation by peptide cleavage, and 9 % showed no product of daptomycin degradation, presumably due to extensive peptide bond cleavage. Other actinomycetes, including *Nocardia* sp. and fast growing *Mycobacterium* sp., show high-level resistance to daptomycin (Bastian et al. 2010; Lai et al. 2009). Thus, actinomycetes have a propensity to destroy daptomycin activity by deacylation of the fatty acid or cleavage of ester and peptide bonds in the tridecapeptide.

6.4.3 Resistance in Gram-positive Bacteria with Low G+C Content

Unlike what has been observed in the high G+C Gram-positive actinomycetes, no high-level resistance to daptomycin has been observed within the low G+C Gram-positive bacteria (see Rossolini et al., this volume). Futhermore, the DapR phenotypes are not associated with transport or hydrolysis of acyl, ester, or peptide bonds. Instead, DapR development is incremental, and caused by spontaneous mutations in genes that influence membrane charge or cell wall thickness.

6.4.3.1 Resistance in *S. aureus*

The MICs for *S. aureus* strains susceptible to daptomycin (DapS) range from about 0.25 to 1.0, and strains with MICs > 1.0 are considered to be non-susceptible (Moise et al. 2009). For the purposes of this chapter, I refer to daptomycin non-susceptible as DapR. The incidence of DapR in clinical isolates of *S. aureus* is <1 per 1000 (Moise et al. 2009), and the DapR strains tend to be isolated from patients with deep-seated infections associated high numbers of infecting organisms (e.g., endocarditis). To understand how *S. aureus* becomes resistant to daptomycin, Friedman et al. (2006) passaged *S. aureus* MW2 (starting MIC = 0.5 μg/ml) in laboratory media containing increasing levels of daptomycin, then analyzed the mutations by NimbleGen Technology. Three DapR strains had *mprF* [lysyl-phosphatidylglycerol (LPG) synthase] mutations by six days, and these were coupled with mutations *rpoB*, *rpoC,* or *yycG*. RpoB and RpoC comprise major subunits of RNA polymerase, and YycG is a sensor histidine kinase the couples with the YycF response regulator in signal transduction. The final genotypes and order of mutation acquisition were: *mprF rpoC*; *mprF rpoB yycG*; and *mprF yycG rpoB rpoC*. Each mutation accounted for about a 2- to 4-fold increase in MIC, and the strain with all four mutations had an MIC of 10 μg/ml. The strain carrying the *mprF rpoB yycG* mutations was later shown to have increased transcription of *mprF* and the *dlt* operon, increased conversion of PG to LPG, increased cell wall thickness, and increased resistance to vancomycin (Mishra et al. 2009). Friedman et al. (2006) also examined three isogenic pairs of clinical MRSA isolates for

mutations in these genes. Two of the strains had mutations in *mprF*, and the other had mutations in *mprF* and *yycG*. Shortly after the Friedman publication, *mprF* mutations were documented in other clinical isolates of *S. aureus* expressing incremental increases in MICs for daptomycin (Julian et al. 2007; Murthy et al. 2008). More recently, Patel et al. (2011) used a gradient plate selection for DapR starting with three different clinical strains and a laboratory strain. They sequenced the *mprF* and *yycG* genes from five mutants, and two had *mprF* mutations, one had a *yycG* mutation, two had *mprF* plus *yycG* mutations. The three strains with *yycG* mutations had thickened cell walls, reduced autolysis, and increased expression of the *vraSR* two-component signal transduction system.

Sakoulas et al. (2008) described an isogenic pair of methicillin-susceptible *S. aureus* (MSSA) strains isolated before and after daptomycin treatment failure from a patient with endocarditis. The DapR strain had a single nucleotide substitution in the *mprF* gene causing a serine to leucine amino acid substitution at position 295 (S295L) in the MprF protein (Yang et al. 2009). This mutation is identical to one of the mutations identified in an MRSA clinical isolate (Friedman et al. 2006). Mishra et al. (2011) also noted that DapR *S. aureus* clinical isolates often contain *mprF* mutations, and these were sometimes coupled with *yycG* mutations. The DapR strains were generally associated with increased membrane fluidity and reduced susceptibility to host defense cationic peptides (HDPs), and sometimes coupled with thickened cell walls.

Another study compared six pairs of *S. aureus* strains collected before and after daptomycin treatment, and five of the DapR strains had mutations in *mprF*. Deletion and complimentation studies confirmed that *mprF* mutations were associated with part of the decreased susceptibility to daptomycin (Mehta et al. 2012). Upregulation of the *vraSR* two-component signal transduction system was also involved in decreased susceptibility to daptomycin, and was associated with a thickened cell wall. In a whole genome sequencing study, 12 DapR clinical isolates derived from nine DapS starting strains were sequenced along with their isogenic partners. Nine additional DapR strains derived from three DapS strains were selected in the laboratory and sequenced (Peleg et al. 2012). On average, the clinical DapR mutants had six mutations in structural genes. Ten of the strains had *mprF* mutations, and three of those also had mutations in *cls2*, which encodes cardiolipin (CL) synthase. Three had *rpoB* mutations and one had *yycG* mutation. Camargo et al. (2008) carried out serial selection for increased daptomycin resistance and recovered an *S. aureus* strain with an MIC of 3 versus 0.5 μg/ml of the starting strain. Notably, the DapR strain had a thickened cell wall, reduced negative charge, and up-regulated *vraSR*. Subsequent whole genome sequencing identified an *rpoB* mutation that accounted for the cell wall thickening (Cui et al. 2010). The reduction of the negative cell surface charge was associated with increased transcription of the *dlt* operon, presumably giving rise to increased D-alanylation of cell wall teichoic acid (WTA), which results in reduction of negative charge. A DapR phenotype has also been correlated with increased expression of the *tag* and *dlt* operons leading to increased production of WTA and increased D-ananylation of WTA (Bertsche et al. 2011; Fischer et al. 2011). The

DapR strain also had elevated transcription of *vraRS* and certain cell wall biosynthetic genes, but the chromosomal mutation(s) leading to this phenotype were not reported.

Staphylococcus aureus can become incrementally resistant to vancomycin after exposure to vancomycin in the clinic. Two types of resistance have been described as vancomycin-intermediate *S. aureus* (VISA) or hetero-VISA (hVISA) (see Rossolini et al., Marcone and Marinelli, this volume). A common feature of VISA strains is a thickened cell wall relative to parental strains (Reipert et al. 2003). VISA strains often show cross-resistance to daptomycin (Kosowska-Shick et al. 2009), and the the level of DapR correlates with the level of cell wall thickness (Cui et al. 2006). Jansen et al. (2007) studied a clinical VISA strain and noted that it was upregulated for the expression of the *yycFGHI* operon; upregulation was caused by the insertion of IS*256* in the *yycFG* promoter region. Howden et al. (2011) sequenced five clinical pairs of VSSA and VISA strains and observed *yycG* or *yycF* mutations in four VISA strains and an *rpoB* mutation in the other strain, all from patients treated with vancomycin but not daptomycin. All five strains had thickened cell walls, vancomycin MICs of 4 μg/ml, and daptomycin MICs 0.5–2 μg/ml, the latter corresponding to 2- to 6-fold elevations in MICs relative to the starting VSSA strains.

Mutations in *rpoB* have been observed in VISA strains in other studies, and they include those within the rifampin resistance determining region (RRDR) and outside of the RRDR (Watanabe et al. 2011). Mutations within the RRDR were associated with a RifR phenotype, and most appeared to be associated with the VISA phenotype. The MICs for daptomycin were not reported, so it remains to be seen what fraction of the *rpoB* mutations give cross-resistance to daptomycin.

From the forgoing extensive studies, it is now clear that *mprF* mutations are the most common of the DapR mutations in *S. aureus*, followed by *yycG* and *rpoB* mutations. Their mechanisms will be discussed in Sects. 6.4.3.4–6.4.3.6.

6.4.3.2 Resistance in *Enterococci*

Enterococcus faecalis and *Enterococcus faecium* are susceptible to daptomycin at MIC_{90} values of 2–4 μg/ml, respectively (Steenbergen et al. 2005). Daptomycin is not approved for the treatment of enterococci, but it is often used to treat severe enterococcal infections (Arias et al. 2011, see Rossolini et al., this volume). The spontaneous mutation to DapR at 2 times the MIC in *E. faecalis* and *E. faecium* is very low ($<10^{-10}$), but mutants with incremental increases in MIC can be obtained after serial passage in increasing concentrations of daptomycin (Arias et al. 2011; Palmer et al. 2011).

Palmer et al. (2011) used whole genome resequencing to follow the development of DapR in a multi-drug-resistant *E. faecalis* strain during serial passaging. They observed no mutations in *mprF*, *yycG*, *rpoB* of *rpoC*, but observed mutations in *cls* (CL synthase) arising early in the development of each of three lines of selection. Two of the mutations led to single amino acid substitutions, and the

other led to an in frame deletion of three amino acids, suggesting that the CL synthase enzymes have enhanced function. Mutations in gene EF1797 encoding a membrane spanning protein of unknown function were also observed in each of the strains, and the nature of the mutations (IS*256* insertion, 75 bp deletion, and one bp deletion leading to a reading frame-shift) suggest loss-of-function. Mutations in a small number of other genes were noted, including a base-pair substitution in *rpoN*, which encodes the transcription factor σ^{54}. They also surveyed six clinical isolates of *E. faecalis* with daptomycin MICs of 8–32 μg/ml for *cls* mutations; one had a base-pair substitution and the other had the same 9 bp deletion observed in the serial passage experiment (Palmer et al. 2011). In addition, they sequenced the *cls* gene from two isogenic pairs of DapS and DapR *E. faecium* strains isolated before and after daptomycin treatment of two patients, and each had single bp substitutions leading to amino acid substitutions in Cls. They also expressed the mutant alleles of the *cls* gene *in-trans* in the DapS strain, and showed that they conferred a DapR phenotype. This supports the interpretation that these mutations confer gain-of-function, increasing the conversion of PG to CL by CL synthase. It appears that conversion of PG to CL by is a critical step in the progression to DapR in *E. faecalis* and *E. faecium.*

Arias et al. (2011) used whole genome sequencing to study an isogenic pair of strains isolated before and after daptomycin treatment of a patient with fatal *E. faecalis* bacteremia. The initial strain had an MIC of 1 μg/ml, and the final strain, which had a thickened cell wall, had an MIC of 12 μg/ml. They observed mutations in *cls*, *liaF*, and *gdpD*. Gene replacement studies confirmed the contributions of *liaF* and *gdpD* mutations to the increased MIC to daptomycin, but they did not report on gene replacement studies with the mutated *cls* gene. They noted that the *liaF* mutation probably results in loss-of-function, leading to increased transcription of the *liaRS* two-component signal transduction system. They surveyed eight other isogenic pairs of DapS and DapR enterococci, and found combinations of *liaF*, *liaR* or *liaS,* and *cls* mutations in three; *cls* alone in two; and *liaF*, *liaR,* or *liaS* in one each, and no *liaFRS* or *cls* mutations in a single strain. It is noteworthy that the *liaRS* signal transduction system in enterococci is orthologous to the *vraRS* system in *S. aureus*.

6.4.3.3 Resistance in *B. subtilis*

In *B subtilis*, exposure to daptomycin strongly induces the LiaRS signal transduction system and genes regulated by the extracytoplasmic function (ECF) σ factors, σ^{W}, and σ^{M}, all of which participate in the cell envelope stress response triggered by cell wall active antibiotics (Hachmann et al. 2009). Deletion of the *liaIHGFRS* operon reduced the daptomycin MIC from 1 to 0.3 μg/ml. Deletion of *mprF* reduced the daptomycin MIC to 0.5 μg/ml, whereas reduced expression of *pgsA*, which encodes PG synthase, increased the MIC to 8.0 μg/ml. Serial passage of *B. subtilis* in the presence of increasing daptomycin concentrations enriched for a mutant (MIC ~ 20 μg/ml) that contained *pgsA*, *mreB,* and *relA* mutations

(Hachmann et al. 2011). Gene replacement experiments indicated that the *pgsA* mutation, which caused about a 5-fold reduction in PG in the membrane, accounted for the increase in MIC; the other mutations were likely compensatory, providing viability to the strain carrying an otherwise greatly debilitating or lethal *pgsA* mutation.

6.4.3.4 The Role of *mprF*, *cls2*, and *pgsA* Mutations in Resistance

Two common themes for partial resistance to daptomycin have emerged: (i) thickening of the cell wall; and (ii) reducing the overall negative charge on the membrane (Yang et al. 2009, 2010). It is noteworthy that vancomycin-intermediate *S. aureus* (VISA) strains also have thickened cell walls as a common theme (Reipert et al. 2003; Cui et al. 2006). The *mprF*, *cls2,* and *pgsA* genes encode membrane spanning enzymes involved in the formation of membrane phospholipids. PgsA (PG synthase) catalyzes the initial step in phospholipid biosynthesis by converting CDP-diacylglycerol (CD-DG) into PG. PG is converted to cardiolipin (CL) by CL synthase (Cls2 or Cls), and to LPG by LPG synthase (MprF). In *S. aureus*, the major phospholipids contain PG (~65 %), LPG (~20 %), CL (~10 %), and all others (~5 %) (Mukhopadhyay et al. 2007). Furthermore, each of the three major phospholipids is normally distributed asymmetrically between the inner and outer membrane leaflets. The ratios of inner to outer membrane localization are: PG (~1:3); LPG (~5:1); and CL (~1:4). Importantly, PG, CL and LPG have net charges of −2, −1, and +1, respectively, so their distribution in the membrane influences the net surface charge encountered by the cationic Ca^{2+}-bound daptomycin.

The *mprF* gene was initially identified by a transposition mutation that caused *S. aureus* to become more susceptible to cationic peptides (Peschel et al. 2001). Hence the gene was named *mprF* for multiple peptide resistance factor. In addition to its LPG synthase activity, MprF also has flippase activity that translocates LPG from the inner membrane to the outer membrane leaflet (Ernst and Peschel 2011). Membranes lacking LPG (in strains deleted for *mprF*) are more acidic than those containing PG and LPG, and have stronger electrostatic interactions with cationic peptides. They are also more susceptible to daptomycin, and have MICs about 4-fold lower than wild type *S. aureus* (Jones et al. 2008; Rubio et al. 2012). Therefore, *mprF* mutations expressing increased MICs to daptomycin must express gain-of-function(s) that could act by increasing the overall LPG content or by "flipping" more LPG to the membrane outer leaflet. Some DapR strains with *mprF* mutations have membranes with increased ratios of LPG/PG and some do not (Jones et al. 2008; Rubio et al. 2012; Yang et al. 2010). Mutants with *mprF* mutations in the LPG synthase domain have increased ratios of LPG/PG, whereas those with a mutation in the flippase domain yielding S295L amino acid substitutions have a normal LPG/PG ratio, but a 2-fold increased proportion of LPG in the outer membrane leaflet (Jones et al. 2008). Antisense RNA expression studies with wild type and DapR mutants containing *mprF* mutations in either LPG

synthase or flippase domains also support the notion that each function by gain of overall charge on the outer membrane leaflet: (i) by simply increasing in overall LPG in synthase mutants and maintaining normal distribution between the inner and outer membrane leaflets, or (ii) by increasing the translocation of LPG into the outer leaflet of the membrane bilayer in flippase mutants. Expression of *mprF* antisense RNA caused a 4-fold reduction in MIC for wild type and each of the *mprF* synthase and flippase mutants (Rubio et al. 2011). Furthermore, expression of *mprF* antisense RNA caused a dramatic increase in the binding of the cationic protein cytochrome C, indicating that gain-of-function mutations in LPG synthase or flippase cause a reduction in negative charge on the membrane outer leaflet. These results are also consistent with the mode of action of daptomycin where Ca^{2+}-bound daptomycin acts as a cationic peptide that interacts with membranes enriched for PG (see Sect. 6.5.1). The reduced negative charge imparted by LPG apparently interferes with daptomycin penetration by reducing the electrostatic interaction.

The mutations in *cls2* (*cls*) observed in *S. aureus* and enterococci may result in gain-of-function. Increased conversion of PG to CL would cause a reduction in PG, the target for daptomycin insertion into the membrane, and a reduction in the negative charge on the membrane. The *pgsA* mutation in *B. subtilis* caused a substantial reduction in PG, thus reducing the negative charge on the membrane and interfering with daptomycin binding to the cells (Hackmann et al. 2011).

Studies on DapR mutants have not demonstrated a causal relationship between *mprF* mutations and thickened cell wall. However, there are examples of *mprF* synthase and flippase mutants that do not have thickened cell walls relative to parental strains (Yang et al. 2010). For example, *S. aureus* CB184, which has a mutation in the *mprF* flippase domain (S295L), but no mutations in *yycG*, *rpoB,* or *rpoC* (Friedman et al. 2006), has the same cell wall thickness as its isogenic parent strain (Yang et al. 2010). Likewise, *S. aureus* REF2145, which has an *mprF* mutation in the synthase domain (T345A) and increased LPG synthase activity leading to increased LPG/PG ratio, has the same cell wall thickness as its parent strain (Yang et al. 2010). These studies suggest that the primary MORs imparted by *mprF* mutants are mediated by increased conversion of PG to LPG or increased flippage of LPG to the membrane outer leaflet, and these mechanisms do not cause thickened cell walls.

6.4.3.5 The Roles of RpoB, RpoC, and LiaRS (VraRS) in Resistance

The *rpoB* and *rpoC* genes encode the β and β' subunits of RNA polymerase, a multienzyme complex that interacts with many different sigma factors to modulate transcription under different environmental conditions. In one example, an *rpoB* mutation was associated with upregulation the *vraRS*, upregulation of the *dlt* operon, upregulation of several genes involved in cell envelope biosynthesis, decreased negative surface charge and thickened cell envelope (Camargo et al. 2008; Cui et al. 2010). Upregulation of *vraSR* in strains with *yycG* mutations is

also associated with thickened cell walls (see Sect. 6.4.3.6). The thickened cell wall and reduced negative charge in the *rpoB* mutant undoubtedly interfere with the penetration of daptomycin into the membrane. It remains to be seen if these are general effects of *rpoB* and *rpoC* mutations.

6.4.3.6 The Role of YycG in Resistance

YycG is a membrane spanning sensor/histidine kinase that partners with the YycF response regulator as the sole two-component system required for viability in *S. aureus* and other low G+C Gram-positive bacteria. YycFG functions as a master regulatory system for cell wall metabolism and biofilm formation, and is also referred to as VicRK and WalRK (Dubrac et al. 2007, 2008; Winkler and Hock 2008). Enhanced expression of YycFG in *S. aureus* caused increased peptidoglycan biosynthesis and turnover, and increased biofilm formation; depletion of YycFG caused cell death without lysis (Dubrac et al. 2007). YycG is localized to the cell division septum and in the helical region along the long axis associated with peptidoglycan biosynthesis in *B. subtilis,* where it co-ordinates cell wall remodeling with cell division as a positive regulator of the expression of autolysins and other enzymes involved in cell wall restructuring, and a repressor of expression of inhibitors of these functions (Fukushima et al. 2008, 2012). YycG is co-localized with FtsZ, and precipitates with FtsZ by treatment with anti-FtsZ antibody (Fukushima et al. 2008). Dubrac et al. (2008) have proposed that YycFG plays a critical role in the divisome at the nascent cell wall septum, and that the extracytoplasmic YycG sensing loop might physically interact with extracytoplasmic Lipid II to monitor the cell division process. In non-dividing *B. subtilis* cells, YycG interacts with YycH and YycI to block kinase activity (Szurmant et al. 2007, 2008; Fukushima et al. 2011). During active cell division, YycG no longer binds to YycH and YycI, but interacts with Pbp2B and proteins that are part of the divisome scaffold at the cell division septum (DivIB, FtsL, and possibly FtsW; Fukushima et al. 2011). YycG misregulation (i.e., no kinase activity) was observed only when both DivIB and FtsL proteins were missing, suggesting that YycG also senses the integrity of the divisome. Thus, mutations in YycG giving enhanced DapR phenotype in *S. aureus* cause gain-of-function or modification of function leading to thickened cell walls. It is also conceivable that some of the *yycG* mutations yielding a DapR phenotype might interfere directly with Ca^{2+}-bound daptomycin oligomers at the divisome (see Sect. 6.5).

6.5 Mechanisms of Action of Daptomycin

Early studies on the mechanism(s) of action reported that daptomycin inhibited the biosynthesis of cell wall, protein, DNA, RNA, and lipoteichoic acid, and that it caused dissipation of membrane potential (for reviews, see Baltz 2009a, b;

Baltz et al. 2005). More recent studies have led to the interpretation that these diverse effects are initiated by the disruption of membrane function (Jung et al. 2008; Muraih et al. 2011, 2012; Scott et al. 2007; Silverman et al. 2003; Straus and Hancock 2006).

6.5.1 Daptomycin Interactions with Membranes

The antibacterial activity of daptomycin requires Ca^{2+}, which facilitates its penetration into bacterial membranes (Baltz 2009a, b; Baltz et al. 2005). Daptomycin is anionic, with one basic and four acidic amino acids, but Ca^{2+}-bound daptomycin acts as a cationic peptide, and binds acidic phospholipids by an electrostatic interaction (Jung et al. 2008; Straus and Hancock 2006). Ca^{2+} neutralizes the acidic charge of PG, and bridges the acidic charges of daptomycin (Jung et al. 2008). Ca^{2+}-bound daptomycin forms oligomers in solution at millimolar concentrations (Ball et al. 2004; Jung et al. 2004; Rotondi and Gierasch 2005), and two models on daptomycin MOA are based upon the formation of oligomers. Silverman et al. (2003) proposed that Ca^{2+}-bound daptomycin inserts into the bacterial membrane, oligomerizes and forms pores, disrupts, and depolarizes the membrane, and triggers leakage of K^+ ions causing cell death. Another model proposed that daptomycin binds Ca^{2+}, oligomerizes to form micelles which deliver daptomycin to the membrane in a detergent-like form; oligomers dissociate and insert into the membrane then possibly reform oligomers, then cause leakage and cell death (Scott et al. 2007; Straus and Hancock 2006). However, more recent studies carried out with daptomycin at clinically relevant micromolar concentrations with and without Ca^{2+} showed no oligomerization in solution or in lipid membranes containing only the neutral phosphatidylcholine (PC). Daptomycin formed oligomers in *B. subtilis* membranes and in liposomes containing PC and negatively charged PG only in the presence of Ca^{2+} (Muraih et al. 2011). They proposed a model in which daptomycin binds to Ca^{2+}, inserts into membrane, interacts with PG, then forms oligomers which serve as the functional membrane lesion (Muraih et al. 2011). Muraih et al. (2012) carried out additional studies demonstrating that daptomycin interacts with PG in 1:1 stoichiometry, and that the extent of oligomer formation exceeds 90 % in membranes containing 50 % PG.

Other studies suggest that the MOA is more complex than simple membrane disruption/depolarization by oligomeric daptomycin. First, clinical isolates of *S. aureus* with reduced susceptibility to daptomycin, and containing elevated LPG in the membrane outer leaflet, did not display membrane depolarization at a dose higher than the MIC (Jones et al. 2008). Second, membrane depolarization and K^+ leakage do not precede, but follow cell death in *S. aureus* (Hobbs et al. 2008). Third, transcriptome studies in *S. aureus* and in *B. subtilis* suggest an additional primary mechanism, inhibition of cell wall biosynthesis (Dengler et al. 2011; Muthaiyan et al. 2008; Hackmann et al. 2009; Wecke et al. 2011). Finally, in vitro antibacterial activities of daptomycin, A54145, and hybrid lipopeptides against an

E. coli imp mutant defective in outer membrane assembly (Nguyen et al. 2006, 2010) do not support a single MOA mediated by membrane disruption/depolarization (see Sects. 6.5.2 and 6.5.3).

6.5.2 Transcriptome Studies and Cell Wall Biosynthesis

In *S. aureus*, daptomycin induces the cell wall stress stimulon (Dengler et al. 2011; Muthaiyan et al. 2008), including the *vraSR* two-component signal transduction system that positively regulates cell wall biosynthesis (Belcheva and Golemi-Kotra 2008; Gardete et al. 2006; Kuroda et al. 2003). The *vraSR* genes are strongly induced by the cell wall targeted antibiotics vancomycin, teicoplanin, fosfomycin, oxacillin, bacitracin, and D-cycloserine (Dengler et al. 2011; Kuroda et al. 2003; Muthaiyan et al. 2008), but not by the membrane-targeted compounds CCCP or nisin (Muthaiyan et al. 2008). Daptomycin also induced a set of genes induced by CCCP but not by vancomycin or oxacillin (Muthaiyan et al. 2008), suggesting that daptomycin has cell envelope assembly target(s) as well as the membrane PG target.

In *B. subtilis*, daptomycin strongly induces transcription of *liaRS*, a signal transduction system orthologous to *vrsRS* (Hachmann et al. 2009; Wecke et al. 2009). Daptomycin preferentially inserts into membrane at the division septum, a region enriched for PG. Depletion of PG causes reduced concentration of daptomycin at the septum and at regions of peptidoglycan biosynthesis, consistent with mechanisms of resistance. It should be noted that friulimicin, a Ca^{2+}-dependent cyclic ten-membered ring lipopeptide related to daptomycin (Baltz et al. 2005), has a specific target in cell envelope assembly, the binding to bactoprenol phosphate (Schneider et al. 2009). However, daptomycin and friulimicin have different specific mechanisms of disrupting cell envelop assembly based upon different, but overlapping patterns of induction of cell envelop stress responses (Wecke et al. 2009).

6.5.3 Antibacterial Activity Against E. coli imp (lptD)

Daptomycin, like vancomycin, is generally not active against Gram-negative bacteria, because it cannot penetrate the outer membrane effectively. The impermeability of *E. coli* to hydrophobic dyes, hydrophobic antibiotics and detergents is due to lipopolysaccharide (LPS) on the outer leaflet of the outer membrane bilayer (Chimalakonda et al. 2011). The final stages of assembly of LPS in the outer membrane require a complex of two outer membrane proteins, LptD, an essential outer membrane protein (Braun and Silhavy 2002), and LptE (Chimalakonka et al. 2011).

The *E. coli lptD* gene was originally called *imp* for increased membrane permeability (Sampson et al. 1989). The *E. coli imp*-4312 mutation conferred sensitivity to large hydrophobic antibiotics such as rifampin and erythromycin, among

other compounds. MICs for rifampin and erythromycin were reduced from >64 and >128 μg/ml in the parental strain to <0.5 and <0.5 μg/ml, respectively, in *imp*-4312 (Sampson et al. 1989). Similarly, vancomycin does not penetrate the outer membrane of wild type *E. coli* strains, but it has an MIC of 0.8 μg/ml against *E. coli imp*-4123 (Eggert et al. 2001). Futhermore, chlorobiphenyl vancomycin, which is bulkier than vancomycin, had an MIC of 0.2 μg/ml.

The data obtained with rifampin, erythromycin, vancomycin, and chlorobiphenyl vancomycin suggest that daptomycin should penetrate the defective outer membrane of *E. coli* imp-4213. If so, and if its target(s) are present in *E. coli*, then daptomycin should display antibacterial activity against *E. coli imp*-4213. PG is the most abundant anionic phospholipid in *E. coli* membranes (De Siervo 1969; Kikuchi et al. 2000) and is required for viability in wild type *E. coli* strains. The *pgsA* mutation blocking the formation of PG and cardiolipin (CL) is lethal in *E. coli* unless it is coupled with a mutation in the *lpp* gene blocking the production of a major outer membrane protein (Kikuchi et al. 2000). Therefore, if the presence of PG in *E. coli* membranes provides the sole target for daptomycin to form oligomers to disrupt membrane function/permeability, then daptomycin should display antibacterial activity against *E. coli imp*-4213. Nguyen et al. (2006) demonstrated that daptomycin has an MIC of 128 μg/ml on *E. coli imp*-4213, and >256 μg/ml on wild type *E. coli* 35. On the other hand, A54145E, a lipopeptide related to daptomycin (Fig. 6.1), had an MIC of 16 μg/ml (Nguyen et al. 2010; Table 6.2), indicating that lipopeptide antibiotics can penetrate *E. coli imp*-4213 and interact with antibacterial target(s). The data imply that A54145E has a different antibacterial target(s) than daptomycin that is present in *E. coli*. Interestingly, a hybrid lipopeptide (CB-182,290) containing 12 amino acids from the daptomycin core peptide and a single amino acid from A54146E (D-asn$_{11}$ substituted for D-ser$_{11}$), had an MIC of 32 μg/ml on *E. coli imp*-4213 (Nguyen et al. 2010). This suggests that the hybrid lipopeptide has altered target specificity and now interacts with a lethal target in *E. coli*. The combined data suggest that interaction of Ca^{2+}-bound daptomycin with membranes containing PG, followed by oligomerization, is not sufficient to account for the antibacterial activity. It also supports the interpretation that daptomycin oligomers must interact with another target or targets associated with cell division/cell wall assembly in Gram-positive pathogens not present in *E. coli*. So, are there any candidate targets?

6.5.4 Potential Role of YycG

It has been suggested that YycG should be considered as a possible target for daptomycin action (Baltz 2009a, b, 2010a). Functional YycFG is required for viability in low G+C Gram-positive pathogens, but *yycFG* orthologs are not present in Gram-negative bacteria. If daptomycin binds directly to YycG to interfere with signal transduction, some of the *yycG* (DapR) mutants may express histidine kinases with altered daptomycin binding affinities. Alternatively,

daptomycin oligomers in the PG rich cell division septum might act by interfering with the coordination of the membrane-associated multienzyme divisome (Dubrac et al. 2008) by a "sand in the gearbox" mechanism (Schneider and Sahl 2010), which has been suggested as the MOA of human β-defensin 3 (Sass et al. 2010).

If YycG is a target for daptomycin action, either by direct binding or by general disruption in the divisome, then interference with YycG function might mimic the effects of daptomycin treatment of cells (Baltz 2009a). The key observations on YycG are that: (i) YycG co-localizes with FtsZ at the cell division septum *in B. subtilis* (Fukushima et al. 2008); (ii) YycG depletion leads to rapid cell death without lysis in *S. aureus* (Dubrac et al. 2007); (iii) YycF depletion causes formation of aberrant cell division septa in *S. pneumoniae* (Ng et al. 2004); and (iv) YycFG positively regulates biofilm formation in *S. aureus* (Dubrac et al. 2007). In comparison, daptomycin (i) inserts preferentially into PG-enriched membranes at cell division septa in *B. subtilis* (Hackmann et al. Hachmann et al. 2009); (ii) causes rapid cell death without lysis in *S. aureus* (Cotroneo et al. 2008); (iii) causes the formation of aberrant cell division septa in *S. aureus* (Cotroneo et al. 2008); and (iv) treats *S. aureus* in an in vitro catheter-infection biofilm model (Raad et al. 2007). Thus, the effects of YycG depletion mimic the effects of daptomycin treatment of Gram-positive bacteria with low G+C content. Furthermore, many of the *S. aureus* mutants partially resistant to daptomycin have mutations in the *yycG*. If YycG is a target for daptomycin action, then daptomycin should display elevated antibacterial activity against *B. subtilis* CNM2000, which contains a temperature-sensitive mutation in *YycF (walR)*, than against *B. subtilis* 168 (Okada et al. 2010). *B. subtilis* CNM2000 is more sensitive to killing by inhibitors of YycG than its parental strain, but not to other antibiotics, including inhibitors of cell wall biosynthesis (ampicillin and vancomycin).

6.6 Approaches to Improve the Properties or Extend the Spectrum of Lipopeptide Antibiotics Related to Daptomycin

A21978C and daptomycin are complex molecules that have limited possibilities for medicinal chemical and chemoenzymatic approaches to generate novel derivatives. The medicinal chemistry approaches to A21978C have been limited to substitutions for the lipid side chain and additions to the δ-amino group of ornithine (Debono et al. 1988; Hill et al. 2003; Siedlecki et al. 2003). Chemoenzymatic approaches to generate A21978C and A54145 analogs have been limited by the lack of availability of 3mGlu and other modified amino acids (Grünewald et al. 2004; Kopp et al. 2006). An alternative approach was to generate novel cyclic lipopeptides biosynthetically by combinatorial biosynthesis. Before undertaking this technically challenging approach, it was important to define some specific targets for lipopeptides with improved activities.

6.6.1 Other Potential Clinical Indications for Lipopeptides

Although daptomycin had successful clinical trials that culminated in approved indications to treat skin and skin structure infections caused by Gram-positive pathogens, and bacteremia and right-sided endocarditis caused by *S. aureus*, it failed to show non-inferiority in a clinical trial targeting community-acquired pneumonia (CAP) caused by *S. pneumoniae* (Pertel et al. 2008), in spite of the fact that daptomycin is very active against *S. pneumonia* strains in vitro (Nguyen et al. 2006, 2010; Table 6.2). Silverman et al. (2005) demonstrated that daptomycin activity is inhibited by pulmonary surfactant. Therefore, one approach to improve activity against CAP was to screen for novel derivatives of A21978C or A54145 that retained the potent antibacterial activity and low toxicity of daptomycin, while reducing the inhibitory interaction with surfactant. Daptomycin is also active against strains of *C. difficile*, but the MICs range from 1 to 2 times those of vancomycin (Goldstein et al. 2004; Nóren et al. 2010; Tyrrell et al. 2006), which is used clinically to treat *C. difficile*-associated diarrhea (CDAD) (see Rossolini et al., this volume). From a mechanistic perspective, nearly 100 % of membrane phospholipids in *C. difficile* contain PG (Drucker et al. 1996), the target for daptomycin insertion and oligomerization; and *C. difficile* has a *yycF* ortholog, but not a *yycG* ortholog, based upon BLASTP analysis.

Targeting indications for CAP and CDAD required modifying the core peptides or lipid side chains of the A21978C or A54145. Early studies on the A54145 factors indicated that the compounds containing Glu_{12} and Ile_{13} were much less inhibited by surfactant than daptomycin. Although these compounds were less active than daptomycin, they were also much less toxic than A54145 factors containing $3mGlu_{12}$ (Counter et al. 1990).

6.6.2 Approaches to Generate or Discover Novel Lipopeptide Antibiotics

Four approaches to improve lipopeptide efficacy against CAP were pursued at Cubist: (i) to screen an existing library of lipopeptides related to daptomycin (Hill et al. 2003; Siedlecki et al. 2003) and to generate additional compounds; (ii) to generate A54145D derivatives containing different lipid or aryl side chains; (iii) to modify the core peptide of A21978C by combinatorial biosynthesis; and (iv) to modify the core peptide of A54145 by gene disruptions and combinatorial biosynthesis. Disruption of the *lptI* gene encoding a methyltransferase involved in the formation of $3mGlu_{12}$ generated a strain for the production of A54145D for approach (ii). Highly active compounds generated by these approaches were also screened for antibacterial activities against *C. difficile*.

Another future approach is to use the genetic information from the lipopeptide antibiotic biosynthetic pathways as probes to explore cryptic lipopeptide pathways

identified in genome sequencing projects. Whereas this approach is in its infancy, a cryptic pathway highly related to the A21978C pathway has already been identified (Baltz 2010b).

6.7 Combinatorial Biosynthesis to Generate Novel Lipopeptides

The lipopeptides A21978C and A54145 presented attractive starting materials for combinatorial biosynthesis for the following reasons: (i) The molecules are similar in overall chemical structure and amino acid chirality, yet they differ substantially in primary amino acid sequence in the tridecapepties (Fig. 6.1); (ii) the peptide cores are encoded by three (A21978C) or four (A54145) related non-ribosomal peptide synthetase (NRPS) genes (Miao et al. 2005; 2006a, b) that could be engineered independently and reassorted in combinatorial fashion; (iii) modifications of the lipid side chains can be made by precursing different fatty acids or amino acids (Boeck and Wetzel 1990; Huber et al. 1988); (iv) additional amino acid modifications might be made by deleting genes encoding enzymes involved in amino acid modifications; and (v) combinatorial biosynthesis might be achieved by combining engineered NRPS genes, gene deletions for amino acid modifications, and variations in lipid side chains.

6.7.1 Development of Expression Hosts, Vectors, and Engineering Strategies

In order to realize the potential for combinatorial biosynthesis of lipopeptides related to A21978C and A54145, several molecular genetic tools were developed. These included: (i) *S. roseosporus* and *S. fradiae* expression hosts containing different sets of deletions of lipopeptide biosynthetic genes; (ii) vectors to facilitate rapid engineering in *E. coli* followed by conjugal transfer to streptomycete expression hosts and site-specific insertion into the host chromosome; and (iii) regulatory elements for the expression of engineered genes from ectopic loci.

The development of combinatorial biosynthesis systems in *S. roseosporus* and *S. fradiae* was facilitated by cloning the complete A21978C and A54145 biosynthetic gene clusters in BAC vectors (Miao et al. 2005, 2006a, b). A number of mutants lacking one or more A21978C or A54145 biosynthetic genes (Baltz et al. 2010a; Coëffet-Le Gal et al. 2006; Nguyen et al. 2006; Alexander et al. 2010) were generated by cloning the crossover segments into the temperature-sensitive plasmid vector pRHB514 (Hosted and Baltz 1997) and screening for deletions generated by double crossovers. Most of the engineering steps were facilitated by λ-Red-mediated recombination in *E. coli* in BAC or other vectors that contained

oriT from RP4 to drive conjugation into the streptomycete hosts, and ϕC31, ϕBT1, or IS117 attachment/integration (*att/int*) functions to insert site-specifically into the streptomycete chromosomes (Alexander et al. 2010; Alexander et al. 2011; Baltz et al. 2006a, b, 2010a, b; Nguyen et al. 2006, 2010). In addition, ectopic expression of engineered NRPS and other genes was facilitated by the insertion of the strong constitutive *ermE** promoter upstream of the gene or set of genes to be expressed.

6.7.2 Hybrid Lipopeptides Generated by Combinatorial Biosynthesis

Using the methods discussed above, many lipopeptides related to A21978C or A54145 were generated (Alexander et al. 2010, 2011; Baltz et al. 2010a, Miao et al. 2006a, b; Nguyen et al. 2006, 2010). During the process, a number of "rules" for productive expression of engineered NRPS genes were developed (Baltz et al. 2010b). These mainly dealt with maintaining functional domain interactions and choosing appropriate interdomain linker regions for coupling heterologous domains in the NRPS modules.

6.7.2.1 Chemical Structures Generated by Combinatorial Biosynthesis and Antibacterial Activities

Table 6.1 summarizes the antibacterial activities against *S. aureus* of a subset of novel lipopeptides containing different amino acid substitutions in the peptide cores of A21978C and A54145 generated by combinatorial biosynthesis. The molecules at the top are more related to daptomycin, and those at the bottom are more related to A54145E. Among the daptomycin analogs, it was first noted that substitutions for Kyn_{13} reduced the inhibition by bovine surfactant, but also influenced the MICs in the absence of surfactant negatively with the addition of amino acids with smaller hydrophobic side chains (MIC = Kyn < Trp < Ile < Val), and antibacterial activity was totally lost with the substitution of Asn. Amino acid substitutions at positions 8 and 11 yielded moderately improved activity in the presense of surfactant. Substitution of Glu_{12} for $3mGlu_{12}$ reduced the ratio of MICs in the presence and absense of surfactant substantially, but at the expense of overall antibacterial activity. Combinations of other amino acid changes with the change at Glu_{12} generally led to further loss in antibacterial activity.

The substitution of Glu_{12} for $3mGlu_{12}$ in the context of A54145 gave a more positive outcome. A54145D (containing Glu_{12}) had an MIC of 4 μg/ml in the presense of surfactant, and was only 2-fold less active than A54145E in the absence of surfactant (Table 6.1). Importantly, A54145D is much less toxic in mice than A54145E (Counter et al. 1990). Several examples of combinatorial

changes coupled with $3mGlu_{12}$ or Glu_{12} are shown in Table 6.1. There were three ways to improve the activity of A54145D in the presence of surfactant. Substitutions at positions 8 or 11 were not helpful, but the substitution of Kyn_{13} from the daptomycin pathway (coupled with Glu_{12}) improved the activity by 2-fold. Substitution of Asn_3 and Asp_9 for $hAsn_3$ and $moAsp_9$ or substitution of D-Asn_2 and Asp_3 (from the daptomycin pathway) for D-Glu_2 and $hAsn_3$ also improved the activity 2-fold (MIC = 2 with surfactant).

Table 6.2 shows the extended antibacterial spectrum of a few recombinant molecules. Two daptomycin analogs, CB-182,122 (D-Ala_{11}) and CB-182,290 (D-Asn_{11}), which showed moderately improved antistaphylococcal activity in the presence of surfactant (Table 6.1) displayed good antibacterial activities against other Gram-positive pathogens. CB-182,290 showed improved activity against DapR strains of *S. aureus*, *E. faecium*, and *E. faecalis* relative to daptomycin, and also had an MIC of 32 against *E. coli* BAS849 (*imp-4213*), suggesting that the substitution of Asn_{11} for Ser_{11} modifies the MOA. A54145E and the three derivatives with improved activity in the presence of surfactant (CB-182,561, CB-182,549, and CB-182,548) had similar antibacterial activities relative to daptomycin and A54145E. It is noteworthy that A54145E and the three derivatives all had MICs of 16 against *E. coli* (*imp-4213*), suggesting that these lipopeptides differ from daptomycin in MOA, and each can interfer with some target(s) in *E. coli (imp-4213)* that daptomycin does not inhibit. The MICs of the A54145 analogs none-the-less show cross-resistance with daptomycin in DapR *S. aureus* and enterococcal strains (Table 6.2), indicating that they have similar MORs. This was not surprising, because all are Ca^{2+}-dependent and require penetration of the cell envelope.

CB-182,561, CB-182,549, and CB-182,548 were tested for in vivo efficacy in a mouse model for *S. pneumonia* pneumonia (Nguyen et al. 2010), and none were as active as the vancomycin control. Other chemically modified derivatives of A54145D containing lipid side chain substitutions were also tested in this model, and none were as active as vancomycin (see Alexander et al. 2010). Without a candidate superior to vancomycin, the CAP project was discontinued at Cubist.

6.8 Clinical Development of CB-183,315 for CDAD

Daptomycin was shown to be active against *C. difficile* in a hamster model for pseudomembranous colitis (Dong et al. 1987). More recent studies indicated that daptomycin is active against a wide variety of clinical isolates of *C. difficile* in vitro, displaying MIC_{50} and MIC_{90} values of 0.5–1.0 and 1.0–2.0 μg/ml, respectively (Goldstein et al. 2004; Tyrrell et al. 2006; Nóren et al. 2010). In general, the MIC values for daptomycin are about 2-fold higher than those reported for vancomycin. Cubist explored lipopeptides derived from the A21978C and A54145D, and identified CB-183,315, a A21978C derivative with a long chain length, unsaturated, methyl-branched fatty acid with an internal benzyl group as a

Table 6.1 Lipopeptide antibiotics generated by combinatorial biosynthesis

Compound[a]	Amino acid at position									*S. aureus* MIC (μg/ml)		
	2	3	5	6	8	9	11	12	13	−Surf	+Surf (1 %)	Ratio (±)
Daptomycin	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	3mGlu	Kyn	0.5	64	128
CB-181,220	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	3mGlu	Kyn	0.5	64	128
CB-182,098	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	3mGlu	***Trp***	1	32	32
CB-182,107	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	3mGlu	***Ile***	2	8	4
CB-182,106	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	3mGlu	***Val***	4	8	2
A21978C1(Asn13)	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	3mGlu	***Asn***	128	ND	ND
CB-182,130	D-Asn	Asp	Gly	Orn	D-Ala	Asp	D-Ser	***Glu***	Kyn	8	16	2
CB-182,122	D-Asn	Asp	Gly	Orn	D-Ala	Asp	***D-Ala***	3mGlu	Kyn	1	16	16
CB-182,290	D-Asn	Asp	Gly	Orn	D-Ala	Asp	***D-Asn***	3mGlu	Kyn	1	16	16
CB-182,166	D-Asn	Asp	Gly	Orn	***D-Ser***	Asp	D-Ser	3mGlu	Kyn	1	32	32
CB-182,257	D-Asn	Asp	Gly	Orn	***D-Asn***	Asp	D-Ser	3mGlu	Kyn	8	ND	ND
CB-182,251	D-Asn	Asp	Gly	Orn	D-Ala	Asp	***D-Asn***	***Glu***	Kyn	32	ND	ND
CB-182,269	D-Asn	Asp	Gly	Orn	***D-Asn***	Asp	D-Ser	***Glu***	Kyn	128	ND	ND
A54145E	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	D-Asn	3mGlu	Ile	1	32	32
CB-182,548	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	***D-Ala***	3mGlu	Ile	1	16	16
CB-182,332	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	***D-Ser***	3mGlu	Ile	2	16	8
CB-182,571	D-Glu	hAsn	Sar	Ala	***D-Ala***	moAsp	D-Asn	3mGlu	Ile	1	32	32
CB-182,549	D-Glu	hAsn	Sar	Ala	***D-Ser***	moAsp	D-Asn	3mGlu	Ile	1	16	16
CB-182,443	D-Glu	hAsn	Sar	Ala	D-Lys	***Asp***	D-Asn	3mGlu	Ile	2	4	2
CB-182,363	D-Glu	***Asn***	Sar	Ala	D-Lys	moAsp	D-Asn	3mGlu	Ile	2	16	8
CB-182,575	***D-Asn***	hAsn	Sar	Ala	D-Lys	moAsp	D-Asn	3mGlu	Ile	2	4	2
CB-182,390	D-Glu	***Asn***	Sar	Ala	D-Lys	***Asp***	D-Asn	3mGlu	Ile	2	2	1
CB-182,561	***D-Asn***	***Asp***	Sar	Ala	D-Lys	moAsp	D-Asn	3mGlu	Ile	1	2	2
A54145D	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	D-Asn	***Glu***	Ile	2	4	2

(continued)

Table 6.1 (continued)

Compound[a]	Amino acid at position									*S. aureus* MIC (μg/ml)		
	2	3	5	6	8	9	11	12	13	−Surf	+Surf (1 %)	Ratio (±)
CB-183,296	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	***D-Ala***	***Glu***	***Kyn***	1	2	2
CB-182,509	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	***D-Ser***	***Glu***	Ile	8	16	2
CB-182,336	D-Glu	hAsn	Sar	Ala	D-Lys	moAsp	***D-Asn***	***Glu***	Ile	64	128	2
CB-182,333	D-Glu	hAsn	Sar	Ala	D-Lys	***Asp***	D-Asn	***Glu***	Ile	32	64	2
CB-182,567	D-Glu	hAsn	Sar	Ala	***D-Ala***	moAsp	D-Asn	***Glu***	Ile	4	8	2
CB-182,532	D-Glu	hAsn	Sar	Ala	***D-Ser***	moAsp	D-Asn	***Glu***	Ile	8	8	1

[a] Daptomycin has an *N*-decanoyl side chain. All others compounds have *anteiso*-undecanoyl side chains. MICs against *S. aureus* 42 were determined with and without the addition of 1 % bovine surfactant. The data on compounds related to daptomycin (CB-181,220 - CB-182,290) are from (Miao et al. 2006a, b; Nguyen et al. 2006, 2010). The data on compounds related to A54145 are from (Nguyen et al. 2010; Alexander et al. 2011)

Table 6.2 Antibacterial spectrum of hybrid lipopeptides

Strain	MIC (μg/ml)						
	Daptomycin	CB-182,122	CB-182,290	A54145E	CB-182,561	CB-182,549	CB-182,548
S. aureus 42 (MSSA, DapS)	1	1	1	0.5	1	1	1
S. aureus 399 (MRSA, DapS)	0.5	1	1	2	0.5	1	1
S. aureus MW2 (MRSA, DapS)	1	0.5	1	1	1	0.5	1
S. aureus 1695 (DapR)	4	4	4	4	4	4	8
S. aureus 1616 (DapR)	16	16	8	16	16	8	16
E. faecium 14 (DapS)	2	4	2	4	4	4	4
E. faecium 384 (DapR)	32	16	8	32	16	32	32
E. faecalis 201 (DapS)	2	4	1	4	4	4	4
E. faecalis 312 (DapR)	128	128	32	64	64	128	128
S. pneumoniae 402 (DapS)	0.25	0.25	0.125	0.5	1	0.25	0.5
E. coli 35	>256	>256	>256	NR	NR	NR	NR
E. coli BAS849 (*imp)*	128	128	32	16	16	16	16

DapS daptomycin susceptible, *DapR* daptomycin resistant, *MSSA* methicillin-susceptible *S. aureus*, *MRSA* methicillin resistant *S. aureus*, *NR* not reported. Data from Nguyen et al. (2006, 2010)

promising candidate (Patino et al. 2011). It displayed an MIC range of 0.06–2.0 μg/ml, and MIC_{90} of 0.5 μg/ml against 556 clinical isolates of *C. difficile* (Citron et al. 2012), and has demonstrated in vivo efficacy in Phase II clinical trials (Patino et al. 2011).

6.9 Conclusions

Daptomycin is a clinically important antibiotic approved to treat skin and skin structure infections caused by Gram-positive pathogens, and bacteremia and right-sided endocarditis caused by *S. aureus* strains. However, it was not approved to treat community-acquired pneumonia (CAP), even though *S. pneumonia* is susceptible to daptomycin in vitro. Scientists at Cubist discovered that daptomycin becomes sequestered in lung surfactant and loses its antibacterial activity. To address this shortcoming, medicinal chemistry and combinatorial biosynthesis approaches were undertaken to generate novel lipopeptides related to A21978C and A54145D.

The chemical modification of A54145D was enabled by generating a highly productive mutant of *S. fradiae* blocked in the formation of $3mGlu_{12}$ (Alexander et al. 2010). This provided a fermentation route to produce the key starting materials containing Glu_{12}, and lacking the more toxic A54145 factors containing $3mGlu_{12}$. Many compounds were generated by both approaches, and several of the A54145D derivatives had good antibacterial activities in the presence of pulmonary surfactant and low mammalian toxicity, but none were equivalent to vancomycin in a mouse pneumonia model. Daptomycin is also active against *C. difficile*, but not as active as vancomycin (see Rossolini et al., this volume). Compounds generated in the CAP project were tested against *C. difficile* to address CDAD, and the A21978C derivative, CB-183,315, had antibacterial activity superior to daptomycin and vancomycin. CB-183,315 is currently under clinical development by Cubist.

The work at Cubist demonstrated that complex lipopeptides can be structurally modified by combinatorial biosynthesis, and that antibacterial activity, toxicity, and interactions with surfactant can be modulated through subtle changes in the peptide core. Only a small fraction of the chemical space was addressed in the relatively modest program at Cubist, and many combinations of amino acid substitutions could be addressed in the future, particularly with advances in genome mining to identify NRPS modules for unusual amino acids. The "rules" for successful genetic engineering of NRPS modules and domains are now fairly well defined, and the fermentation yields from engineered strains are sufficient for early stage development (Baltz 2009b; Baltz et al. 2010a, b). In addition, little work has been done to couple combinatorial biosynthesis with medicinal chemistry, so this is another avenue that could be further explored. It is unlikely that novel derivatives of A21978C or A54145 will circumvent the now well-established mechanisms of incremental DapR caused by changes in surface change (e.g., *mprF* mutations) or by changes in cell wall thickness (e.g., *yycG* or *rpoB* mutations). However, it is reasonable to envision generating lipopeptide derivatives that are more active than daptomycin and vancomycin against difficult-to-treat Gram-positive infections (e.g., *S. aureus* bacteremia and endocarditis).

References

Alexander D, Davies J, Miao V, Baltz RH (2004) Identification of a resistance gene within the daptomycin biosynthetic gene cluster from *Streptomyces roseosporus.* In: Abstracts of the joint 8th symposium on genetics and molecular biology of industrial microorganisms (GMBIM), and 8th international conference on the biotechnology of microbial products (BMP), San Diego, CA, 14–18 November

Alexander DC, Rock J, He X, Miao V, Brian P, Baltz RH (2010) Development of genetic system for lipopeptide combinatorial biosynthesis in *Streptomyces fradiae* and heterologous expression of the A54145 biosynthetic gene cluster. Appl Environ Microbiol 76:6877–6887

Alexander DC, Rock J, Gu J-Q, Mascio C, Chu M, Brian P, Baltz RH (2011) Production of novel lipopeptide antibiotics related to A54145 by *Streptomyces fradiae* mutants blocked in biosynthesis of modified amino acids and assignment of *lptI*, *lptK* and *lptL* gene functions. J Antibiot 64:79–87

Arbeit RD, Maki D, Tally FP, Campanaro E, Eisenstein BI (2004) The safety and efficacy of daptomycin for the treatment of complicated skin and skin-structure infections. Clin Infect Dis 38:1673–1681

Arias CA, Panesso D, McGrath DM et al (2011) Genetic basis for in vivo daptomycin resistance in enterococci. N Eng J Med 365:892–900

Ball LJ, Goult CM, Donarski JA, Micklefield J, Ramesh V (2004) NMR structure determination and calcium binding effects of lipopeptide antibiotic daptomycin. Org Biomol Chem 2:1872–1878

Baltz RH (2005) Natural product discovery and development at Eli Lilly and Company: one scientist's view. SIM News 55:5–16

Baltz RH (2008) Biosynthesis and genetic engineering of lipopeptide antibiotics related to daptomycin. Curr Top Med Chem 8:618–638

Baltz RH (2009a) Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Curr Opin Chem Biol 13:144–151

Baltz RH (2009b) Chapter 20. Biosynthesis and genetic engineering of lipopeptides in *Streptomyces roseosporus*. Methods Enzymol 458:511–531

Baltz RH (2010a) Daptomycin. In: Buss AD, Butler MS (eds) Natural product chemistry for drug discovery. RSC Publishing, Cambridge, pp 395–409

Baltz RH (2010b) Genomics and the ancient origins of the daptomycin biosynthetic gene cluster. J Antibiot 63:506–511

Baltz RH (2010c) *Streptomyces* and *Saccharopolyspora* hosts for heterologous expression of secondary metabolite gene clusters. J Ind Microbiol Biotechnol 37:759–772

Baltz RH, Miao V, Wrigley SW (2005) Natural products to drugs: daptomycin and related lipopeptide antibiotics. Nat Prod Rep 22:717–741

Baltz RH, Nguyen KT, Alexander DC (2010a) Genetic engineering of acidic lipopeptide antibiotics. In: Baltz RH, Davies JE, Demain AL (eds) Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington DC, pp 391–410

Baltz RH, Nguyen KT, Alexander DC (2010b) Reprogramming daptomycin and A54145 biosynthesis to produce novel lipopeptide antibiotics. In: Yeh W-K, Yang H-C, McCarty JR (eds) Enzyme technologies: metagenomics, evolution, biocatalysis, and biosynthesis. Wiley, Hoboken, pp 285–308

Bastian S, Brossier F, Wichlacz C, Jarlier V, Veziris N (2010) Daptomycin is not active against rapidly growing mycobacteria. J Med Microbiol 59:135–136

Belcheva A, Golemi-Kotra D (2008) A close-up view of the VraSR two-component system: a mediator of *Staphylococcus aureus* response to cell wall damage. J Biol Chem 283:12354–12364

Bertsche U, Weidenmaier C, Kuehner D, Yang S-J, Baur S, Wanner S, Francois P, Schrenzel J, Yeaman MR, Bayer AS (2011) Correlation of daptomycin resistance in a clinical *Staphylococcus aureus* strain with increased cell wall teichoic acid production and D-alanylation. Antimicrob Agents Chemother 55:3922–3928

Boeck LD, Wetzel RW (1990) A54145, a new lipopeptide antibiotic complex: factor control through precursor directed biosynthesis. J Antibiot 43:607–615

Boeck LD, Papiska HR, Wetzel RW, Mynderse JS, Fukuda DS, Mertz FP, Berry DM (1990) A54145, a new lipopeptide antibiotic complex: discovery, taxonomy, fermentation and HPLC. J Antibiot 43:587–593

Boeck LD, Fukuda DS, Abbott BJ, Debono M (1988) Deacylation of A21978C, an acidic lipopeptide antibiotic complex, by *Actinoplanes utahensis*. J Antibiot 41:1085–1092

Braun M, Silhavy TJ (2002) Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. Mol Microbiol 45:1289–1302

Camargo IL, Neoh H-M, Cui L, Hiramatsu K (2008) Serial daptomycin selection generates daptomycin-nonsusceptible *Staphylococcus aureus* strains with heterogenous vancomycin-intermediate phenotype. Antimicrob Agents Chemother 52:4289–4299

Chimalakonda G, Ruiz N, Chng SS, Garner RA, Kahne D, Silhavy TJ (2011) Lipoprotein LptE is required for the assembly of LptD by the β-barrel assembly machine in the outer membrane of *Escherichia coli*. Proc Natl Acad Sci U S A 108:2492–2497

Citron DM, Tyrrell KL, Merriam V, Goldstein EJC (2012) In vitro activity of CB-183,315, vancomycin and metronidazole against 556 strains of *Clostridium difficile*, 445 other intestinal anaerobes and 56 *Enterobacteriaceae* species. Antimicrob Agents Chemother 56:1613–1615

Coëffet-Le Gal M-F, Thurson L, Rich P, Miao V, Baltz RH (2006) Complementation of daptomycin *dptA* and *dptD* deletion mutations *in-trans* and production of hybrid lipopeptide antibiotics. Microbiology 152:2993–3001

Counter FT, Allen NE, Fukuda DS, Hobbs JN, Ott J, Ensminger PW, Mynderse JS, Preston DA, Wu CY (1990) A54145 a new lipopeptide antibiotic complex: microbiological evaluation. J Antibiot 43:616–622

Cotroneo N, Harris R, Perlmutter N, Beveridge T, Silverman JA (2008) Daptomycin exerts bactericidal activity without lysis of *Staphylococcus aureus*. Antimicrob Agents Chemother 52:2223–2225

Cui L, Isii T, Fukuda M et al (2010) An *rpoB* mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother 54:5222–5233

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. Science 311:374–377

D'Costa VM, Mukhtar TA, Patel T, Koteva K, Waglechner N, Hughes DW, Wright GD, De Pascale G (2012) Inactivation of the lipopeptide antibiotic daptomycin by hydrolytic mechanisms. Antimicrob Agents Chemother 56:757–764

De Siervo AJ (1969) Alterations in the phospholipid composition of *Escherichia coli* B during growth at different temperatures. J Bacteriol 100:1342–1349

Debono M, Barnhart M, Carrell CB, Hoffmann JA, Occolowitz JL, Abbott BJ, Fukuda DS, Hamill RL, Biemann K, Herlihy WC (1987) A21978C, a complex of new acidic peptide antibiotics: isolation, chemistry, and mass spectral structure elucidation. J Antibiot 40:761–777

Debono M, Abbott BJ, Molloy RM, Fukuda DS, Hunt AH, Daupert VM, Counter FT, Ott JL, Carrell CB, Howard LC, Boeck LD, Hamill RL (1988) Enzymatic and chemical modifications of lipopeptide antibiotic A21978C: the synthesis and evaluation of daptomycin (LY146032). J Antibiot 41:1093–1105

Dengler V, Meier PS, Heusser R, Berger-Bachi B, McCallum N (2011) Induction kinetics of the *Staphylococcus aureus* cell wall stress stimulon in response to different cell wall active antibiotics. BMC Microbiol 11:16

Dong M-Y, Chang T-W, Gorbach SL (1987) Treatment of *Clostridium difficile* colitis in hamsters with a lipopeptide antibiotic, LY146032. Antimicrob Agents Chemother 31:1135–1136

Drucker DB, Wardle HM, Boote V (1996) Phospholipid profiles of *Clostridium difficile*. J Bacteriol 178:5844–5846

Dubrac S, Boneca IG, Poupel O, Msadek T (2007) New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. J Bacteriol 189:8257–8269

Dubrak S, Bisicchia P, Devine KM, Msadek T (2008) A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. Mol Microbiol 70:1307–1322

Dugourd D, Yang H, Elliott M, Siu R, Clement JJ, Straus SK, Hancock REW, Rubinchik E (2011) Antimicrobial properties of MX-2401, an expanded-spectrum lipopeptide active in the presence of lung surfactant. Antimicrob Agents Chemother 55:3720–3728

Eggert US, Ruiz N, Falcone BV, Branstrom AA, Goldman RC, Silhavy TJ, Kahne D (2001) Genetic basis for activity differences between vancomycin and glycolipid derivatives of vancomycin. Science 294:361–364

Eisenstein BI, Oleson FB Jr, Baltz RH (2010) Daptomycin: from the mountain to the clinic, with the essential help of Francis Tally, MD. Clin Inf Dis 50:S10–S15

Ernst CM, Peschel A (2011) Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. Mol Microbiol 80:290–299

Fischer A, Yang S-J, Bayer AS et al (2011) Daptomycin resistance mechanisms in clinically derived *Staphylococcus aureus* assessed by a combined transcriptomics and proteomics approach. J Antimicrob Chemother 66:1696–1711

Fowler VG, Boucher HW, Corey GR et al (2006) Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. N Engl J Med 355:653–655

Friedman L, Alder JD, Silverman JA (2006) Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother 50:2137–2145

Fukuda DS, Du Bus RH, Baker PJ, Berry DM, Mynderse JS (1990a) A54145, a new lipopeptide antibiotic complex: isolation and characterization. J Antibiot 43:594–600

Fukuda DS, Debono M, Molloy RM, Mynderse JS (1990b) A54145, a new lipopeptide antibiotic complex: microbial and chemical modification. J Antibiot 43:601–606

Fukushima T, Szurmant H, Kim E-J, Perego M, Hoch JA (2008) A sensor histidine kinase co-ordinates cell wall architecture with cell division in *Bacillus subtilis*. Mol Microbiol 69:621–632

Fukushima T, Furihata I, Emmins R, Daniel RA, Hoch JA, Szurmant H (2011) A role for the essential YycG sensor histidine kinase in sensing cell division. Mol Microbiol 79:503–522

Gardete S, Wu SW, Gill S, Tomasz A (2006) Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. Antimicrob Agents Chemother 50:3424–3434

Goldstein EJC, Citron DM, Merriam CV, Warren YA, Tyrrell KL, Fernandez HT (2004) In vitro activities of the new semisynthetic glycopeptides televancin (TD-6424), vancomycin, daptomycin, linezolid, and four comparator agents against anaerobic gram-positive species and *Corynebacterium* spp. Antimicrob Agents Chemother 48:2149–2152

Grünewald J, Seiber SA, Mahlert C, Linne U, Marahiel MA (2004) Synthesis and derivatization of daptomycin: a chemoenzymatic route to acidic lipopeptide antibiotics. J Am Chem Soc 126:17025–17031

Hachmann A-B, Angert ER, Helmann JD (2009) Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. Antimicrob Agents Chemother 53:1598–1609

Hachmann AB, Sevim E, Gaballa A, Popham DL, Antelmann H, Helmann JD (2011) Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. Antimicrob Agents Chemother 55:4326–4337

Hill J, Siedlecki J, Parr I et al (2003) Synthesis and biological activity of N-acylated ornithine analogues of daptomycin. Bioorg Med Chem Lett 13:4187–4191

Hobbs JK, Miller K, O'Neill AJ, Chopra I (2008) Consequences of daptomycin-mediated membrane damage in *Staphylococcus aureus*. J Antimicrob Chemother 62:1003–1008

Hojati Z, Milne C, Harvey B et al (2002) Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. Chem Biol 9:1175–1187

Hosted TJ, Baltz RH (1997) Use of *rpsL* for dominance selection and gene replacement in *Streptomyces roseosporus*. J Bacteriol 179:180–186

Howden BP, McEvoy CRE, Allen DL et al (2011) Evolution of multidrug resistance during *Staphylococcus aureus* infection involves mutation of the essential two component regulator WalKR. PLoS Pathog 7:e1002359

Huber FM, Pieper RL, Tietz AJ (1988) The formation of daptomycin by supplying decanoic acid to *Streptomyces roseosporus* cultures producing the antibiotic complex A21978C. J Biotechnol 7:283–292

Jansen A, Türck M, Szekat C, Nagel M, Clever I, Bierbaum G (2007) Role of insertion elements and *yycFG* in the development of decreased susceptibility to vancomycin in *Staphylococcus aureus*. Int J Med Microbiol 297:205–215

Jones T, Yeaman MR, Sakoulas G, Yang S-J, Procter RA, Sahl H-G, Schrenzel J, Xiong YQ, Bayer AS (2008) 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 52:269–278

Julian K, Kosowska-Shick K, Whitener C, Roos M, Labishinski H, Rubio A, Parent L, Ednie L, Koeth L, Bogdanovich T, Applebaum PC (2007) Characterization of a daptomycin-nonsusceptible vancomycin-intermediate *Staphylococcus aureus* strain in a patient with endocarditis. Antimicrob Agents Chemother 51:3445–3448

Jung D, Rozek A, Oken M, Hancock RE (2004) Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. Chem Biol 11:949–957

Jung D, Powers JP, Straus SK, Hancock REW (2008) Lipid-specific binding of the calcium-dependent antibiotic daptomycin leads to changes in lipid polymorphism of model membranes. Chem Phys 154:120–128

Kikuchi S, Shibuta I, Matsumoto K (2000) Viability of an *Escherichia coli pgsA* null mutant lacking detectable phosphatidylglycerol and cardiolipin. J Bacteriol 182:371–376

Kirst HA, Thompson DG, Nicas TI (1998) Historical yearly usage of vancomycin. Antimicrob Agents Chemother 42:1303–1304

Kopp F, Grünewald J, Mahlert C, Marahiel MA (2006) Chemoenzymatic design of acidic lipopeptide hybrids: new insights into the structure-activity relationships of daptomycin and A54145. Biochemistry 45:10474–10481

Kosowska-Shick K, Clark C, Pankuch GA, McGhee P, Dewasse B, Beachel L, Applebaum PC (2009) Activity of televancin against staphylococci and enterococci determined by MIC and resistance selection. Antimicrob Agents Chemother 53:4217–4224

Kreuzman AJ, Hodges RL, Swartling JR, Pohl TE, Ghag SK, Baker PJ, McGilvray D, Yeh WK (2000) Membrane-associated echinocandin B deacylase of *Actinoplanes utahensis*: purification, characterization, heterologous cloning and enzymatic deacylation reaction. J Ind Microbiol Biotechnol 24:1730180

Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol Microbiol 49:807–821

Lai CC, Tan CK, Lin SH, Liao CH, Chou CH, Hsu HL, Huang YT, Hsueh PR (2009) Comparative in vitro activities of nemonoxicin, doripenem, tigecycline and 16 other antimicrobials against *Nocardia braziensis*, *Nocardia asteroides* and unusual *Nocardia* species. J Antimicrob Chemother 64:73–78

Mehta S, Cuirolo AX, Plata KB, Riosa S, Silverman JA, Rubio A, Rosato RR, Rosato AE (2012) 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 56:92–102

Miao V, Coëffet-LeGal M-F, Brian P, Brost R, Penn J, Whiting A, Martin S, Ford R, Parr I, Bouchard M, Silva CJ, Wrigley SK, Baltz RH (2005) Daptomycin biosynthesis in *Streptomyces roseosporus*: cloning and analysis of the gene cluster and revision of peptide stereochemistry. Microbiology 151:1507–1523

Miao V, Brost R, Chapple J, She K, Coëffet-Le Gal M-F, Baltz RH (2006a) The lipopeptide antibiotic A54145 biosynthetic gene cluster from *Streptomyces fradiae*. J Ind Microbiol Biotechnol 33:129–140

Miao V, Coëffet-Le Gal M-F, Nguyen K, Brian P, Penn J, Whiting A, Steele J, Kau D, Martin S, Ford R, Gibson T, Bouchard M, Wrigley SK, Baltz RH (2006b) Genetic engineering in *Streptomyces roseoporus* to produce hybrid lipopeptide antibiotics. Chem Biol 13:269–276

Mishra NN, Yang SJ, Sawa A, Rubio A, Nast CC, Yeaman MR, Bayer AS (2009) Analysis of cell membrane characteristics of in vitro-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53:2312–2318

Mishra NN, McKinnell J, Yeaman MR, Rubio A, Nast CC, Chen L, Kreiswirth BN, Bayer AS (2011) In vitro cross-resistance to daptomycin and host defense cationic antimicrobial

peptides in clinical methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob Agents Chemother 55:4012–4018

Moise PA, North D, Steenbergen JN, Sakoulas G (2009) Susceptibility relationship between vancomycin and daptomycin in *Staphylococcus aureus*: facts and assumptions. Lancet Infect Dis 9:617–624

Mukhopadhyay K, Whitmore W, Xiong YQ et al (2007) In vitro susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry. Microbiology 153:1187–1197

Muraih JK, Pearson A, Silverman J, Palmer M (2011) Oligomerization of daptomycin on membranes. Biochim Biophys Acta 1808:1154–1160

Muraih JK, Harris J, Taylor SD, Palmer M (2012) Characterization of daptomycin oligomerization with perylene excimer fluorescence: stoichiometric binding of phosphatidylglycerol triggers oligomer formation. Biochim Biophys Acta 1818:1154–1160

Murthy MH, Olson ME, Wickert RW, Fey PD, Jalali Z (2008) Daptomycin non-susceptible meticillin-resistant *Staphylococcus aureus* USA 300 isolate. J Med Microbiol 57:1036–1038

Muthaiyan A, Silverman JA, Jayaswal RK, Wilkerson BJ (2008) Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. Antimicrob Agents Chemother 52:980–990

Ng W-L, Kasmierczak KM, Winkler ME (2004) Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. Mol Microbiol 53:1161–1175

Nguyen K, Ritz D, Gu J-Q, Alexander D, Chu M, Miao V, Brian P, Baltz RH (2006) Combinatorial biosynthesis of lipopeptide antibiotics related to daptomycin. Proc Natl Acad Sci USA 103:17462–17467

Nguyen K, He X, Alexander DC, Li C, Gu J-Q, Mascio C, Van Praagh A, Morton L, Chu M, Silverman JA, Brian P, Baltz RH (2010) Genetically engineered lipopeptide antibiotics related to A54145 and daptomycin with improved properties. Antimicrob Agents Chemother 54:1404–1413

Nóren T, Alriksson I, Åkerlund T, Burman LG, Unemo M (2010) In vitro susceptibility to 17 antimicrobials of clinical *Clostridium difficile* isolates collected in 1993-2007 in Sweden. Clin Microbiol Infect 16:1104–1110

Okada A, Igarashi M, Okajima T et al (2010) Walkmycin B targets WalK (YycG), a histidine kinase essential for bacterial cell growth. J Antibiot 63:89–94

Oleson FB Jr, Berman CL, Kirkpatrick JB, Regan KS, Lai JJ, Tally FP (2000) Once-daily dosing in dogs optimizes daptomycin safety. Antimicrob Agents Chemother 44:2948–2953

Palmer KL, Daniel A, Hardy C, Silverman J, Gilmore MS (2011) Genetic basis for daptomycin resistance in enterococci. Antimicrob Agents Chemother 55:3345–3356

Patel D, Husain M, Vidaillac C, Steed ME, Rybak MJ, Seo SM, Kaatz GW (2011) Mechanisms of in vitro-selected daptomycin-non-susceptiblity in *Staphylococcus aureus*. Int J Antimicrob Agents 38:442–446

Patino H, Stevens C, Louie T et al (2011) Efficacy and safety of lipopeptide CB-183,315 for the treatment of *Clostridium difficile* infection. In: Abstracts of the 51st interscience conference on antimicrobial agents and chemotherapy, Chicago, IL, 17–20 September

Peleg AY, Miyakis S, Ward DV et al (2012) Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of *Staphylococcus aureus*. PLoS ONE 7:e28316

Penn J, Li X, Whiting A, Latif M, Gibson T, Silva CJ, Brian P, Davies J, Miao V, Wrigley SK, Baltz RH (2006) Heterologous production of daptomycin in *Streptomyces lividans*. J Ind Microbiol Biotechnol 33:121–128

Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, van Kessel KPM, van Strijp JAG (2001) *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 193:1067–1076

Pertel PE, Bernardo P, Fogerty C, Matthews P, Northland R, Benvenuto M, Thorne GM, Luperchio SA, Arbeit RD, Alder J (2008) Effects of prior effective therapy on the efficacy of daptomycin and cephtriaxone for the treatment of community-acquired pneumonia. Clin Infect Dis 46:1142–1151

Raad I, Hanna H, Jiang Y, Dvorak T, Reitzel R, Chaiban G, Sherertz R, Hachem R (2007) Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant *Staphylococcus* bacteremic isolates embedded in biofilm. Antimicrob Agents Chemother 51:1656–1660

Reipert A, Ehlert K, Kast T, Bierbaum G (2003) Morphological and genetic differences in two isogenic *Staphylococcus aureus* strains with decreased susceptibilities to vancomycin. Antimicrob Agents Chemother 47:568–576

Richardson MA, Kuhstoss S, Huber ML, Ford L, Godfrey O, Turner JR, Rao RN (1990) Cloning of spiramycin biosynthetic genes and their use in constructing *Streptomyces ambofaciens* mutants defective in spiramycin biosynthesis. J Bacteriol 172:3790–3798

Rotondi KS, Gierasch LM (2005) A well-defined amphipathic conformation for the calcium-free cyclic lipopeptide antibiotic, daptomycin, in aqueus solution. Biopolymers 80:374–385

Rubinchik E, Schneider T, Elliott M, Scott WRP, Pan J, Ankin C, Yang H, Dugourd D, Müller A, Gries K, Straus SK, Sahl HG, Hancock REW (2011) Mechanism of action and limited cross-resistance of new lipopeptide MX-2401. Antimicrob Agents Chemother 55:2743–2754

Rubio A, Conrad M, Haselbeck RJ, Kedar GC, Brown-Driver V, Finn J, Silverman JA (2011) Regulation of *mprF* antisense RNA restores daptomycin susceptibility to daptomycin-resistant isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother 55:364–367

Rubio A, Moore J, Varaglu M, Conrad M, Chu M, Shaw W, Silverman JA (2012) LC-MS/MS characterization of phospholipid content in daptomycin-susceptible and -resistant isolates of *Staphylococcus aureus* with mutations in *mprF*. Mol Membr Biol 29:1–8

Sakoulas G, Rose W, Rybak MJ, Pillai S, Alder J, Moellering RC, Eliopoulos GM (2008) Evaluation of endocarditis caused by methicillin-susceptible *Staphylococcus aureus* developing nonsusceptibility to daptomycin. J Clin Microbiol 46:220–224

Sass V, Schneider T, Wilmes M, Körner C, Tossi A, Novikova N, Shamova O, Sahl HG (2010) Human β-defensin 3 inhibits cell wall biosynthesis in staphylococci. Infect Immun 78:2793–2800

Schneider T, Sahl H-G (2010) An oldie but goodie – cell wall biosynthesis as antibiotic target pathway. Int J Med Microbiol 300:161–169

Schneider T, Gries K, Wiedemann I, Pelzer S, Labischinski H, Sahl HG (2009) The lipopeptide antibiotic Friulimicin B inhibits cell wall biosynthesis through complex formation with bactoprenol phosphate. Antimicrob Agents Chemother 53:1610–1618

Scott WRP, Baek S-B, Jung D, Hancock REW, Straus SK (2007) NMR structural studies of the antibiotic lipopeptide daptomycin in DHPC micelles. Biochim Biophys Acta 1768:3116–3146

Siedlecki J, Hill J, Parr I et al (2003) Array synthesis of novel lipodepsipeptide. Bioorg Med Chem Lett 13:4245–4249

Silverman JA, Perlmutter NG, Shapiro HM (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother 47:2538–2544

Silverman JA, Morton LI, Vanpraagh AD, Li T, Alder J (2005) Inhibition of daptomycin by pulmonary surfactant: in vitro modeling and clinical impact. J Infect Dis 191:2149–2152

Steenbergen JN, Alder J, Thorne GM, Tally FP (2005) Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J Antimicrob Chemother 55:283–288

Straus SK, Hancock REW (2006) Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. Biochim Biophys Acta 1758:1215–1223

Strieker M, Marahiel MA (2009) The structural diversity of acidic lipopeptide antibiotics. ChemBioChem 10:607–616

Szurmant H, Mohan MA, Imus M, Hoch JA (2007) YycH and YycI interact to regulate the essential YycFG two-component system in *Bacillus subtilis*. J Bacteriol 189:3280–3289

Szurmant H, Bu L, Brooks CL, Hoch JA (2008) An essential sensor histidine kinase controlled by transmembrane helix interactions with its auxiliary proteins. Proc Natl Acad Sci U S A 105:5891–5896

Tyrrell KL, Citron DM, Warren YA, Fernandez HT, Merriam CV, Goldstein EJC (2006) In vitro activities of daptomycin, vancomycin, and penicillin against *Clostridium difficile*, *C. perfringens*, *Finegoldia magna*, and *Propionibacterium acnes*. Antimicrob Agents Chemother 50:2728–2731

Vértesy L, Ehlers E, Kogler H, Kurz M, Meiwes J, Seibert G, Vogle M, Hammann P (2000) Friulimicins: novel lipopeptide antibiotics with peptidoglycan synthesis inhibiting activity from *Actinoplanes friuliensis* sp. nov. II. Isolation and structural characterization. J Antibiot 53:816–827

Watanabe Y, Cui L, Katayama Y, Kozue K, Hiramatsu K (2011) Impact of *rpoB* mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. J Clin Microbiol 49:2680–2684

Wecke T, Zühlke D, Mäder U et al (2009) Daptomycin versus friulimicin B: in-depth profiling of *Bacillus subtilis* cell envelope stress response. Antimicrob Agents Chemother 53:1619–1623

Winkler ME, Hock JA (2008) Essentiality, bypass, and targeting of YycFG (VicRK) two-component regulatory system in Gram-positive bacteria. J Bacteriol 190:2645–2648

Yang S-J, Xiong YQ, Dunman PM, Schrenzel J, Francois P, Pechel A, Bayer AS (2009) Regulation of *mprF* in daptomycin-nonsusceptible *Staphylococcus aureus* strains. Antimicrob Agents Chemother 53:2636–2637

Yang S-J, Nast CC, Mishra NN, Yeaman MR, Fey PD, Bayer AS (2010) Cell wall thickening is not a universal accompaniment of the daptomycin nonsusceptibility phenotype in *Staphylococcus aureus*: evidence for multiple resistance mechanisms. Antimicrob Agents Chemother 54:3079–3085

Chapter 7
Lantibiotics and Similar Peptides Produced by and Active on Gram-Positives: Discovery, Development and Perspectives

Jesus Cortes

Abstract Lantibiotics are a group of ribosomally synthesised peptides that contain post-translational modifications consisting of (methyl)lanthionine residues forming bridges that confer them characteristic structures. Most lantibiotics are antibacterials that bind to the cell wall precursor lipid II. They can be rod shaped or globular depending on the distribution of the lanthionine residues. Their biosynthetic pathway is relatively simple when compared to other secondary metabolites. Due to this simplicity, genetic manipulation of the pathways is a very attractive tool to obtain variants that might have improved properties. Mutagenesis programmes have shown that the biosynthetic machinery of lantibiotics has relaxed specificity allowing the production of large collections of variants. Lantibiotic gene clusters are a common feature within bacteria, particularly Gram-positive organisms. Genome mining and in vitro synthesis experiments suggest that there is a great potential for discovery of new lantibiotics. With the increasing need for effective antibiotics against multidrug-resistant pathogens, lantibiotics are an attractive option for a new class of molecules. There are two lantibiotics in late preclinical development for use against systemic Gram-positive infections, one lantibiotic in Phase 1 clinical trials for the treatment of *Clostridium difficile* infections and another lantibiotic in Phase 2b for the treatment of cystic fibrosis. The potential of lantibiotics for the treatment of bacterial infections should become a reality in the next few years with the current compounds going through the corresponding drug development stages and new compounds joining the collection of useful compounds in the fight against multidrug-resistant bacteria.

J. Cortes (✉)
Entrechem SL, Edificio Severo Ochoa, Campus El Cristo. 33006 Oviedo, Spain
e-mail: jesus.cortes@entrechem.com

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_7,

7.1 Introduction

The emergence and prevalence of antibiotic resistant pathogens demands an increasing effort to identify, develop and design new antibiotics. The discovery of new antimicrobial agents has been outpaced by the occurrence of new antibiotic resistance mechanisms underpinning the need for new classes of antimicrobial compounds, especially those with new modes of action. There is a particular interest and need for antibiotics of novel structures expecting them to interact with their targets via a new mechanism. The lantibiotics, to this effect, offer an exciting alternative to conventional antibiotics as they do not seem to show cross-resistance with any of the major classes of antibiotics currently used in the clinical set-up. Like the antibiotic vancomycin (see Marcone and Marinelli, this volume), most lantibiotics target the peptidoglycan precursor lipid II, an essential intermediate for bacterial cell wall synthesis. Vancomycin resistant strains are still susceptible to lantibiotics, which interact with a different part of lipid II. Vancomycin binds to the terminal dipeptide D-ala-D-ala and the lantibiotics to the pyrophosphate moiety (Hsu et al. 2006). Lantibiotics are a large and diverse group of modified peptides produced by Gram-positive bacteria sharing the common feature of lanthionine or methyl-lanthionine ring structures (Sahl and Bierbaum 1998). This group of compounds is derived from ribosomally synthesized peptides that are subject to post-translational modifications. Serine or threonine residues are dehydrated to dehydroalanine (Da) or dehydrobutyrine (Db) respectively. These residues undergo Michael addition with the thiol group of cysteine residues from the same polypeptide to form the characteristic (methyl)lanthionine rings (Fig. 7.1). Apart from these distinctive rings, some lantibiotics also contain other modified residues or ring structures, such as the 2–thioethenamine moiety in mersacidin (Chatterjee et al. 1992) or epidermin (Allgaier et al. 1986), the lysinoalanine ring and hydroxyaspartate in cinnamycin (Kessler et al. 1987), D–alanine in lactocin S (Skaugen et al. 1994) and lacticin 3147 (Ryan et al. 1999), an N–terminal oxobutyryl group in Pep5 (Kellner et al.1989) and lacticin 3147–A2 (Ryan et al. 1999) and dihydroxyproline and chlorotryptophan in microbisporicin (Castiglione et al. 2008). Reports on lantibiotics date back to 1928 when nisin, the prototypical lantibiotic, was first described as a compound with antibacterial activity (Rogers and Whittier 1928) and was isolated from *Lactococcus lactis* cultures by Mattick and Hirsch in 1947. Further research on this lantibiotic and its properties has become key for the development and widespread use as a food preservative sold in more than 40 countries. It is currently in the list of authorized food additives by the European Union as additive number E234 (EEC. 1983 *EEC Commission Directive* 83/463/EEC.) and has been approved by the US Food and Drug Administration (Delves-Broughton 1990). The lantibiotics have been classified into two types depending on their structure.

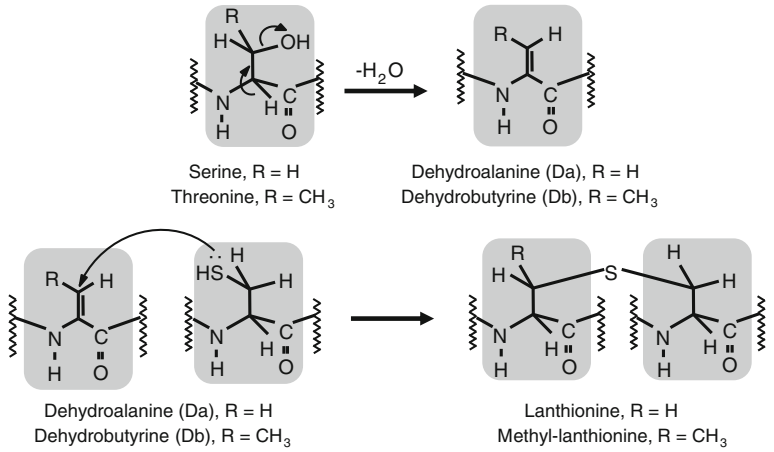


Fig. 7.1 Modification of peptides by lantibiotic synthetases. Ribosomally synthesized peptides are dehydrated by LanB or LanM enzymes, serine and threonine residues are converted into dehydroalanine and dehydrobutyrine, respectively. Subsequently, LanC or LanM catalyse the intramolecular addition of cysteine thiols onto the dehydroamino acids in a stereo- and regioselective manner to form lanthionine and methyl-lanthionine

7.1.1 Type A Lantibiotics

Type A lantibiotics are elongated amphiphilic cationic peptides that form pores in bacterial membranes, leading to cell death. The most representative lantibiotics of this group are the nisins, epidermins and the lacticin 481-nukacin group (Fig. 7.2). Pore formation of type A lantibiotics has been studied in detail using various membrane systems. Driessen et al. (1995) suggested a "wedge model" for pore formation in which nisin induces perturbation of the membrane bilayer in a surface-bound configuration. However, micromolar concentrations are required to provoke perturbation. In contrast, many Gram-positive bacteria have MICs in the nanomolar concentration range. An explanation for this discrepancy was provided when lipid II was identified as a docking molecule enabling pore formation in model membranes at nanomolar concentrations of nisin (Brötz et al. 1998a, b). Further work on their mode of action has shown that some type A lantibiotics actually can exert a dual mode of action as many of them bind to lipid II and disturb the membrane. A lipid II binding domain has been identified for nisin consisting in the first two lanthionine rings that interact with the pyrophosphate cage of lipid II (Hsu et al. 2004); these rings are a common feature in the nisins, subtilin and epidermins (Fig. 7.2), while the lacticin 481-nukacin group seems to bind lipid II by docking with the first lanthionine ring (Islam et al. 2012). Not all type A lantibiotics are pore formers, the members of the lacticin 481-nukacin group are not membrane disruptors and in the case of the epidermins, membrane disruption seems to depend on cell wall thickness of the target organism.

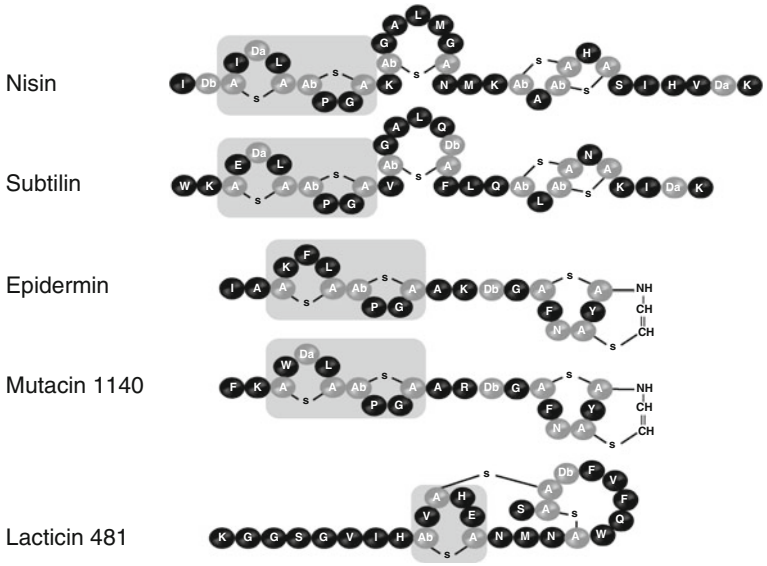


Fig. 7.2 Structures of representative Type A lantibiotics. Modified residues have been coloured *grey*. Da: dehydroalanine; Db: dehydrobutyrine; Ab: 2-aminobutyrate. The *grey boxes* show the lanthionine rings involved in binding to lipid II

7.1.2 Type B Lantibiotics

Type B lantibiotics are a diverse group of compounds containing, in general, overlapped lanthionine rings conferring them a globular structure (Fig. 7.3). In contrast to type A lantibiotics the members of this group do not have a specific overall charge. The main representatives of this group are mersacidin, the actagardines and the cinnamycins. The most studied member of this group is mersacidin, a tetracyclic peptide (Fig. 7.3) containing 20 amino acid residues produced by *Bacillus* sp. at late stages of growth and stationary phase (Chatterjee et al. 1992). Mersacidin has antibacterial activity against Gram-positive pathogens by binding to lipid II. Binding experiments of mersacidin to lipid II in the presence of vancomycin suggest that mersacidin binds to a different part of the molecule (Brötz et al. 1998a) hence no cross-resistance has been observed. Significantly, in vivo studies have shown that mersacidin subcutaneously administered, cures systemic multidrug resistant *Staphylococcus aureus* (MRSA) infections in mice and abscesses in rats at concentrations comparable to vancomycin (Chatterjee et al. 1992). The activity of mersacidin against MRSA makes it a potential candidate for development as a therapeutic agent. The actagardine group comprises actagardine A (Zimmermann et al. 1995), deoxyactagardine B (Boakes et al. 2010) and michiganin A (Holtsmark et al. 2006). This group of compounds has in vitro antibacterial activity comparable to mersacidin and shares the mechanism of action by binding to lipid II, mediated very likely, by the ring containing the

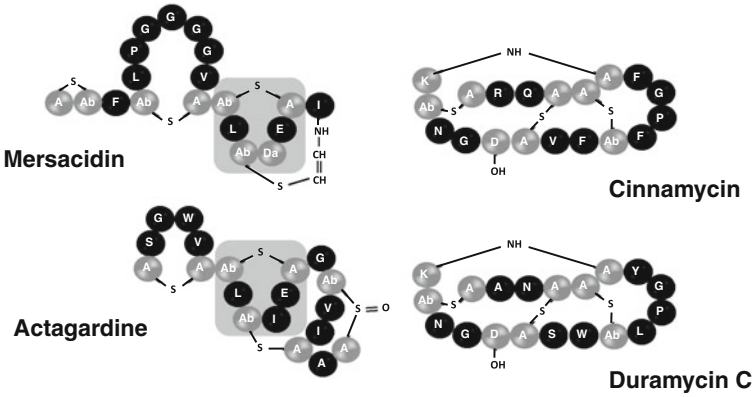


Fig. 7.3 Structures of representative Type B lantibiotics. Modified residues have been coloured *grey*. Da: dehydroalanine; Db: dehydrobutyrine; Ab: 2-aminobutyrate. The *grey boxes* show the lanthionine rings involved in binding to lipid II

glutamate residue (Fig. 7.3) as proposed by Islam et al. (2012). The cinnamycin group comprises cinnamycin, the duramycins and ancovenin and they are all produced by actinomycetes. These compounds are different from the lantibiotics mentioned above as their antibacterial activity is not mediated by binding to lipid II; this group displays a number of activities mediated by binding to phosphatidylethanolamine, a lipid found in biological membranes.

7.1.3 Two-component Lantibotics: Lacticin 3147

A separate group is formed by the two-component lantibiotics that consist of two post-translationally modified peptides with weak antibacterial activity but synergistically displaying strong antibacterial action. From the structure point of view, one of the peptides, the α peptide, seems to have a type B conformation with structural homology to mersacidin. The β peptide has an elongated shape that reminds of type A lantibiotics (Fig. 7.4). It is believed that the α peptide binds lipid II and the β peptide is a membrane disruptor. This group of compounds includes lacticin 3147, haloduracin, lichenicidin, staphylococcin C55 and plantaricin W, among others.

7.1.4 The Microbisporicin–Planosporicin Group

This group contains two lantibiotics with mixed structural features, microbisporicin and planosporicin. The N-terminus of these compounds has two distinctive lanthionine rings involved in lipid II binding, similar to nisin and epidermin

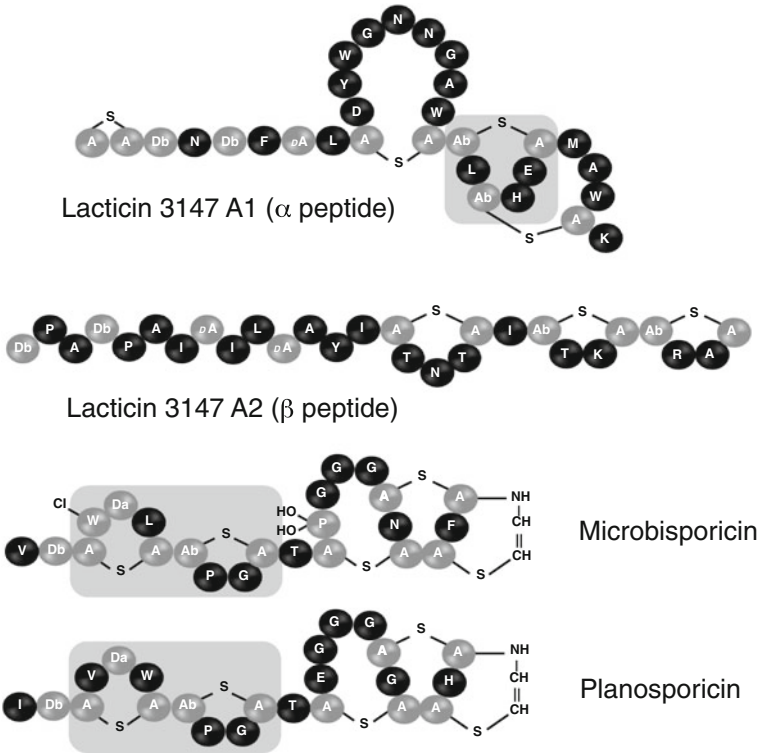


Fig. 7.4 Structures of the two-component lantibiotic lacticin 3147 A1 and A2 peptides, microbisporicin and planosporicin. Modified residues have been coloured *grey*. Da: dehydroalanine; Db: dehydrobutyrine; Ab: 2-aminobutyrate; DA: D-alanine. The *grey boxes* show the lanthionine rings involved in binding to lipid II

(Fig. 7.4) and presumably would have a rod-shaped conformation. The C-terminus is composed of a mixture of overlapping rings more characteristic of type B lantibiotics and an epidermin-like carboxy-terminal ring. These compounds were discovered during screening of natural products that selectively block peptidoglycan biosynthesis applied to uncommon actinomycetes (Castiglione et al. 2007, 2008). The members of this group are potent antibacterial agents active against a wide variety of Gram-positive pathogens, in particular microbisporicin, considered the most potent antibacterial among known lantibiotics.

7.2 Biosynthesis

The genes encoding the biosynthetic pathways for a considerable number of lantibiotics have been cloned and sequenced and their functions have been inferred based on homology with genes of known function, gene disruptions, enzymatic

studies and heterologous expression of biosynthetic clusters. Like many other antibiotics or secondary metabolites in general, the biosynthetic genes are clustered together. The lantibiotic genes have been given the generic locus name *lan.* They are assigned *lanA* for the gene encoding the lantibiotic prepeptide, *lanB* for the gene encoding the polypeptide responsible for serine or threonine dehydration and *lanC* for the gene responsible for the lanthionine cycle formation. In some cases the activities of LanB and LanC are part of a bifunctional enzyme encoded by *lanM* (Sahl and Bierbaum 1998). Depending on whether the lantibiotics are produced by a LanB/LanC combination or a LanM bifunctional enzyme, they are classified as class I or class II respectively. The biosynthetic gene clusters of representative lantibiotics are presented in Fig. 7.5. The products of the *lanP*, *lanT* and *lanH* genes are involved in processing and/or transport of the prepeptide and *lanI*, *lanE*, *lanF* and *lanG* encode proteins involved in immunity or resistance. Other genes specific for particular features of some lantibiotics have been identified as well, like *lanD* involved in the oxidative decarboxylation of the C-terminal cysteine of epidermin, named *epiD* (Kupke et al. 1992), or of mersacidin, named *mrsD* (Majer et al. 2002); *cinX* responsible for the hydroxylation of aspartate in cinnamycin (Okesli et al. 2011); *garO* responsible for the sulfoxide formation in actagardine (Boakes et al. 2009) or *mibH* and *mibO* responsible for the chlorination of tryptophan and hydroxylation of proline in microbisporicin (Foulston and Bibb 2010). The gene *lanA* encodes the peptide containing the lantibiotic sequence preceded of a leader peptide; this leader sequence varies in length independently of the type of lantibiotic and it does not contain cysteine

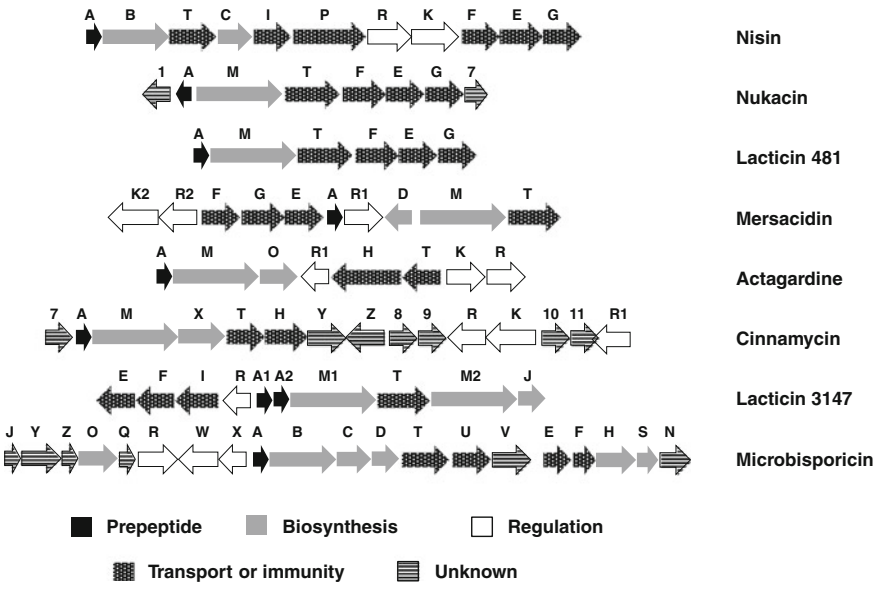


Fig. 7.5 Organisation of representative lantibiotic biosynthetic gene clusters

residues. Consensus motifs have been proposed for lantibiotic leader sequences like FNLD (Chatterjee et al. 2005) or DLD (Marsh et al. 2010) present in most of class I lantibiotics or PAG in some class II lantibiotics (Begley et al. 2009). The leader sequences of class II lantibiotics, in general, contain a double glycine GG or GA proposed to be the protease recognition/cleavage sequence (Chatterjee et al. 2005). The role of the leader sequence is not clear. It may play a role in protection of the host cell against the antibacterial activity of the lantibiotic; a possible role in recognition for the transport machinery outside the producer has been suggested; finally a possible molecular recognition role for the enzymes involved in processing of the peptide by LanB/C or LanM has been proposed. Experimental evidence using the lacticin 481 system suggests that the leader sequence is required for the formation of the lanthionine bridges although changes in specific amino acid sequence did not affect the activity of the processing enzymes but substitutions using proline abolished processing (Patton et al. 2008). Perhaps single changes do not alter the overall conformation of the leader peptide apart from the known disruptive effect of proline. Considerable progress concerning the biochemistry of the modification reactions of lantibiotics has been reported in recent years. In vitro modification systems using LctM from lacticin 481, HalM1/HalM2 from haloduracin, NisC from nisin and CinM from cinnamycin have been successfully established, and the mechanism and residues involved in the active sites identified (Xie et al. 2004; Li et al. 2006; You et al. 2007; Paul et al. 2007; Helfrich et al. 2007; McClerren et al. 2006). It has been shown, using the lacticin 481 in vitro system, that LctM utilises ATP and Mg^{2+} to selectively phosphorylate hydroxy amino acids of the prepeptide before double bond formation (Chatterjee et al. 2005). There is evidence that lanthionine formation is not necessarily the first step on lantibiotics biosynthesis, the mersacidin prepeptide MrsA is decarboxylated and oxidised by MrsD producing a 2-thioethenamine functionality at the C-terminal cysteine. This modified MrsA is proposed to be the substrate for MrsM that produces the tetracyclic structure (Majer et al. 2002). Once the lanthionine rings have been formed, other processing or tailoring enzymes modify the prepeptide and the transport machinery LanT exports the prepeptide outside the cell. Cleavage of the leader peptide is carried out by LanP but in some cases the transport polypeptides LanT are bifunctional, containing a protease domain responsible for the removal of this leader peptide like MrsT in the mersacidin pathway (Altena et al. 2000). There are biosynthetic pathways lacking genes encoding for proteases to remove the leader peptide like cinnamycin (Widdick et al. 2003), actagardine (Boakes et al. 2009) or deoxyactagardine B (Boakes et al. 2010); in these cases it is believed that unspecific proteases from the producer organism remove the leader peptide. The requirement of the leader peptide for further processing of the lantibiotic by the tailoring enzymes remains unclear: it is likely that processing enzymes can accept both, the lantibiotic containing the leader peptide or the lantibiotic itself as substrate. As example, CinX —an aspartate hydroxylase —and CinM— the lanthionine synthetase— from the cinnamycin biosynthetic pathway do not have a specific order of reaction and CinX can hydroxylate CinA and a leaderless CinA as well (Okesli et al. 2011).

7.3 Regulation

Lantibiotic production in many organisms is controlled by a two-component system comprised of a receptor histidine kinase LanK and a transcriptional response regulator LanR (Fig. 7.5). The histidine kinase receptors are located on the cellular surface and are involved in detecting extracellular signals or changes. A phosphorylation-mediated cascade is initiated by modification of a histidine residue in this receptor that is transferred to an aspartate residue of the regulator, which is the mediator of the activation of specific *lan* operons. For the nisin regulation network, the phosphoryl group from *nisK* is transferred to *nisR* which subsequently binds to the *nisA* and *nisF* operators (Fig. 7.5). This in turn activates transcription of the *nisABTCIP* operon involved in nisin biosynthesis as well as *nisFEG* involved in immunity (Chatterjee et al. 2005). The genes *nisR* and *nisK* are constitutively expressed and the effector that produces the phosphorylation cascade is nisin itself (Lubelski et al. 2008). The regulation network of mersacidin is different, the sensor/kinase system *mrsR2/mrsK2* can be deleted in the producer organism without affecting mersacidin production (Guder et al. 2002); this system regulates the immunity genes of the operon *mrsFEG*. The biosynthetic genes are regulated by *mrsR1*, an OmpR like protein with the distinctive HTH DNA binding domain but without a kinase partner. Mersacidin, like nisin, is an inducer of its own biosynthesis (Schmitz et al. 2006). Lacticin 481 does not have any regulatory genes in the biosynthetic cluster (Fig. 7.5); it is believed that general regulatory mechanisms may control its biosynthesis like pH control (Hindré et al. 2004). A single gene *epiQ* is the regulator of epidermin biosynthesis, this gene product binds to the *epiA* operator and induces the synthesis of all the biosynthetic genes required to produce epidermin (Peschel et al. 1993). For the two-component lantibiotic lacticin 3147, the transcriptional repressor LtnR regulates its own transcription and that of the downstream immunity genes *ltnIFE*. The biosynthesis of lacticin 3147 is not regulated by LtnR, expression from the promoter preceding the biosynthetic genes appears to be constitutive (McAuliffe et al. 2001). In the case of microbisporicin, the biosynthetic gene cluster encodes several putative regulatory proteins, including an extracytoplasmic function σ factor MibX, a likely cognate anti-σ factor MibW, and a helix-turn-helix DNA binding protein, *mibR*. S1 nuclease protection assays were used to determine transcriptional start sites in the microbisporicin gene cluster and confirmed the presence of the likely ECF sigma factor of -10 and -35 sequences in five out of six promoters. The promoter of *mibA*, encoding the microbisporicin prepropeptide, has a typical *Streptomyces* vegetative sigma factor consensus sequence. MibX interacts with the anti-sigma factor MibW and autoregulates its own expression but does not regulate expression of *mibA* (Foulston and Bibb 2011). On the basis of quantitative reverse transcriptase PCR data, a model for the biosynthesis of microbisporicin has been proposed in which MibR functions as an essential master regulator and the ECF sigma factor/anti-sigma factor pair, σ(MibX)/MibW, induces feedforward biosynthesis of microbisporicin and immunity, adding this lantibiotic to the list of

autoinducers of their own biosynthetic pathway. Lantibiotics biosynthesis can be regulated, like other secondary metabolites, by general regulation systems like cell density, growth phase or carbon catabolite repression, but pleiotropic regulators associated to the control of lantibiotic pathways have not been identified so far. A clearer understanding of regulatory contributors in lantibiotics biosynthesis will be required in the future to increase production of these compounds for their application in therapeutic or food preservation areas.

7.4 Drug Discovery and Development

Several studies have been carried out to manipulate the structure and biological activity of lantibiotics by site-directed mutagenesis of the *lanA* genes (Cotter et al. 2006). Some specific mutations for different lantibiotics are tolerated and fully processed lantibiotics are synthesised, but others result in partially processed lantibiotics or abolish production altogether. Since the first systematic mutant analysis of the two-component lantibiotic lacticin 3147 (Cotter et al. 2006) by alanine scanning mutagenesis suggesting that there are particular areas within the peptides that are amenable to changes and areas that are essential for production of the final compound (Cotter et al. 2006), extensive investigations have been carried out involving the lantibiotic nisin (Rink et al. 2007; Field et al. 2008), mersacidin (Appleyard et al. 2009c), actagardine (Dawson et al. 2011), lacticin 3147 (Field et al. 2007) and nukacin (Islam et al. 2009). Lantibiotic variants with improved antibacterial properties have been found for these compounds. Further studies including multiple mutations, construction of hybrid molecules and the making of new lantibiotic variants with improved pharmacokinetics will define the potential to develop this type of antibiotics for therapeutic use. The activity of lantibiotics is not necessarily restricted to Gram-positive bacteria; there is a report of a lantibiotic produced by *Bifidobacterium longum* active against *Escherichia coli*, *Serratia*, *Proteus* and *Salmonella* spp. (O′Sullivan and Lee 2009).

Regarding the discovery of new lantibiotics, genome mining studies searching for *lanB-lanC* gene combinations (Marsh et al. 2010) or *lanM* genes (Begley et al. 2009) within sequences of published bacterial genomes have shown that the presence of lantibiotic gene clusters in bacterial genomes is a relatively frequent feature and the sequence of a variety of putative new lantibiotics has been proposed. Analysis of the *Streptomyces venezuelae* chromosome and in vitro production of a predicted encoded lantibiotic lead to the discovery of venezuelin, a new type of lantibiotic produced by a new type of kinase/lyase/cyclase system called LanL (Goto et al. 2010). DNA amplification screening of actinomycetes using oligonucleotides encoding conserved regions of *lanM* has shown that class II lantibiotic biosynthetic genes are present in over 20 % of the tested actinomycete strains (Dodd et al. 2006). Finding expression conditions of these clusters for the characterization of the encoded lantibiotics remains a challenge. An alternative system that has been successful for isolating and the characterization of

lantibiotics from 'cryptic' pathways has been the heterologous expression of operons encoding genes responsible for the synthesis of lantibiotics in *E. coli* and/or in vitro production of the target lantibiotic (McClerren et al. 2006). Regarding drug discovery, lantibiotics have just emerged and it is likely that plenty of new structures will be available in the next few years. There are reports of three lantibiotics in late preclinical or early clinical development stages for the treatment of bacterial infections and one lantibiotic in clinical development for the treatment of cystic fibrosis and dry eye syndrome.

7.4.1 Mutacin 1140

Mutacin 1140 (MU1140) is a lantibiotic produced by *Streptococcus mutans* with activity against a broad spectrum of Gram-positive bacteria (Fig. 7.2). It is a structural analogue of epidermin. Like many of the lipid II binding antibiotics, sensitive species of bacteria tested were unable to acquire stable, spontaneous resistance. After 21 sequential passages in sub-inhibitory concentrations of mutacin 1140, there was only a slight ($\leq$4 fold) increase in the MIC against *S. pneumoniae* or *S. aureus*.

Time-kill experiments using *S. aureus, Streptococcus pneumoniae,* and *Enterococcus faecalis* show that mutacin 1140 is bactericidal against *S. aureus* and *S. pneumoniae* and bacteriostatic against *E. faecalis*. The addition of human or rat serum to MIC evaluation of mutacin 1140 causes a reduction of activity against *S. pneumoniae,* presumably due to significant binding to serum proteins (92 %), but serum was found to cause an unexplained increase in activity against *S. aureus.* Other aspects of safety and efficacy that have been tested, including maximum tolerated dose, immunogenicity, cytotoxicity, efficacy in *S. aureus* sepsis models suggest the potential usefulness of mutacin 1140 for the treatment of Gram-positive infectious diseases (Smith and Hillman 2008). The half-life of this lantibiotic is approximately 1.5 h in a rat model. Rapid injection of MU1140 is associated with a hypersensitivity reaction that can be blocked by premedication with diphenhydramine (Ghobrial et al. 2010). Oni Biopharma Inc. (Oragenics Inc.) is currently conducting preclinical trials on this compound.

7.4.2 Microbisporicin

The lantibiotic microbisporicin (Fig.7.4) also known as NAI-107 is produced by the actinomycete *Microbispora corallina* (Castiglione et al. 2008). It has an antibacterial spectrum that covers all Gram-positive pathogens, including methicillin-resistant *S. aureus*, glycopeptide-intermediate *S. aureus*, and vancomycin-resistant enterococci and shows activity against fastidious Gram-negative bacteria, such as *Neisseria* spp., *Haemophilus influenzae*, and *Moraxella catarrhalis* (Jabes et al. 2009).

NAI-107 is very effective in different animal models of infection induced by multidrug resistant bacteria. In a *S. aureus* induced rat endocarditis model, its bactericidal activity resulted in 70 % of treated animals carrying no detectable bacteria in the heart vegetation, in comparison with no sterile vegetations observed with vancomycin at therapeutic doses. In acute lethal infections induced with *S. pneumoniae* in immunocompetent mice, or with *S. aureus* or enterococci strains in neutropenic mice, the ED_{50} values of NAI-107 were comparable or lower than those of reference compounds. In an *S. aureus*-induced granuloma pouch model in rats, NAI-107 showed a dose-proportional bactericidal activity that, at a single 40 mg/kg dose, compared with two 20 mg/kg doses at a 12h or 24h interval, caused a $3\log_{10}$ CFU/ml reduction of viable bacterial counts in exudates that persisted for more than 72 h. In an *S. aureus* endocarditis model induced in rats, NAI-107 was effective in reducing the bacterial load in heart vegetations in a dose-dependent manner. The rapid bactericidal activity of NAI-107 observed in vitro correlates well with the efficacy in several models of infection induced by Gram-positive pathogens regardless of its high protein binding (>93%) observed for rat and human serum (Jabes et al. 2011). Sentinella Pharmaceuticals is developing NAI-107 for the treatment of multi-drug resistant Gram-positive infections in hospitalized patients.

7.4.3 NVB302

NVB302 is a semisynthetic derivative of deoxyactagardine B (Boakes et al. 2010), currently under development by Novacta Biosystems. NVB302 is prepared by the selective peptide coupling of 1, 7-diaminoheptane to the terminal carboxylate of deoxyactagardine B (Appleyard et al. 2009a). NVB302 inhibits the growth of clinical isolates *of Clostridium difficile* at concentrations comparable to vancomycin with MICs of 2 to 1 μg/ml (Wadman et al. 2009). NVB302 and is not active against most of the tested Gram-negative strains with MICs >64 μg/ml. NVB302 is active against most of the not-spore-forming Gram-positive rods, though only poorly against *Eubacterium* and *Lactobacillus* and it is active against other clostridia. Low antibacterial activity against other microbes in the gastrointestinal tract is a desirable profile as it is believed that recurrence of *C. difficile* infection is due to unbalanced intestinal flora after antibiotic treatment (see Rossolini et al., this volume). NVB302 is stable in simulated gastric fluid and simulated intestinal fluid over clinically relevant periods and is excreted unchanged in faeces after oral dosing in rats; it is as efficacious as vancomycin in the Syrian hamster cecitis model for *C. difficle* infection (Appleyard et al. 2009b), suggesting that therapeutic doses of NVB302 suitable for the treatment of *C. difficile* infection can be achieved by oral dosing without requiring enteric formulations. NVB302 is well tolerated and did not cause systemic toxicity in animal models. Novacta Biosystems has announced the starting of Phase 1 clinical trials of NVB302 in November 2011, becoming the first molecule of a novel therapeutic class of lantibiotics commencing Phase 1 trials.

7.4.4 *Duramycin A*

Duramycin A (Moli1901) is a structural analogue of cinnamycin differing only in a single substitution of R to K in position 2 of cinnamycin. Duramycin is produced by *Streptomyces cinnamoneum* and is also known as lancovutide or Moli1901. This lantibiotic is undergoing clinical evaluation for its alteration of mammalian cell membranes affecting fluid homeostasis. This phospholipid-targeting activity seems to be relevant in the treatment of cystic fibrosis, dry eye syndrome and is also of potential interest in antiviral and anticancer therapy (Dawson 2006). After a successful Phase 1 clinical trial of duramycin A for treatment of cystic fibrosis, a Phase 2 trial was carried out to assess the safety and tolerability of multiple doses of the medication. This study, completed in 2007, showed a significant improvement in the forced expiratory volume of patients dosed with Moli1901, compared to those who received placebo (Grasemann et al. 2007). A Phase 2b clinical trial began in 2008 to further analyse the change in the predicted forced expiratory volume of participants, establish the minimum effective dose, the optimal dose, and the maximum safe dose of Moli1901. Moli1901 is under co-development by Lantibio Inc. and AOP Orphan Pharmaceuticals.

7.5 Conclusions

Since the discovery of nisin (Fig. 7.2) in 1928, more than 50 different lantibiotics have been characterized from different bacteria, including mainly lactic acid-producing bacteria, actinomycetes and *Bacillus* spp. The lantibiotics are divided into two groups, type A and type B lantibiotics, depending on the structure: type A are peptides with straight-chain rod shaped and type B are globular structures. Taking into account biosynthesis, the lantibiotics were initially divided into class I and class II depending on whether the lanthionine bridges were formed by the LanB/LanC enzyme systems or the LanM bifunctional enzymes. In recent years, two new types of enzymes capable of forming lanthionine bridges have been discovered, the RamC type (kinase/cyclase) and LanL (kinase/ lyase/cyclase) and compounds produced by these enzymes, are grouped as class III and IV lantibiotics. New gene clusters keep appearing coming from different types of organisms. For example, the collection of lantipeptides recently described from *Prochlorococcus* and *Synechococcus* species, produced by a single LanM like enzyme (Li et al. 2010), constitute a new organization with permissive enzymes capable of processing several natural variants encoded by different *lanA* genes. These enzymes with apparently low specificity have the potential to generate more variants from lantibiotics made either naturally or by genetically modifying the *lanA* genes of the producing strains. Surprisingly, this system comes from Gram-negative bacteria where lantibiotics production has not been described before.

The biosynthetic enzymes of lantibiotic pathways have shown remarkably relaxed specificities: hundreds of lantibiotic variants have been produced in different systems and improved antibacterial activity for nisins (Field et al. 2008), mersacidins (Appleyard et al. 2009c) and actagardines (Dawson et al. 2011) have been described. The simplicity in generating lantibiotic variants due to the ribosomal origin of these compounds offers an invaluable tool for generating large collections of lantibiotics. New hybrid compounds can be generated with the regions responsible for different activities like pore forming and lipid II binding from different compounds using enzymes with low specificity. Determination and comparison of the specificity of lanthionine synthetases will be very helpful to obtain new compounds with improved properties. It should be taken into consideration that most lantibiotics are autoinducers; so the new compounds should function as inducers of their own pathway or the biosynthetic genes should be engineered and placed under the control of strong promoters so that expression can take place. The engineered lantibiotics can have improved properties for their use as therapeutic agents. Apart from increased antimicrobial activity, properties like solubility, stability and pharmacokinetics can be improved by changing the amino acid sequence. The remarkable stability of lantibiotics make them an easy target for improvement by semi-synthesis as well. Other unexplored possibility is the introduction of amino acids with the appropriate functionalities in specific positions within the peptide to facilitate medicinal chemistry programmes. The lantibiotic modification enzymes can be used for introduction of lanthionine bridges in other peptides apart from lantibiotics. The introduction of these bridges, for example, instead of natural disulfide bridges in proteins and peptides, is likely to confer more stability and resistance to proteolysis. Cycle formation of therapeutically important peptides is an efficient method to create stable peptides with improved pharmacodynamic properties. Introduction of thioether bridges in peptides has been applied in the past by synthetic approaches. Production of a modified angiotensin using a lanthionine synthetase conferring it enhanced proteolytic resistance with improved activity compared with its natural counterpart, has been described (Kluskens et al. 2009). Lanthio Pharma is a Biopharmaceutical Company specialized in the use of *Lactococcus lactis*, the nisin producer, for recombinant expression of peptides and uses the lantibiotic enzyme system of this strain to introduce lanthionine bridges in specific locations in peptides. It is clear that the potential application of lantibiotics and their biosynthetic pathways in drug discovery and development will become a reality in the next few years; it is likely that more lantibiotics will be incorporated to the antibacterial pipeline and peptides with lanthionine residues will be tested and used as stable substitutes of the corresponding natural analogues. Considering the advances made in the last few years, it is not too optimistic to anticipate a vast increment of lantibiotics in preclinical and clinical stages of drug development with applications for the treatment of systemic and topical bacterial infections in the near future, filling the current gap due to the increasing emergence of multidrug-resistant pathogens.

References

Allgaier H, Jung J, Werner RG, Schneider U, Zahner H (1986) Epidermin: sequencing of a heterodet tetracyclic 21-peptide amide antibiotic. Eur J Biochem 160:9–22

Altena K, Guder A, Cramer C, Bierbaum G (2000) Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. Appl Env Microbiol 66:2565–2571

Appleyard AN, Ayala T, Boakes S, Cortes J, Dawson MJ, Choi S, Lightfoot A, Read D, Todd M, Wadman SN (2009a) NVB302: a narrow spectrum antibiotic under development for the treatment of *Clostridium difficile* infection. 49th Interscience conference on antimicrobial agents and chemotherapy, San Francisco, Poster F1-1517

Appleyard AN, Candiani G, Carman R, Dawson MJ, Read D, Wadman SN (2009b) NVB302: gastrointestinal stability and in vivo activity in the hamster cecitis model for *Clostridium difficile* infection. 49th Interscience conference on antimicrobial agents and chemotherapy, San Francisco, Poster F1-1520

Appleyard AN, Choi S, Read DM, Lightfoot A, Boakes S, Hoffmann A, Chopra I, Bierbaum G, Rudd BA, Dawson MJ, Cortes J (2009c) Dissecting structural and functional diversity of the lantibiotic mersacidin. Chem Biol 16:490–498

Begley M, Cotter PD, Hill C, Ross P (2009) Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for Lan M proteins. Appl Environ Microbiol 75:5451–5460

Boakes S, Cortes J, Appleyard AN, Rudd BA, Dawson MJ (2009) Organization of the genes encoding the biosynthesis of actagardine and engineering a variant generation system. Mol Microbiol 72:1126–1136

Boakes S, Appleyard AN, Cortes J, Dawson MJ (2010) Organization of the biosynthetic genes encoding deoxyactagardine B (DAB), a new lantibiotic produced by *Actinoplanes liguriae* NCIMB41362. J Antibiot 63:351–358

Brötz H, Bierbaum G, Leopold K, Reynolds P, Sahl HG (1998a) The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrob Agents Chemother 42:154–160

Brötz H, Josten M, Wiedemann I, Schneider U, Götz F, Bierbaum G, Sahl HG (1998b) Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol Microbiol 30:317–327

Castiglione F, Cavaletti L, Losi D, Lazzarini A, Carrano L, Feroggio M, Ciciliato I, Corti E, Candiani G, Marinelli F, Selva E (2007) A novel lantibiotic acting on bacterial cell wall synthesis produced by the uncommon actinomycete *Planomonospor*a sp. Biochemistry 46:884–895

Castiglione F, Lazzarini A, Carrano L, Corti E, Ciciliato I, Gastaldo L, Candiani P, Losi D, Marinelli F, Selva E, Parenti F (2008) Determining the structure and mode of action of microbisporicin, a potent lantibiotic active against multiresistant pathogens. Chem Biol 15:22–31

Chatterjee C, Paul M, Xie L, van der Donk WA (2005) Biosynthesis and mode of action of lantibiotics. Chem Rev 105:633–684

Chatterjee S, Chatterjee S, Lad SJ, Phansalkar MS, Rupp BN, Ganguli BN, Fehlhaber HW, Kogler H (1992) Mersacidin, a new antibiotic from *Bacillus*. Fermentation, isolation, purification and chemical characterization. J Antibiot 45:832–838

Cotter PD, Deegan LH, Lawton EM, Draper LA, O'Connor PM, Hill C, Ross RP (2006) Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. Mol Microbiol 62:735–747

Dawson MJ (2006) The lantibiotics: an underexploited class of natural products with broad potential. Chem Today 24:25–27

Dawson MJ, Appleyard AN, Cortes-Bargallo J, Wadman SN (2011) Actagardine derivatives and pharmaceutical use thereof. WO 2011/095769

Delves-Broughton J (1990) Nisin and its uses as a food preservative. Food Technol 44:100–117

Dodd H, Gasson MJ, Mayer M, Narbad A (2006) Identifying lantibiotic gene clusters and novel lantibiotic genes. WO 2006/111743

Driessen AJ, van den Hooven HW, Kuiper W, van de Kamp M, Sahl HG, Konings RN, Konings WN (1995) Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry 34:1606–1614

Field D, Collins B, Cotter PD, Hill C, Ross RP (2007) A system for the random mutagenesis of the two-peptide lantibiotic lacticin 3147: analysis of mutants producing reduced antibacterial activities. J Mol Microbiol Biotechnol 13:226–234

Field D, Connor PM, Cotter PD, Hill C, Ross RP (2008) The generation of nisin variants with enhanced activity against specific gram-positive pathogens. Mol Microbiol 69(1):218–230

Foulston LC, Bibb MJ (2010) Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes. Proc Natl Acad Sci USA 107:13461–13466

Foulston LC, Bibb MJ (2011) Feed-forward regulation of microbisporicin biosynthesis in *Microbispora corallina*. J Bacteriol 193:3064–3071

Ghobrial O, Derendorf H, Hillman JD (2010) Pharmacokinetic and pharmacodynamic evaluation of the lantibiotic MU1140. J Pharm Sci 99:2521–2528

Goto Y, Li B, Claesen J, Shi Y, Bibb MJ, van der Donk WA (2010) Discovery of unique lanthionine synthetases reveals new mechanistic and evolutionary insights. PLoS Biol 8:e1000339

Grasemann H, Stehling F, Widmann R, Laliberte TW, Molina L, Döring G, Ratjen F (2007) Inhalation of Moli 1901 in patients with cystic fibrosis. Chest 131:1461–1466

Guder A, Schmitter T, Wiedemann I, Sahl HG, Bierbaum G (2002) Role of the single regulator MrsR1 and the two-component system MrsR2/K2 in the regulation of mersacidin production and immunity. Appl Env Microbiol 68:106–113

Helfrich M, Entian KD, Stein T (2007) Structure-function relationships of the lanthionine cyclase SpaC involved in biosynthesis of the *Bacillus subtilis* peptide antibiotic subtilin. Biochemistry 46:3224–3233

Hindré T, Le Pennec JP, Haras D, Dufour A (2004) Regulation of lantibiotic lacticin 481 production at the transcriptional level by acid pH. FEMS Microbiol Lett 231:291–298

Holtsmark I, Mantzilas D, EijsinkV GH, Brurberg MB (2006) Purification, characterization, and gene sequence of michiganin A, an actagardine-like lantibiotic produced by the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis*. Appl Environ Microbiol 72:5814–5821

Hsu SD, Breukink E, Tischenko E, Lutters MAG, de Kruijff B, Kaptein R, Bonvin MJJ, van Nuland NAJ (2004) The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nature Struc Mol Biol 11:963–967

Islam MR, Nishie M, Nagao J, Zendo T, Keller S, Nakayama J, Kohda D, Sahl HG, Sonomoto K (2012) Ring A of Nukacin ISK-1: a lipid II binding motif for type-A(II) lantibiotic. J Am Chem Soc 134:3687–3690

Islam MR, Shioya K, Nagao J, Nishie M, Jikuya H, Zendo T, Nakayama J, Sonomoto K (2009) Evaluation of essential and variable residues of nukacin ISK-1 by NNK scanning. Mol Microbiol 72:1438–1447

Jabes D, Brunati C, Guglierame P and Donadio S (2009) In vitro antibacterial profile of the new lantibiotic NAI-107. 49th Interscience conference on antimicrobial agents and chemotherapy, San Francisco, Abs F1- 1502

Jabes D, Brunati C, Candiani G, Riva S, Romanó G, Donadio S (2011) Efficacy of the new antibiotic NAI-107 in experimental infections induced by multidrug-resistant Gram-positive pathogens. Antimicrob Agents Chemother 55:1671–1676

Kellner R, Jung G, Josten M, Kaletta C, Entian KD, Sahl HG (1989) Pep5: structure elucidation of a large lantibiotic. Angew Chemie Int Ed English 28(5):616–619

Kessler H, Steuernagel S, Gillessen D, Kamiyama T (1987) Complete sequence determination and localisation of one imino and three sulfide bridges of the nonadecapapetide Ro 09–0198 by homonuclear 2D–NMR spectroscopy. The DQF-relayed-NOESY-Experiment. Helv Chim Acta 70:726–741

Kluskens LD, Nelemans SA, Rink R, de Vries L, Meter-Arkema A, Wang Y, Walther T, Kuipers A, Moll GN, Haas M (2009) Angiotensin-(1-7) with thioether bridge: an angiotensin-converting enzyme-resistant, potent angiotensin-(1-7)analog. J Pharmacol Exper Ther 328:849–854

Kupke T, Stevanovic S, Sahl HG, Gotz F (1992) Purification and characterization of EpiD, a flavoprotein involved in the biosynthesis of the lantibiotic epidermin. J Bacteriol 174:5354–5361

Li B, Yu JP, Brunzelle JS, Moll GN, van der Donk WA, Nair SK (2006) Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. Science 311:1464–1467

Li B, Sher D, Kelly L, Shi Y, Huang K, Knerr PJ, Joewono I, Rusch D, Chisholm SW, van der Donk WA (2010) Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. Proc Natl Acad Sci U S A 107:10430–10435

Lubelski J, Rink R, Khusainova R, Moll GN, Kuipers OP (2008) Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. Cell Mol Life Sci 65:455–476

Majer F, Schmid DG, Altena K, Bierbaum G, Kupke T (2002) The flavoprotein MrsD catalyzes the oxidative decarboxylation reaction involved in formation of the peptidoglycan biosynthesis inhibitor mersacidin. J Bacteriol 184:1234–1243

Mattick ATR, Hirsch A (1947) Further observations on an inhibitory substance (Nisin) from lactic streptococci. Lancet 253(2):5–8

Marsh AJ, O'Sullivan O, Ross RP, Cotter PD, Hill C (2010) *In silico* analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria. BMC Genomics 11:679

McAuliffe O, Ross RP, Hill C (2001) Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev 25:285–308

McClerren AL, Cooper LE, Quan C, Thomas PM, Kelleher NL, van der Donk WA (2006) Discovery and in vitro biosynthesis of haloduracin, a two-component lantibiotic. Proc Natl Acad Sci USA 103:17243–17248

Okesli A, Cooper LE, Fogle EJ, van der Donk WA (2011) Nine post-translational modifications during the biosynthesis of cinnamycin. J Am Chem Soc 133(34):13753–13760

O′Sullivan DJ, Lee JH (2009) Lantibiotics and uses thereof. WO2009/058440

Paul M, Patton GC, van der Donk WA (2007) Mutants of the zinc ligands of lacticin 481 synthetase retain dehydration activity but have impaired cyclization activity. Biochemistry 46:6268–6276

Patton GC, Paul M, Cooper LE, Chatterjee C, van der Donk WA (2008) The importance of the leader sequence for directing lanthionine formation in lacticin 481. Biochemistry 47:7342–7351

Peschel A, Augustin J, Kupke T, Stevanovic S, Götz F (1993) Regulation of epidermin biosynthetic genes by EpiQ. Mol Microbiol 9:31–39

Rink R, Wierenga J, Kuipers A, Kluskens LD, Driessen AJM, Kuipers OP, Moll GN (2007) Dissection and modulation of the four distinct activities of nisin by mutagenesis of rings A and B and by C-terminal truncation. Appl Env Microbiol 73:5809–5816

Rogers LA, Whittier EO (1928) Limiting factors in the lactic fermentation. J Bacteriol 16:211–229

Ryan MP, Jack RW, Josten M, Sahl HG, Jung G, Ross RP, Hill C (1999) Extensive post-translational modification, including serine to *D*-alanine conversion, in the two-component lantibiotic, lacticin 3147. J Biol Chem 274:37544–37550

Sahl HG, Bierbaum G (1998) Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria. Ann Rev Microbiol 52:41–79

Schmitz S, Hoffmann A, Szekat C, Rudd B, Bierbaum G (2006) The lantibiotic mersacidin is an autoinducing peptide. Appl Env Microbiol 72:7270–7277

Skaugen M, Nissen–Meyer J, Jung G, Stevanovic S, Sletten K (1994) In vivo conversion of L–serine to D–alanine in a ribosomally synthesized polypeptide. J Biol Chem 269(44): 27183–27185

Smith L, Hillman JD (2008) Therapeutic potential of type A(I) lantibiotics, a group of cationic peptide antibiotics. Curr Opin Microbiol 11:401–408

Wadman SN, Citron DM, Choi S, Cortes J, Goldstein EJC (2009) NVB302: in vitro activity against *Clostridium difficile* and intestinal strains of anaerobic bacteria. 49th Interscience conference on antimicrobial agents and chemotherapy, San Francisco, abs F1- 1518

Widdick DA, Dodd HM, Barraille P, White J, Stein TH, Chater KF, Gasson MJ, Bibb MJ (2003) Cloning and engineering of the cinnamycin biosynthetic gene cluster from *Streptomyces cinnamoneus cinnamoneus* DSM40005. Proc Natl Acad Sci USA 100:4316–4321

Xie L, Miller LM, Chaterjee C, Averin O, Kelleher NL, van der Donk WA (2004) Lacticin 481: in vitro reconstitution of lantibiotic synthetase activity. Science 303:679–681

You YO, van der Donk WA (2007) Mechanistic investigations of the dehydration reaction of lacticin 481synthetase using site directed mutagenesis. Biochemistry 46:5991–6000

Zimmermann N, Metzger JW, Jung G (1995) The tetracyclic lantibiotic actagardine ^{1}H-NMR and ^{13}C-NMR assignments and revised primary structure. Eur J Biochem 228:786–797

Chapter 8
Old and Novel Polymyxins Against Serious Gram-Negative Infections

Martti Vaara

Abstract Polymyxins (polymyxin B and colistin) are bactericidal pentacationic cyclic lipodecapeptides that act specifically on Gram-negative bacteria. They were largely abandoned because of their toxicity to kidney proximal tubuli. Now they have been reinstated, in spite of their toxicity, in the therapy of severe infections caused by extremely multiresistant strains. Such strains are now rapidly emerging and spreading. The nephrotoxicity of polymyxins does complicate the therapy, may even require its discontinuation, and must be weighed against the beneficial effects on patient survival. Furthermore, in the recent years it has become increasingly clear that the current dosage regimens are suboptimal in critically ill patients. Clinicians are advised to use larger doses, but this further increases nephrotoxicity. Since there is notably synergy between polymyxins and several other antibiotics, combination therapies may be useful, and clinical evidence for their advantages is currently accumulating. Novel, less nephrotoxic compounds that have strong antibacterial activity would be very welcome. The nephrotoxicity of polymyxins might be related to their very highly cationic nature. In contrast to the old polymyxins, which carry five positive charges, NAB739 carries three positive charges only. The activity of NAB739 against *Escherichia coli* and *Klebsiella pneumoniae* is quite close to that of polymyxin B. Pieces of indirect evidence suggest that NAB739 might be less nephrotoxic than the old polymyxins. Ongoing studies compare the efficacy and nephrotoxicity of NAB739 and polymyxin B in animal models. Useful compounds might also include NAB7061 and NAB741, both carrying three positive charges. They lack potent direct action but sensitize Gram-negative bacteria to other antibiotics by facilitating their entry inside the cell.

M. Vaara (✉)
Northern Antibiotics Ltd, FI-00720 Helsinki, Finland
e-mail: martti.vaara@northernantibiotics.com

M. Vaara
Division of Clinical Microbiology,
Helsinki University Hospital,
FI-00029 Helsinki, Finland

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_8,

8.1 Introduction

Escherichia coli and *Klebsiella pneumoniae* cause almost 40 % of all community-acquired bacteremias and approximately one-third of all healthcare-associated bacteremias. Out of all Gram-negative bacteremias they cause approximately 60–75 %. *E. coli* and *K. pneumoniae* strains that elaborate extended-spectrum β-lactamases and especially CTX-M type enzymes have spread at a stunning pace everywhere both in the hospitals and in the community worldwide. In most countries the situation has long been out of control (see Paitan and Ron, this volume).

Now, the largest worry is the carbapenemase-producing strains of *E. coli*, *K. pneumoniae* and other *Enterobacteriaceae* (especially the NDM and KPC strains) that are resistant to almost all antibiotics except polymyxins (polymyxin B, colistin) and tigecycline (see Genilloud and Vicente, this volume). Their ongoing spread might follow the path of the CTX-M strains. This would be disastrous since only extremely few antibiotics with a novel mode of action against Gram-negatives have entered the clinical phases of development.

Most of the extremely resistant Gram-negative "superbacteria" are still susceptible to polymyxins. This antibiotic group was discovered in the mid-1940s, and subsequently used in the intravenous therapy. However, polymyxins were soon largely abandoned because of toxicity, especially toxicity to kidney proximal tubuli. Today they have been reinstated as the last-line intravenous therapy to treat infections caused by strains that are resistant to practically all other agents.

Polymyxin B and colistin (i.e., polymyxin E) (Fig. 8.1) are rapidly bactericidal pentacationic cyclic lipodecapeptides that act on Gram-negative bacteria. Gram-positives, eukaryotic microbes, and mammalian cells are typically resistant. Polymyxins interact with the anionic lipopolysaccharide (LPS) molecules located in the outermost cell structure, the outer membrane (OM) (Vaara 1992, 2010). As shown for polymyxin B nonapeptide (PMBN) (Tsubery et al. 2000), their action is

Polymyxin B	MHA/MOA	-Dab$^+$	-Thr	-Dab$^+$	-cy[Dab	-Dab$^+$	-DPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
Colistin (polymyxin E)	MHA/MOA	-Dab$^+$	-Thr	-Dab$^+$	-cy[Dab	-Dab$^+$	-DLeu	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
CB-182,804	2-CPAC	-Dab$^+$	-Thr	-Dab$^+$	-cy[Dab	-Dab$^+$	-DPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
NAB739	OA	-	-Thr	-DSer	-cy[Dab	-Dab$^+$	-DPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
NAB7061	OA	-	-Thr	-Abu	-cy[Dab	-Dab$^+$	-DPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]
NAB741	Ac	-	-Thr	-DSer	-cy[Dab	-Dab$^+$	-DPhe	-Leu	-Dab$^+$	-Dab$^+$	-Thr]

Fig. 8.1 Structure of polymyxin B, colistin, CB-182,804, NAB739, NAB7061, and NAB741. Boxed parts indicate locations where the compounds are not identical. Abbreviations for the nontrivial amino acyl residues: *Dab* diaminobutyryl, *Abu* aminobutyryl. Other abbreviations: *MHA/MOA* the mixture of methyl octanoyl and methyl heptanoyl, *OA* octanoyl, *2-CPAC* 2-chloro-phenylaminocarbonyl, *cy* the cyclic portion indicated with brackets. The positive charge of the free γ-amino group is also shown

determined by proper three-dimensional conformation, since the enantiomer is completely inactive. Polymyxins permeabilize the OM and then damage the cytoplasmic membrane (Nikaido 2003; Vaara 1992). Several recent general or focused reviews on polymyxins are available (Bergen et al. 2011; Landman et al. 2008; Lim et al. 2010; Michalopoulos and Falagas 2011; Nation and Li 2009; Vaara 2010; Yahav et al. 2011; Zawascki et al. 2007).

Both polymyxin B and colistin are mixtures of related lipodecapeptides. Most of the variation is due to various fatty acyl residues linked to the *N*-terminus of the peptides. Altogether 39 and 36 different components have been detected in commercial preparations of polymyxin B and colistin, respectively (Van den Bosche et al. 2011). However, as shown for the most predominant components of polymyxin B, their antibacterial activity appears to be identical (Tam et al. 2011). Furthermore, the activities of polymyxin B and colistin are identical (Gales et al. 2001).

Polymyxin B is used as such but colistin is used as its prodrug, colistin methanesulfonate (CMS). In CMS, the free amino groups are blocked by sulfomethylation to yield an uncharged, inactive derivative that in aqueous solutions and in vivo slowly hydrolyzes to free colistin.

Besides the intravenous use of CMS, aerosolized CMS is used in the therapy of Gram-negative lung infections in cystic fibrosis and, to a limited degree, in other patients (Nation and Li 2009; Yahav et al. 2011). Polymyxin B bound to polystyrene fibers is used in hemoperfusion as a means to remove endotoxins (LPS) from the blood of septic patients (Davies and Cohen 2011; Nation and Li 2009; Yahav et al. 2011).

In a few recent years, two pharmaceutical companies, both aiming to develop polymyxin derivatives that are less nephrotoxic, have published data on multiple properties of their derivatives.

8.2 Antimicrobial Activities of Novel Polymyxins

8.2.1 CB-182,804

The first and thus far the only novel derivative to enter the clinical phase I was CB-182,804, developed by Cubist Pharmaceuticals, Inc. (Keith et al. 2010). It is otherwise identical to polymyxin B, but the fatty acyl moiety linked to the *N*-terminus of the polymyxin B peptide is 2-chloro-phenylamino-carbonyl (Fig. 8.1). The MIC_{90} of CB-182,804 (i.e., the concentration of CB-182,804 that inhibits growth of 90 % of the strains) for *E. coli*, *K. pneumoniae*, *and Pseudomonas aeruginosa* are four-fold, two-fold, and four-fold that of polymyxin B, respectively (Quale et al. 2012).

In the neutropenic mouse thigh infection model, the PD_{50} value of CB-182,804 (i.e., the dose of CB-182,804 that is required to protect 50 % of the animals) was

somewhat lower than that of polymyxin B, when *P. aeruginosa* was used as the challenge organism (Arya et al. 2010). When *Acinetobacter baumannii* was used, CB-182,804 was slightly less active than polymyxin B. In the septicemic mouse model with *E. coli*, the PD_{50} of CB-182,804 was significantly higher than that of polymyxin B or colistin (Arya et al. 2010).

8.2.2 NAB739

NAB739 is under development at Northern Antibiotics Ltd. (Vaara 2010). It has its cyclic part identical to that of polymyxin B, but its side chain consists of octanoyl-threonyl-D-serinyl (Fig. 8.1). Accordingly, its linear part lacks the two positively charged diaminobutyryl (Dab) residues present in the linear part of polymyxin B and colistin. Accordingly, NAB739 carries three positive charges only.

In two evaluations (Vaara et al. 2008 and Northern Antibiotics, data on file), the MIC_{90} values of NAB739 and polymyxin B for *E. coli* were identical, in the first one 1 μg/ml and in the second case 2 μg/ml. For *K. pneumoniae*, the MIC_{90} of NAB739 and polymyxin B were 2 μg/ml and 1 μg/ml, respectively. Unlike that of several other cationic drugs, the activity of NAB739 is not affected by divalent cations (Ca^{2+}, Mg^{2+}).

The MIC range of NAB739 and polymyxin B for the polymyxin-susceptible carbapenemase-producing strains of *E. coli* and *K. pneumoniae* ($n = 9$, including KPC, OXA-48, VIM, and IMP-producing strains) were 1–4 μg/ml and 1–2 μg/ml, respectively (Vaara et al. 2010b).

Against *Acinetobacter* NAB739 is 4-fold less active than polymyxin B, but low subinhibitory concentrations of NAB739 sensitize *A. baumannii* to other antibiotics by facilitating their access inside the cell. As low a concentration as 0.5 μg/ml of NAB739 reduces the MIC of rifampin from 4 to 0.05 μg/ml (Vaara et al. 2008). The MIC of clarithromycin is reduced from 8 to 0.5 μg/ml, and the MIC of vancomycin is reduced from 256 to 3 μg/ml (Vaara et al. 2008).

Against *P. aeruginosa* NAB739 is 8-fold less active than polymyxin B and the MIC_{90} is 16 μg/ml (Northern Antibiotics, data on file). Another derivative, NAB740, which carries decanoyl as the fatty acyl tail, is more active against *P. aeruginosa*, but against *Enterobacteriaceae* it is inferior to NAB739 (Vaara et al. 2008). Furthermore, des-fatty acyl derivatives such as PMBN and many others are quite active against *P. aeruginosa* but virtually lack activity against *Enterobacteriaceae* (Katsuma et al. 2009; Sato et al. 2011; Tsubery et al. 2001; Vaara 1992).

The presence of fresh normal human serum (NHS, 20 %) decreases the MIC of NAB739 for *P. aeruginosa* ATCC 27853 by a factor of 8–16 and yields MIC values as low as 0.25–0.5 μg/ml (Northern Antibiotics, data on file). The MIC for *E. coli* ATCC25922 decreases from 1–2 to 0.5 μg/ml. The MIC of polymyxin B in the presence of 20 % NHS is <0.06 μg/ml for both strains. Vaara and coworkers have previously shown that PMBN acts synergistically with the complement against *Enterobacteriaceae* and *P. aeruginosa* (Vaara and Vaara 1983; Vaara et al. 1984).

The combination of PMBN with human, guinea pig, rabbit, and rat serum is strongly bactericidal, but no synergy can be found with mouse serum (Vaara et al. 1984).

Inherently polymyxin-resistant bacterial species as well as strains that have acquired resistance to polymyxin are resistant to NAB739 (Vaara et al. 2008, 2010b).

NAB739 was found to be effective in treating experimental *E. coli* peritoneal and bacteremic infection in mice at a dose identical to that of polymyxin B (Vingsbo Lundberg et al. 2010). To avoid inactivation of the polymyxins by mucin, the polyanionic polymer commonly used to suppress phagocytosis in the mouse peritonitis model, the K1 capsule-elaborating strain IH3080 (O18:K1:H7) was used. It is virulent to mice in the peritonitis model even in the absence of mucin.

8.2.3 The Sensitizer Derivatives NAB7061 and NAB741

NAB7061 and NAB741 (Vaara et al. 2008, 2010a, b; Vaara 2010), both under development at Northern Antibiotics, have their cyclic part identical to that of polymyxin B, but their side chain consists of octanoyl-threonyl-aminobutyryl, and acetyl-threonyl-D-serinyl, respectively (Fig. 8.1). Hence they carry only three positive charges, as does NAB739.

Hydrophobic antibiotics (such as macrolides, rifamycins, and many others) as well as large molecules (such as vancomycin) are widely used against Gram-positive bacteria but inactive against most Gram-negative bacteria, because these drugs do not effectively cross the OM (Nikaido 2003; Vaara 1992). NAB741 and NAB7061 lack potent direct antibacterial activity, but, by disrupting the bacterial OM, they facilitate the access of these antibiotics inside the Gram-negative cell. At 4 μg/ml, NAB7061 decreased the MIC of rifampin for *E. coli* ($n = 11$), other polymyxin-susceptible *Enterobacteriaceae* ($n = 12$), and *A. baumannii* ($n = 3$) by factors of 85–750, 10–2,000, and 25–125, respectively (Vaara et al. 2008). With clarithromycin (see Kirst, this volume), the corresponding factors were 90–750, 10–1,000, and 40–100, respectively. The antibacterial properties of NAB741 (Vaara et al. 2010) are similar to those of NAB7061.

Both agents also sensitize target bacteria to the bactericidal activity of the complement system present in fresh serum (Vaara 2010; Vaara et al. 2010a).

The sensitizer activity of NAB7061 has been demonstrated also in vivo in the therapy of experimental *E. coli* peritoneal infection in mice (Vingsbo Lundberg et al. 2010). In contrast to NAB7061 or erythromycin alone, the combination of NAB7061 and erythromycin was effective in the therapy.

Accordingly, NAB7061 and NAB741 may find value when used as a combination with a suitable partner antibiotic.

8.3 Determining MIC Values of Polymyxins

Determining the MIC values of polymyxin B and colistin can be performed either by broth microdilution or by agar dilution method, both according to the CLSI standard protocol, the most recent version labeled as M07-A9 (CLSI 2012). For broth microdilution testing, CLSI recommends cation-adjusted Mueller–Hinton broth (CAMHB) that contains 20–25 mg of Ca^{2+} per liter and 10–12.5 mg of Mg^{2+} per liter. For agar dilution testing, CLSI recommends Mueller–Hinton agar (MHA) without addition of cation supplements (CLSI 2012). CLSI has also given quality control ranges for polymyxin B and colistin MIC determinations.

Excellent correlation ($r = 0.96$–0.98) has been reported between broth microdilution and agar dilution tests for both polymyxin B and colistin, as tested by using a representative set of clinical isolates (Gales et al. 2001). A trend toward higher MIC results with the agar method was observed but 94.3 % of the MIC results were ± 1 $\log_2$ dilution between the methods used for both compounds. Another study with a larger collection of clinical isolates and determining MICs for colistin only did not find such a trend; 85.4 % of the MIC results were identical and 96.8 % were ± 1 $\log_2$ dilution between the methods (Lo-Ten-Foe et al. 2007).

It should however be remembered that polymyxins are adsorbed to glass, plastic, and sterilization filters. The adsorption of radiolabelled mono-acetyl polymyxin B is in relative terms very intense in dilute solutions and especially in water and in media that have low ionic strength (Vaara et al. 1979 and Vaara, unpublished). In a recent study (Karvanen et al. 2011), altogether 74 % of colistin was lost during dilution steps from stock to 0.125 μg/ml and during subsequent incubation in uninoculated CAMHB in polystyrene wells at 37 °C for 24 h. At higher concentrations of colistin, the loss gradually decreased but was still 22 % at the intended colistin concentration of 8 μg/ml. The adsorption was less intense in polypropylene than in polystyrene.

Also the novel lipoglycopeptides (see Marcone and Marinelli, this volume) are quite sticky. Approximately 90 % of radiolabelled oritavancin was adsorbed to polystyrene in 1 h, when the initial concentration was 1 μg/ml (Arhin et al. 2008). The nonionic detergent polysorbate 80 (Tween 80) at 0.002 % almost completely inhibited the adsorption and reduced the MIC of oritavancin for *Staphylococcus aureus* by a factor of 30 (Arhin et al. 2008). Polysorbate 80 was also used in the dalbavancin susceptibility assays (Rennie et al. 2007). However, the use of polysorbate to inhibit the adsorption of polymyxins in bacterial susceptibility studies will cause problems. Both polysorbate and polymyxins are membrane-active agents and may act synergistically against the Gram-negative OM and cytoplasmic membrane. As low a concentration as 0.001 % polysorbate 80 has been shown to be synergistic with polymyxin B against *P. aeruginosa*, and at higher concentrations of polysorbate 80, the synergy was even more pronounced (Brown and Winsley 1971).

Less polymyxin is probably adsorbed to plastic materials in the agar dilution tests than in microbroth dilution tests, since polymyxins diffuse rather poorly in agar. On the other hand, polymyxins can be expected to bind to the anionic sulfated carbohydrate polymers of agar.

8.4 Acquired Resistance to Polymyxin and to NAB Compounds

Acquired polymyxin resistance in clinical isolates is rare. In the worldwide SENTRY program, susceptibility of 40,625 Gram-negative isolates, collected in 2006–2009, to colistin and polymyxin B isolates was studied (Gales et al. 2010). Susceptibility was interpreted as the MIC $\leq$ 2 μg/ml. Susceptibility rates of colistin for *E. coli*, *K. pneumoniae*, *Acinetobacter* spp., and *P. aeruginosa* were 99.9, 98.6, 99.2, and 99.8 %, respectively. For strains isolated in 2009, the corresponding rates were 99.8, 98.2, 97.9, and 99.5 %, respectively. Polymyxin B displayed almost identical susceptibility rates.

In strains isolated in the CANWARD program from Canadian hospitals during 2007–2009, the colistin susceptibility rates (susceptibility interpreted as above) for *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *A. baumannii*, and *P. aeruginosa* were 99.4, 97.1, 95.4, 83.6, 93.5, and 91.6 %, respectively (Walkty et al. 2009). In a continuation study that included also the strains isolated in 2009, the susceptibility rates for ESBL-producing *E. coli*, *ampC*-producing *E. coli*, *Acinetobacter spp*., and *P. aeruginosa* were 98.7, 100, 94.3, and 90.9 %, respectively (Simner et al. 2011; Zhanel et al. 2011).

In the material collected in 2009 from 16 hospitals in Brooklyn, NY and Staten Island, NY and consisting of 5,489 strains of *E. coli*, *K. pneumoniae*, *Enterobacter* spp., *A. baumannii*, and *P. aeruginosa*, the susceptibility rates for polymyxin B (susceptibility interpreted as the MIC $\leq$ 2 μg/ml) were 99.8, 96, 76, 97, and 99.5 %, respectively (Landman et al. 2010; Quale et al. 2012).

Amongst imipenem nonsusceptible isolates of *K. pneumoniae* in the SENTRY collection, 12 % were resistant to colistin (Sader et al. 2011). Colistin-resistant *K. pneumoniae* strains may develop during therapy (Lee et al. 2009). They may also cause hospital outbreaks. Colistin-resistant KPC-producing *K. pneumoniae* strains belonging to the international epidemic clone ST258 have been encountered and reported to cause hospital infections (Bogdanovich et al. 2011).

Probably, the highest rates of polymyxin resistance are encountered in *P. aeruginosa* strains isolated from cystic fibrosis (Lim et al. 2010).

In *A. baumannii*, *K. pneumoniae,* and *P. aeruginosa*, heteroresistance to polymyxins has been reported (Lim et al. 2010; Yahav et al. 2011). A small subpopulation of cells survive the antibiotic treatment. In broth cultures this is seen as an initially slow but exponential turbidity increase over time, due to the growth of these persisters. While the clinical significance remains unclear, it might still be

advantageous to use polymyxin compounds in combination with another antibiotic that suppresses the development of persister cells, as suggested by many authors (Bergen et al. 2011; Garonzik et al. 2011; Landman et al. 2008; Nation and Li 2009; Yahav et al. 2011; Zawascki et al. 2007). Perhaps some of the novel compounds currently developed by the pharmaceutical industry and active against Gram-positives may find use as a suitable adjunct or "partner" antibiotic to polymyxins. Such compounds could include novel oxazolidinones (see Zappia, this volume), ketolides (see Kirst, this volume), lipopeptides (see Baltz, this volume), peptide deformylase inhibitors (see East, this volume), pleuromutilins (see Kirst, this volume), and inhibitors of FabI (see Genilloud and Vicente, this volume). Furthermore, partners could also include agents that inhibit targets such as LpxC enzymes, present in Gram-negatives only, but do not effectively penetrate their OM.

The mechanism of polymyxin resistance was elucidated by Vaara and his coworkers already more than 30 years ago. They reported, that the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium* have an altered lipopolysaccharide in their OM (Vaara et al. 1981). It is less anionic, due to increased decoration by 4-aminoarabinose and phosphoryl ethanolamine (Vaara et al. 1981). This resistance mechanism was then found universal amongst *Enterobacteriaceae* and *P. aeruginosa* (Gunn 2008; Landman et al. 2008; Lim et al. 2010; Raetz et al. 2007; Vaara 2010). Furthermore, similar changes in lipid A confer the resistance in several species that are intrinsically resistant to polymyxins, such as *Proteus mirabilis*, *Burkholderia cepacia,* and *Chromobacterium violaceum*, as reviewed by Vaara (1992).

The *pmrAB* regulon is best characterized in *Salmonella typhimurium*, where it regulates at least 20 genes including those involved in the decoration of the lipid A by 4-aminoarabinose and phosphoryl ethanolamine (Gunn 2008; Raetz et al. 2007). Conditions that activate *pmrAB* have recently been thoroughly reviewed (Gunn 2008; Raetz et al. 2007).

The mechanisms that mediate polymyxin resistance have profound effects on the structure and function of the bacterial OM. The OM permeability barrier of the polymyxin-resistant enterobacterial mutants is compromised, as evidenced by their increased susceptibility to other agents such as certain antibiotics and bile acids (Froelich et al. 2006; Murata et al. 2007; Vaara 1981; Vaara and Vaara 1994). This might explain why for instance polymyxin-resistant strains of *E. coli* are still extremely scarce (see above).

In *A. baumannii*, polymyxin resistance is mediated by at least two alternative mechanism. One is analogical to the mechanism described above for other bacteria and involves decoration of lipid A with phosphoryl ethanolamine (Beceiro et al. 2011) while the other is a result of complete loss of lipopolysaccharide (Moffatt et al. 2010) Polymyxin-resistant strains of *A. baumannii* are susceptible to many antibiotics normally active on Gram-positives only and they have reduced in vivo fitness and decreased virulence (Fernández-Reyes et al. 2009; Li et al. 2007; Lôpez-Rojas et al. 2011; Rolain et al. 2011).

Regarding novel polymyxins, the polymyxin-resistant strains of *E. coli*, *K. pneumoniae*, *Acinetobacter* spp,. and *P. aeruginosa* as well as the polymyxin-resistant variant strain CL5762B of KPC-producing *K. pneumoniae* are resistant to NAB739 (Vaara et al. 2008, 2010b, and Northern Antibiotics, data on file). On the other hand, the sensitizer compound NAB7061 sensitizes CL5762B to rifampin and clarithromycin by factors of 24 and 12, respectively (Vaara et al. 2010b).

Some of the polymyxin derivatives recently synthesized by Li et al. (2010) have activity against polymyxin-resistant strains of *A. baumannii* and *P. aeruginosa* but the polymyxin-resistant *K. pneumoniae* strains remain resistant. These compounds are more hydrophobic than the old polymyxins and might have detergent-like activity. For instance, in Compound 2, the hydrophobicity of polymyxin is increased by substituting 2-aminodecanoyl (Ada) for leucyl in the heptapeptide ring portion, to yield octyl instead of isobutyl as the R group in this position (position 7 according to the standard numbering of the amino acid residues in the polymyxin decapeptide). The fatty acyl tail consists of an octanoyl residue. For polymyxin-susceptible reference strains *P. aeruginosa* ATCC27853, *A. baumannii* ATCC19606, and *K. pneumoniae* ATCC13883, the MICs of Compound 2 for are 4 μg/ml, 4 μg/ml, and 2 μg/ml, respectively. Unfortunately, the MIC for *E. coli* has not been disclosed. Compounds 3–5 are analogical to Compound 2 in having, besides the fatty acyl tail, larger hydrophobic moieties (hexyl, bisphenyl) than isobutyl at the position 7. All these four compounds carry five positive charges.

Compound 1 resembles NAB739 and carries only three positive charges. It lacks Dab at the position 1, has Thr at the position 3 and nonanoyl as the fatty acid tail, but, in contrast to NAB739, aminodecanoic acid (Ada) at position 7.

Accordingly, the mode of action of the derivatives with increased hydrophobic properties differs from the classic and quite specific mode of action of polymyxins and might merely resemble the rather nonspecific action of cationic detergents. It has long been known that the polymyxin-resistant *pmrA* mutants of *S. typhimurium* and their analogues in *E. coli* with identical alterations in the lipid A are as susceptible as their parents to octapeptin EM49 (Meyers et al. 1974; Vaara 1981). EM49 is structurally very similar to polymyxins but is more hydrophobic, since it lacks two hydrophilic amino acids and carries a fatty acyl tail that is two methylene units longer than the one of polymyxin B. To the cationic detergents benzalkonium chloride and cetyltriammonium chloride, these mutants are even more susceptible than their parents (Vaara 1981; Vaara and Vaara 1994).

8.5 Pharmacokinetic and Pharmacodynamic Considerations

The pharmacokinetics of CMS and colistin released from CMS has extensively been reviewed elsewhere (Couet et al. 2012; Michalopoulos and Falagas 2011; Nation and Li 2009). In the recent years, it has become increasingly clear that the

current dosage regimens of CMS are suboptimal in critically ill patients (Bergen et al. 2011; Couet et al. 2012; Garonzik et al. 2011; Markou et al. 2008; Micalopoulos and Falagas 2011). While clinicians are advised to use larger doses of CMS (Couet et al. 2012), the nephrotoxic potential may cause problems (see below). Another approach is combination therapy (Bergen et al. 2011; Garonzik et al. 2011; Nation and Li 2009; Yahav et al. 2011). The synergy of polymyxins in vitro with many agents, especially with those that are normally excluded by an intact bacterial OM, has thoroughly been reviewed by several authors (Landman et al. 2008; Nation and Li 2009; Yahav et al. 2011; Zawascki et al. 2007). Clinical evidence for the advantages of combination therapies is accumulating (Nation and Li 2009; Yahav et al. 2011).

The serum half-life of polymyxin B in humans with normal renal function and, as determined for one patient with renal insufficiency (serum creatinine, 3.5 mg/dL), has been reported to be 13.6 h and 11.5 h, respectively (Kwa et al. 2008, 2011). Only less than 1 % of the given dose is excreted in urine (Zawascki et al. 2008).

The area under the unbound concentration–time curve to MIC ratio (fAUC/MIC) has been reported as the pharmacokinetic/pharmacodynamic index that best predicts the efficacy of colistin against *P. aeruginosa* and *A. baumannii* in animal models (thigh and lung infections in neutropenic mice) and in one in vitro model (Bergen et al. 2011).

8.6 Nephrotoxicity of Polymyxin Compounds

The nephrotoxicity of polymyxins does complicate the therapy, may even require its discontinuation, and must be weighed against the beneficial effects on patient survival (Falagas and Rafailidis 2009).The nephrotoxicity rate depends on the definitions for toxicity, correlates with the age and cumulative total dose, and increases in patients with abnormal renal function. It varies in the recent literature from 10 to 30 % (Falagas and Kasiakou 2006; Falagas et al. 2010; Landman et al. 2008; Oliveira et al. 2009; Pastewski et al. 2008; Zawascki et al. 2007), and in selected materials may be as high as 43–80 % (Garonzik et al. 2011; Hartzell et al. 2009; Pastewski et al. 2008; Pogue et al. 2011). The nephrotoxic potential of CMS and polymyxin B appear to be similar (Falagas and Kasiakou 2006; Oliveira et al. 2009).

Polymyxins and aminoglycosides (see Kirst and Marinelli, this volume), another group of strongly cationic compounds, are nephrotoxic, because they damage renal proximal tubuli. Both are bound to the megalin macroprotein in the brush-border membrane (BBM) of the epithelial cells of the proximal tubuli and then effectively taken up by these cells (Vaara 2010). This extensive tubular reabsorption results in high concentrations of the drugs inside the cells. This may explain, at least partially, the nephrotoxicity of polymyxins (Zawascki et al. 2008).

The development of two polymyxin derivatives, both carrying five positive charges, has been discontinued. The first of them was PMBN. It lacks the fatty acid tail and the N-terminal amino acyl residue (Dab) of polymyxins but still carries

five positive charges. Its direct antibacterial action is weak albeit against *P. aeruginosa*, but, as first shown by Vaara et al., PMBN has retained the ability to permeabilize the OM (Vaara 1992; Vaara and Vaara 1983). However, in rodent experiments, PMBN was still considered to be too nephrotoxic, and its development for therapeutic purposes was discontinued in 1984 (Vaara 1992; Vaara, unpublished).

CB-182,804 was reported to be less nephrotoxic to cynomolgus monkeys than polymyxin B (Coleman et al. 2010), even though its superiority of CB-182,804 over polymyxin B looked very modest. CB-182,804 entered into a Phase 1 clinical trial in February 2009, and in September 2010 the project was discontinued.

The polymyxin derivatives of the NAB series carry only three positive charges. Their affinity for isolated rat kidney BBM is only approximately one-seventh (NAB739) or one-fifth (NAB7061) of that of polymyxin B and approximately one-third of that of gentamicin (Vaara et al. 2008).

In trivial nonpolarized porcine renal proximal tubular LLC-PK1 cells that express megalin only very poorly (Servais et al. 2006), polymyxin B elicits a marked degree (approx 50 %) of necrosis at 0.5 mM, whereas the NAB compounds are inert even at 1 mM (Mingeot-Leclercq et al. 2012). In artificially permeabilized (electroporated) LLC-PK1 cells, where polymyxins enter freely and unselectively, polymyxin B induces total necrosis at as low a concentration as 0.016 mM, while an approximately 8-fold concentration of NAB739 and NAB7061 and an approximately 32-fold concentration of NAB741 is required for the same effect (Mingeot-Leclercq et al. 2012). Accordingly, the studies showed that NAB739, NAB7061, and NAB741 have a substantially lower necrotic potential toward LLC-PK1 cells than polymyxin B. Polarized cells that express megalin, form BBM and actively bind polymyxins (see above) and internalize them would be more suitable as targets, but the technology is very demanding. In such polarized cells that mimic natural conditions, the fold difference between the cytotoxicity of the NAB compounds and polymyxin B can be expected to be even more larger than that observed with artificially permeabilized cells, since the affinities of the NAB compounds for isolated rat BBM expressing megalin are significantly lower than that of polymyxin B (see above),

The renal clearances of NAB741, NAB739, and NAB7061 after a single intravenous dose in rats are approx. 400-, 50-, and 30-fold higher than that of colistin (Ali et al. 2009; Vaara et al. 2010a). The higher renal clearance indicates a difference in the relative contributions of renal clearance mechanisms (glomerular filtration, tubular secretion, and tubular reabsorption). In contrast to polymyxins, NAB739 and NAB741 do not undergo net tubular uptake (NAB7061 was not included in the study).

Altogether the results indicate that the nephrotoxicity of the NAB compounds might be lower than that of the old polymyxins.

At least in some experimental setups, such as in those using murine and human macrophage cells, colistin appears to be less cytotoxic than polymyxin B (Das et al. 2011). Even though the relevance of these findings in clinical settings

remains unclear, it is still quite possible that an analogue of NAB739 where the side chain is octanoyl-threonyl-D-serinyl and the cyclic peptide part is identical to that of colistin might offer some advantages over NAB739.

8.7 Neurotoxicity of polymyxin compounds

Neurotoxic side effects of intravenously given polymyxin B and CMS in contemporary clinical settings are considered as rare, mild, and reversible (Falagas and Kasiakou 2006; Lim et al. 2010; Nation and Li 2009; Yahav et al. 2011; Zawascki et al. 2007). Severe neurotoxicity, including neuromuscular blockade and apnea, are extremely rare or nonexistent (Falagas and Kasiakou 2006), as are also the other acute reactions such as those mediated by histamine release. Polymyxin B is administered as an infusion over 1 h, whereas CMS, which liberates free colistin slowly, can be given as a shorter infusion.

PMBN lacks the fatty acid tail of polymyxins and is 150 times less active than polymyxin in causing neuromuscular blockade, 15 times less toxic in an acute toxicity assay in mice, and 25 times less active in releasing histamine from rat mast cells (Vaara 1992), and. also other des-fatty acyl derivatives of polymyxins are better tolerated than polymyxin B and colistin in acute toxicity assays (Katsuma et al. 2009; Sato et al. 2011). It could be anticipated that NAB741, the des-fatty acyl derivative of NAB739, resembles PMBN and the other des-fatty acyl derivatives in being better tolerated than fatty acyl-carrying polymyxins in acute toxicity assays.

8.8 Conclusions

Polymyxins are desperately needed in the therapy of severe infections caused by extremely multiresistant strains of Gram-negative bacteria. One can predict that the exponential spread of carbapenemase-producing strains of *E. coli* and *K. pneumoniae* in hospitals and in the community will mimic the triumph already seen in the case of the extended-spectrum betalactamase CTX-M –producing strains.

The nephrotoxicity of the old polymyxins shadows their use. In addition, in the recent years it has become increasingly clear that the current dosage regimens are suboptimal in critically ill patients. Clinicians are advised to use larger doses, but this further increases nephrotoxicity. Since there is notably synergy between polymyxins and several other antibiotics, combination therapies may be useful, and clinical evidence for their advantages is currently accumulating.

Novel, less nephrotoxic compounds that have strong antibacterial activity would be very welcome. Unfortunately, the development of CB-182,804 was discontinued after the clinical Phase I.

The nephrotoxicity of polymyxins might be related to their very highly cationic nature. In contrast to the old polymyxins and CB-182,804, which carry five positive charges, NAB739 carries three positive charges only. Pieces of indirect evidence suggest that it might be less nephrotoxic than the old polymyxins. Ongoing studies compare the efficacy and nephrotoxicity of NAB739 and polymyxin B in animal models.

Useful compounds might also include NAB7061 and NAB741, both carrying three positive charges. They lack potent direct action but sensitize bacteria to other antibiotics.

References

Ali FE, Cao G, Poydal A, Vaara T, Nation RL, Vaara M, Li J (2009) Pharmacokinetics of novel antimicrobial cationic peptides NAB7061 and NAB739 in rats following intravenous administration. J Antimicrob Chemother 64:1067–1070

Arhin FF, Sarmiento I, Belley A, McKay GA, Draghi DC, Grover P, Sahm DF, Parr TR Jr, Moeck G (2008) Effect of polysorbate 80 on oritavancin binding to plastic surfaces: implications for susceptibility testing. Antimicrob Agents Chemother 52:1597–1603

Arya A, Li T, Zhang S, Chuong L, Kang C, Zhang X, Mortin LI (2010) Efficacy of CB-182,804, a novel polymyxin analog, in rat and mouse models of Gram-negative bacterial infections. Abstr 50th Intersci Conf Antimicrob. Agents Chemother, Boston, abstr F1-1627

Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N (2011) Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. Antimicrob Agents Chemother 55:3370–3379

Bergen PJ, Li J, Nation RL (2011) Dosing of colistin-back to basic PK/PD. Curr Opin Pharmacol 11:464–469

Bogdanovich T, Adams-Haduch JM, Tian GB, Nguyen MH, Kwak EJ, Muto CA, Doi Y (2011) Colistin-resistant, *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae* belonging to the international epidemic clone ST258. Clin Infect Dis 53:373–376

Brown MRW, Winsley BE (1971) Synergism between polymyxin and Polysorbate 80 against *Pseudomonas aeruginosa*. J Gen Microbiol 68:367–373

CLSI (2012) M07–A9. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th edn. Clinical and Laboratory Standards Institute, Wayne

Coleman S, Deats T, Pawliuk R, Chavan A, Oleson FB Jr (2010) CB-182,804 is less nephrotoxic as compared to polymyxin B in monkeys following seven days of repeated intravenous dosing. Abstr 50th Intersci Conf Antimicrob Agents Chemother, Boston, abstr F1-1630.

Couet W, Grégoire N, Marchand S, Mimoz O (2012) Colistin pharmacokinetics: the fog is lifting. Clin Microbiol Infect 18:30–39

Das D, Buyk J, Van Bambeke F, Lorent J, Mingeot-Leclercq MP, Gobin P, Couet W, Tulkens PM (2011) Abstr 51th Intersci Conf Antimicrob Agents Chemother, abstr F2-170. Comparative assessment of antibacterial and ccytotoxic effects of colistin (CST) colistin methanesulfonate (CMS) and polymyxin B (PMB) using *Pseudomonas aeruginosa*, macrophages and renal cells, and liposomes

Davies B, Cohen J (2011) Endotoxin removal devices for the treatment of sepsis and septic shock. Lancet Infect Dis 11:65–71

Falagas ME, Kasiakou SK (2006) Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit Care 10:R27

Falagas ME, Rafailidis PI (2009) Nephrotoxicity of colistin: new insight into an old antibiotic. Clin Infect Dis 48:1729–1731

Falagas ME, Rafailidis PI, Ioannidou E, Alexiou VG, Matthaiou DK, Karageorgopoulos DE, Kapaskelis A, Nikita D, Michalopoulos A (2010) Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients. Int J Antimicrob Agents 35:194–199

Fernández-Reyes M, Rodriguez-Falcón M, Chiva C, Pachón J, Andreu D, Rivas L (2009) The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. Proteonomics 9:1632–1645

Froelich JM, Tran K (2006) Wall D (2006) A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. J Bacteriol 188:1180–1183

Gales AC, Reis AO, Jones RN (2001) Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. J Clin Microbiol 39:183–190

Gales AC, Jones RN, Sader HS (2010) Contemporary activity of colistin and polymyxin B against a worldwide collection of gram-negative pathogens: results from the SENTRY antimicrobial surveillance program (2006–09). J Antimicrob Chemother 66:2070–2074

Garonzik SM, Li J, Thamlikitkul V, Paterson DL, Shoham S, Jacob J, Silveira FP, Forrest A, Nation RL (2011) Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. Antimicrob Agents Chemother 55:3284–3294

Gunn JS (2008) The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol 16:284–290

Hartzell JD, Neff R, Ake J, Howard R, Olson S, Paolino K, Vishnepolsky M, Weintraub A, Wortmann G (2009) Nephrotoxicity associated with intravenous colistin (colistimethate sodium) treatment at a tertiary care medical center. Clin Infect Dis 48:1724–1728

Karvanen M, Malmberg C, Mohamed A, Lagerbäck M, Friberg LE, Cars O (2011) Colistin is extensively lost during normal experimental conditions. Abstr 51th Intersci Conf Antimicrob Agents Chemother, Chicago, abstr D-690.

Katsuma N, Sato Y, Ohki K, Okimura K, Ohnishi K, Sakura N (2009) Development of des-fatty acyl-polymyxin B decapeptide analogs with *Pseudomonas aeruginosa*-specific antimicrobial activity. Chem Pharm Bull 57:332–336

Keith DD, Borders D, Curran WV, Leese RA, Mahamoon A, Jarolman H, Francis ND, Zhang Y, Arya A, Cotroneo N, Mortin LI, Silverman J, Brian P (2010) Synthesis and activity of CB-182,804: a novel polymyxin analog active against clinically relevant Gram-negative bacteria. Abstr 50th Intersci Conf Antimicrob Agents Chemother, Boston, abstr F1-1619.

Kwa AL, Lim TP, Low JG, Hou J, Kurup A, Prince RA, Tam VH (2008) Pharmacokinetics of polymyxin B1 in patients with multidrug-resistant Gram-negative bacterial infections. Diagn Microbiol Infect Dis 60:163–167

Kwa AL, Abdelraouf K, Low JG, Tam VH (2011) Pharmacokinetics of polymyxin B in a patient with renal insufficiency: a case report. Clin Infect Dis 52:1280–1281

Landman D, Georgescu C, Martin DA, Quale J (2008) Polymyxins revisited. Clin Microbiol Rev 21:449–465

Landman D, Urban C, Bäcker M, Kelly P, Shah N, Babu E, Bratu S, Quale J (2010) Susceptibility profiles, molecular epidemiology, and detection of KPC-producing *Escherichia coli* isolates from the New York City vicinity. J Clin Microbiol 48:4604–4607

Lee J, Patel G, Huprikar S, Calfee DP, Jenkins SG (2009) Decreased susceptibility to polymyxin B during treatment for carbapenem-resistant *Klebsiella pneumoniae* infection. Antimicrob Agents Chemother 47:1611–1612

Li J, Nation RL, Owen RJ, Wong S, Spelman D, Franklin C (2007) Antibiograms of multidrug-resistant clinical *Acinetobacter baumannii*: promising therapeutic options for treatment of infection with colistin-resistant strains. Clin Infect Dis 45:594–598

Li J, Velkov T, Nation RL, Thompson PE (2010) Patent application WO2010/130007 A1

Lim LM, Ly N, Anderson D, Yang JC, Macander L, Jarkowski A, Forrest A, Bulitta J, Tsuji BT (2010) Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. Pharmacother 30:1279–1291

López-Rojas R, Domínguez-Herrera J, McConnell MJ, Docobo-Peréz F, Smani Y, Fernández-Reyes M, Rivas L, Pachón J (2011) Impaired virulence and in vivo fitness of colistin-resistant *Acinetobacter baumannii*. J Infect Dis 203:545–548

Lo-Ten-Foe JR, de Smet AM, Diederen BMW, Kluytmans JAJ, van Keulen PHJ (2007) Comparative evaluation of the VITEK 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. Antimicrob Agents Chemother 51:3726–3730

Markou N, Markantonis SL, Dimitrakis E, Panidis D, Boutzouka E, Karatzas S, Rafailidis P, Apostolakos H, Baltopoulos G (2008) Colistin serum concentrations after intravenous administration in critically ill patients with serious multidrug-resistant, gram-negative bacilli infections: a prospective, open-label, uncontrolled study. Clin Ther 30:143–151

Meyers E, Parker WL, Brown WE (1974) EM49: a new polypeptide antibiotic active against cell membranes. Ann NY Acad Sci 234:493–501

Michalopoulos AS, Falagas ME (2011) Colistin: recent data on pharmacodynamics properties and clinical efficacy in critically ill patients. Ann Intensive Care 1:30

Mingeot-Leclercq MP, Tulkens PM, Denamur S, Vaara T, Vaara M (2012) Novel polymyxin derivatives are less cytotoxic than polymyxin B to renal proximal tubular cells. Peptides 35:248–252

Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD (2010) Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother 54:4971–4977

Murata T, Tseng W, Guina T, Miller SI, Nikaido H (2007) PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica Serovar typhimurium*. J Bacteriol 189:7213–7222

Nation RL, Li J (2009) Colistin in the 21st century. Curr Opin Infect Dis 22:535–543

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Oliveira MS, Prado GVB, Costa SF, Grinbaum RS, Levin AS (2009) Polymyxin B and colistimethate are comparable as to efficacy and renal toxicity. Diagn Microbiol Infect Dis 65:431–434

Pastewski AA, Caruso P, Parris AR, Dizon R, Kopec R, Sharma S, Mayer S, Ghitan M, Chapnick EK (2008) Parenteral polymyxin B use in patients with multidrug-resistant Gram-negative bacteremia and urinary tract infections: a retrospective case series. Ann Pharmacother 42:1177–1187

Pogue JM, Lee J, Marchaim D, Yee V, Zhao JJ, Chopra T, Lephart P, Kaye KS (2011) Incidence of and risk factors for colistin-associated nephrotoxicity in a large academic health system. Clin Infect Dis 53:879–884

Quale J, Shah N, Kelly P, Babu E, Bäcker M, Rosas-Garcia G, Salamera J, George A, Bratu S, Landman D (2012) Activity of polymyxin B and the novel polymyxin analogue CB-182,804 against contemporary Gram-negative pathogens in New York City. Microb Drug Resist 13:574–581

Raetz CRH, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in Gram-negative bacteria. Annu Rev Biochem 76:295–329

Rennie RP, Koeth L, Jones RN, Fritsche TR, Knapp CC, Killian SB, Goldstein BP (2007) Factors influencing broth microdilution antimicrobial susceptibility test results for dalbavancin, a new glycopeptide agent. J Clin Microbiol 45:3151–3154

Rolain JM, Roch A, Castanier M, Papazian L, Raoult D (2011) *Acinetobacter baumannii* resistant to colistin with impaired virulence: a case report from France. J Infect Dis 204:1146–1147

Sader HS, Farrell DJ, Jones RN (2011) Susceptibility of Klebsiella spp. to colistin and polymyxin B: results from the SENTRY Antimicrobial Surveillance Program (2006–2009). Int J Antimicrob Agents 37:174–175

Sato Y, Shindo M, Sakura N, Uchida Y, Kato I (2011) Novel des-fatty acyl-polymyxin B derivatives with *Pseudomonas aeruginosa*-specific antimicrobial activity. Chem Pharm Bull 59:597–602

Servais H, Jossin Y, Van Bambeke F, Tulkens PM, Mingeot-Leclercq M-P (2006) Gentamicin causes apoptosis at low concentrations in renal LLC-PK1 cells subjected to electroporation. Antimicrob Agents Chemother 50:1213–1221

Simner PJ, Zhanel GG, Pitout J, Tailor F, McCracken M, Mulvey MR, Lagacé-Wiens PR, Adam HJ, Hoban DJ, Canadian Antimicrobial Resistance Alliance (CARA) (2011) Prevalence and characterization of extended-spectrum β-lactamase- and AmpC β-lactamase-producing Escherichia coli: results of the CANWARD 2007-2009 study. Diagn Microbiol Infect Dis 69:326–334

Tam VH, Cao H, Ledesma KR, Hu M (2011) In vitro potency of various polymyxin B components. Antimicrob Agents Chemother 55:4490

Tsubery H, Ofek I, Cohen S, Fridkin M (2000) The functional association of polymyxin B with bacterial lipopolysaccharide is stereospecific: studies on polymyxin B nonapeptide. Biochemistry 39:11838–11844

Tsubery H, Ofek I, Cohen S, Fridkin M (2001) N-terminal modifications of polymyxin nonapeptide and their effect on antibacterial activity. Peptides 22:1675–1681

Vaara M (1981) Increased outer membrane resistance to ethylenediamine tetraacetate and cations in novel lipid a mutants. J Bacteriol 48:426–434

Vaara M (1992) Agents that increase the permeability of the outer membrane. Microbiol Rev 56:395–411

Vaara M (2010) Polymyxins and their novel derivatives. Curr Opin Microbiol 13:574–581

Vaara M, Vaara T (1983) Sensitization of Gram-negative bacteria to antibiotics and complement by a non-toxic oligopeptide. Nature (London) 303:526–528

Vaara M, Vaara T (1994) Ability of cecropin B to penetrate the enterobacterial outer membrane. Antimicrob Agents Chemother 38:2498–2501

Vaara M, Vaara T, Sarvas M (1979) Decreased binding of polymyxin by polymyxin-resistant mutants of *Salmonella typhimurium*. J Bacteriol 139:664–667

Vaara M, Vaara T, Jensen M, Helander I, Nurminen M, Rietschel E, Mäkelä P (1981) Characterization of the lipopolysaccharide from the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*. FEBS Lett 129:145–149

Vaara M, Viljanen P, Vaara T, Mäkelä PH (1984) An outer membrane-disorganizing peptide PMBN sensitizes *E. coli* strains to serum bactericidal action. J Immunol 132:2582–2589

Vaara M, Fox J, Loidl G, Siikanen O, Apajalahti J, Hansen F, Frimodt-Moeller N, Nagai J, Takano M, Vaara T (2008) Novel polymyxin derivatives carrying only three positive charges are effective antibacterial agents. Antimicrob Agents Chemother 52:3229–3236

Vaara M, Siikanen O, Apajalahti J, Fox J, Frimodt-Moeller J, He H, Poydyal A, Li J, Nation RL, Vaara T (2010a) A novel polymyxin derivative that lacks the fatty acid tail and carries only three positive charges has strong synergism with agents excluded by the intact outer membrane. Antimicrob Agents Chemother 54:3341–3346

Vaara M, Siikanen O, Apajalahti J, Frimodt-Møller N, Vaara T (2010b) Susceptibility of carbapenemase-producing strains of *Klebsiella pneumoniae* and *Escherichia coli* to the direct antibacterial activity of NAB739 and to the synergistic activity of NAB7061 with rifampin and clarithromycin. J Antimicrob Chemother 65:942–994

Van den Bosshe L, Van Schedael A, Chopra S, Hoogmartens J, Adams E (2011) Identification of impurities in polymyxin B and colistin bulk sample using liquid chromatography coupled to mass spectrometry. Talanta 83:1521–1529

Vingsbo Lundberg C, Vaara T, Frimodt-Møller N, Vaara M (2010) Novel polymyxin derivatives are effective in treating experimental *E. coli* peritoneal infection in mice. J Antimicrob Chemother 65:981–985

Walkty A, DeCorby M, Nichol K, Karlowsky JA, Hoban DJ, Zhanel GG (2009) In vitro activity of colistin (polymyxin E) against 3,480 isolates of Gram-negative bacilli obtained from patients in Canadian hospitals in the CANWARD study, 2007–2008. Antimicrob Agents Chemother 53:4924–4926

Yahav D, Farbman L, Leibovici L, Paul M (2011) Colistin: new lessons on an old antibiotic. Clin Microbiol Infect 18:18–29

Zawascki AP, Goldani LZ, Li J, Nation RL (2007) Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J Antibiot Chemother 60:1206–1215

Zawascki AP, Goldani LZ, Cao G, Superti SC, Lutz L, Barth AL, Ramos F, Boniatti MM, Nation RL, Li J (2008) Pharmacokinetics of intravenous polymyxin B in critically ill patients. Clin Infect Dis 47:1298–1304

Zhanel GG, Adam HJ, Low DE, Blondeau J, Decorby M, Karlowsky JA, Weshnoweski B, Vashisht R, Wierzbowski A, Hoban DJ, Canadian Antimicrobial Resistance Alliance (CARA) (2011) Antimicrobial susceptibility of 15,644 pathogens from Canadian hospitals: results of the CANWARD 2007-2009 study. Diagn Microbiol Infect Dis 69:291–306

Chapter 9
Uridyl Peptide Antibiotics: Developments in Biosynthesis and Medicinal Chemistry

Guy T. Carter and Leonard A. McDonald

Abstract The Uridyl-Peptide Antibiotics (UPAs) are a diverse group of bacterial metabolites that are characterized by a uridyl moiety linked to a peptidic residue. Included in this group are the pacidamycins, liposidomycins, capuramycins, and muraymycins. Most of these antibiotics are produced by *Streptomyces* species and are naturally found as complexes of closely related congeners. The compounds all bear some resemblance to intermediates involved in cell wall biosynthesis in bacteria and exert their antibacterial action through inhibition of the membrane-bound translocase I. Caprazamycin was the first of the UPAs for which a biosynthetic gene cluster was identified and cloned, which quickly led to the discovery of several others. Preliminary experiments have been reported in which biosynthetic insights were applied to the production of analogs for evaluation of antimicrobial activity. Synthetic chemistry has provided evidence for the core features necessary for target inhibition and antibacterial efficacy. Although many of the UPAs have shown potent antibacterial activity, and are effective in inhibiting a highly selective bacterial target, none have progressed to become commercially viable agents. In this chapter, we will explore recent findings that have clarified the promise and limitations of this class of antibiotics.

9.1 Introduction

This chapter is devoted to a subclass of nucleoside antibiotics that are minimally composed of a uridyl moiety linked to a peptidic residue and that exert their antibacterial activity through inhibition of cell wall biosynthesis. These compounds are

G. T. Carter (✉)
Carter-Bernan Consulting, 350 Phillips Hill Rd, New City, NY 10956, USA
e-mail: gtc9531@gmail.com

L. A. McDonald
Chromocell Corporation, 685 U.S. Highway One, North Brunswick, NJ 08902, USA

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_9,

variously referred to as uridyl peptides, peptidyl nucleosides, nucleoside-lipopeptides, or other similar permutations. In this chapter, we will refer to this group of antibiotics as uridyl peptide antibiotics or UPAs. The majority of UPAs are produced by *Streptomyces* spp. and have been shown to inhibit the later stages of cell wall biosynthesis, in particular the lipid-linked steps associated with membrane passage (see Genilloud and Vicente, this volume). Previous work on this family of antibiotics has been covered in two excellent sequential reviews by Tim Bugg and collaborators (Winn et al. 2010).

The four major structural classes that constitute this set of antibiotics are depicted in Fig. 9.1. The pacidamycin (Chen et al. 1989) subset, including the closely related mureidomycins (Inukai et al. 1989), napsamycins (Chatterjee et al. 1994) and sansanmycins (Xie et al. 2007) are the simplest, consisting only of uridyl and peptidyl moieties. Liposidomycin (Isono et al. 1985) is the prototype for the second group that is characterized by a diazepinone moiety (representing the "peptidyl" portion), an amino ribose unit and substantial lipid substitution. The remaining two structural types are represented by capuramycin (Yamaguchi et al. 1986) and muraymycin (McDonald et al. 2002). The former characteristically contains the caprolactam moiety and no additional lipid constituent, while the muraymycins are considerably more complex with extensive peptidic character.

UPAs exert their growth-inhibitory activity on bacteria by interfering with construction of the bacterial cell wall. In those cases for which detailed mechanism has been explored, the specific inhibition of MraY (translocase I) has been identified as the target of UPA action (Bugg et al. 2011, see Genilloud and Vicente, this volume). MraY catalyzes the transfer of phospho-MurNAc-pentapeptide to the lipid carrier undecaprenyl phosphate (lipid I), a step that mediates the former's eventual passage through the membrane (Bouhss et al. 2008). MraY is an essential enzyme in bacteria, is not found in mammalian cells, and as such it represents an attractive target in the search for novel antibiotics.

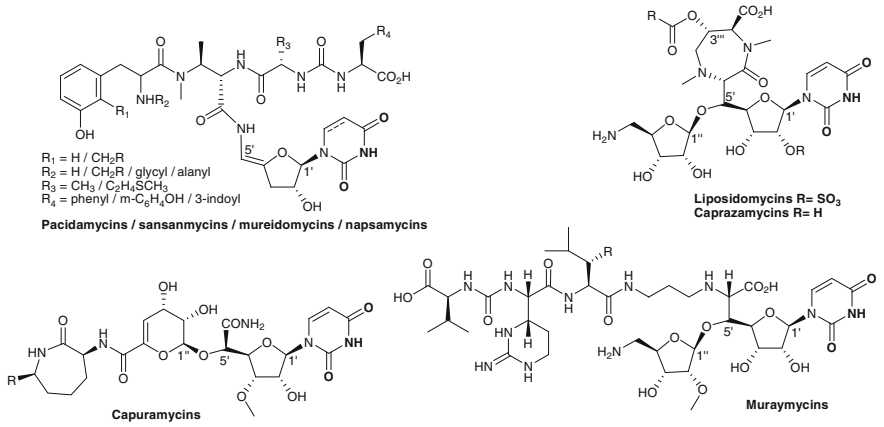


Fig. 9.1 Core structures of representative UPAs

Although many of the UPAs have shown potent antibacterial activity, none have progressed to become commercially viable agents. In this chapter, we will explore recent findings that have clarified the promise and limitations of this class of antibiotics.

9.2 Biosynthetic Developments

Molecular insights into the biosynthetic processes involved in the assembly of the UPAs began with identification, cloning, and expression of the caprazamycin gene cluster from a *Streptomyces* species (Kaysser et al. 2009). In their pioneering work, Gust and co-workers proposed the largely hypothetical pathway shown in Fig. 9.2 for caprazamycin biosynthesis. This hypothesis was evaluated to a certain extent by genetic knockout experiments conducted in the heterologous host *S. coelicolor* M512. The results of these experiments revealed that the genes for the elaboration and transfer of the rhamnosyl moiety were not co-clustered with the bulk of the pathway genes. In subsequent work however, the glycosylation genes were identified and co-expressed demonstrating formation of intact caprazamycins (Kaysser et al. 2010b). Simplified precursors lacking the 3-methylglutarate moiety (**2** in Fig. 9.2) are known to retain substantial antibiotic potency, and production of these was demonstrated by knocking out a putative hydrolase Cpz21. These knockout experiments represent a first step toward biosynthetic investigation of structure-activity relationships (SAR), or biosynthetic medicinal chemistry. Of the intermediates proposed in the pathway shown in Fig. 9.2, two have more general relevance to the biogenesis of other UPAs. The first of these is uridine-5′-aldehyde

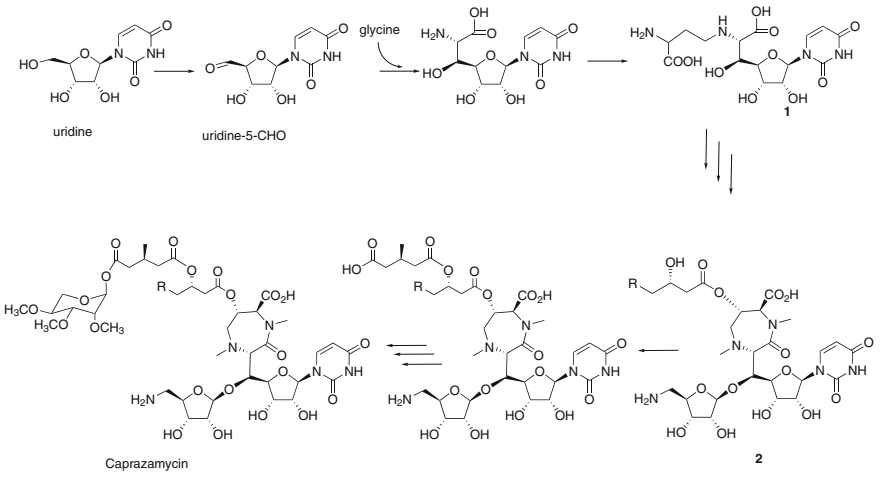


Fig. 9.2 Hypothetical biosynthetic pathway for caprazamycin (from Kaysser et al. 2009)

that appears to be the starting point for all UPAs and this relationship has been thoroughly discussed in a recent overview of the area (Walsh and Zhang 2011). The intermediate that consists of the uridine core plus the precursor for the diazepinone moiety (**1** in Fig. 9.2) may be a common progenitor for liposidomycins and muraymycins as well. Interestingly, the Gust group has demonstrated that the genes for the sulfotransferase reaction that generates the 2″-sulfate group of the liposidomycins are also present in the caprazamycin producer and can function to add a sulfate group to the CPZ aglycones (Kaysser et al. 2010a).

Mass spectrometry (MS) has played a pivotal role in the study of the biosynthetic processes of UPAs. The nature of the chemical framework of UPAs makes them ideal substrates for MS characterization. Specifically, the UPAs consist of catenated building blocks each derived from distinct biosynthetic processes, which generate structurally diagnostic fragment ions by MS that contain sufficient information to characterize pathway intermediates. In our identification of the novel suite of natural muraymycin congeners found in fermentation broths, high-resolution Fourier Transform-Ion Cyclotron Resonance (FT-ICR) MS was an invaluable tool (McDonald et al. 2003). Once the fragmentation patterns are established by high-resolution methods, this knowledge is readily applied to characterize multiple components via liquid chromatography/mass spectrometry (LC/MS) (Siebenberg et al. 2011). LC/MS and LC/MS^n have become the central analytical technique in studying how complex UPAs are assembled from simple precursors, and to quickly ascertain the effects of disrupting the genes encoding various steps in a biosynthetic sequence (Kaysser et al. 2009). Similarly, LC/MS techniques have been employed to detect and characterize the products described in the following sections from various manipulations of UPA biosynthetic pathways. The power of FT MS analysis was further exemplified in the definition of the role of PacB, one of the enzymes involved in pacidamycin biosynthesis. In this work, the Walsh laboratory utilized evidence from the MS analysis of enzyme-bound intermediates to demonstrate that PacB coded for a rare t-RNA-dependent amino acid transferase that catalyzed the addition of the terminal alanyl or glycyl residues to pacidamycin pentapeptides (Zhang et al. 2011b).

Recently, there has been a flurry of research directed at unraveling the mysteries of the pacidamycin biosynthetic pathway. The first report detailing the identification, cloning and expression of the gene cluster came from the Goss laboratory (Rackham et al. 2010). In this paper, the authors were able to demonstrate the production of pacidamycin D and S (shown in Fig. 9.3) in the heterologous host *Streptomyces lividans*. They also realized that in addition to the 22 core genes that were cloned and expressed, other genes must be involved in generating the natural suite of congeners. Shortly thereafter accounts appeared of the napsamycin gene cluster (Kaysser et al. 2011) from *Streptomyces* sp. HIL Y-82 and a second pacidamycin cluster from the identical *S. coerulrubidus* strain (Zhang et al. 2010). The Walsh laboratory subsequently examined the enzymology of the pathway in some detail (Zhang et al. 2011a) and has proposed the chemical logic depicted in Fig. 9.4 to account for the formation of pacidamycin congeners (Walsh and Zhang 2011).

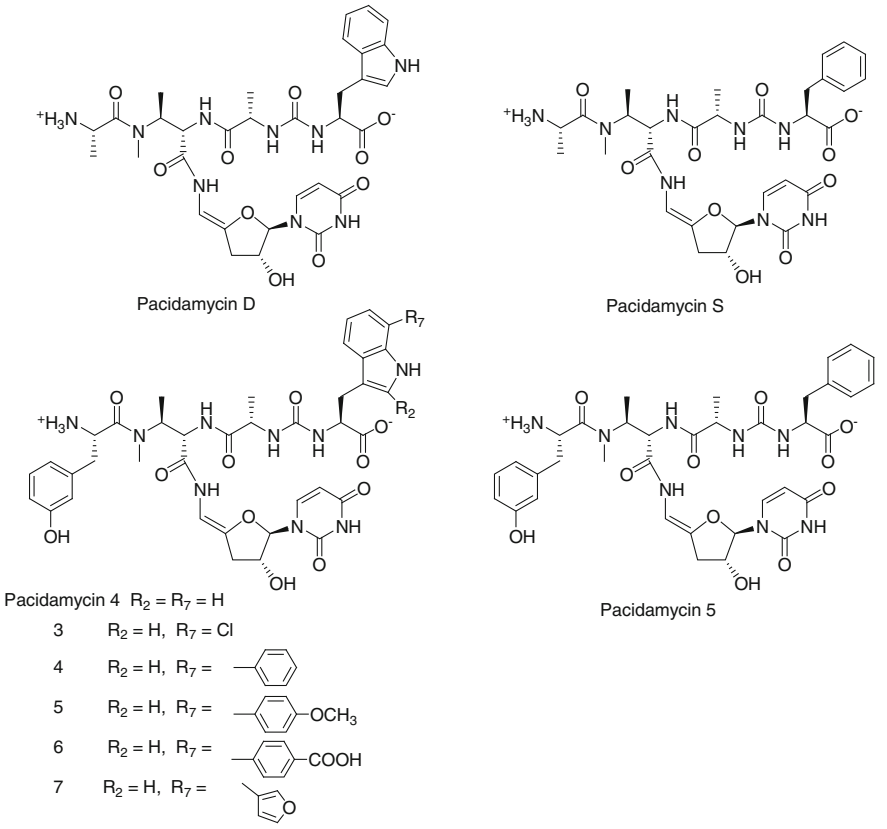


Fig. 9.3 Structures of pacidamycin D, S, 4 (plus biosynthetic analogs) and 5

As the mechanisms involved in the biosynthetic pathways of the UPAs are clarified, it will be increasingly feasible to use this knowledge to rationally create analogs for the exploration of SAR. Before the biosynthetic results noted above appeared, it was possible to design incorporation experiments that would take advantage of the apparent promiscuity of selected steps in pacidamycin biosynthesis (Gruschow et al. 2009). Given the natural variations observed in the C-terminal residue (tryptophan, phenylalanine, or *meta*-tyrosine) this site was targeted for modification through incorporation of synthetic tryptophan analogs by precursor-directed biosynthesis. In this study, it was found that 2 and 7-substituted tryptophan analogs were quite readily incorporated and the yields of the resulting pacidamycin 4 analogs were equivalent to the parent compounds. Although a detailed analysis of SAR was not done, it was found that 7-chloro substituents had subtle effects on potency against *Pseudomonas aeruginosa*, which provided encouragement for further experimentation.

An alternate route to structural diversification at the pacidamycin C-terminal involved introduction of a 7-chloro substituent on the indole via a genetically

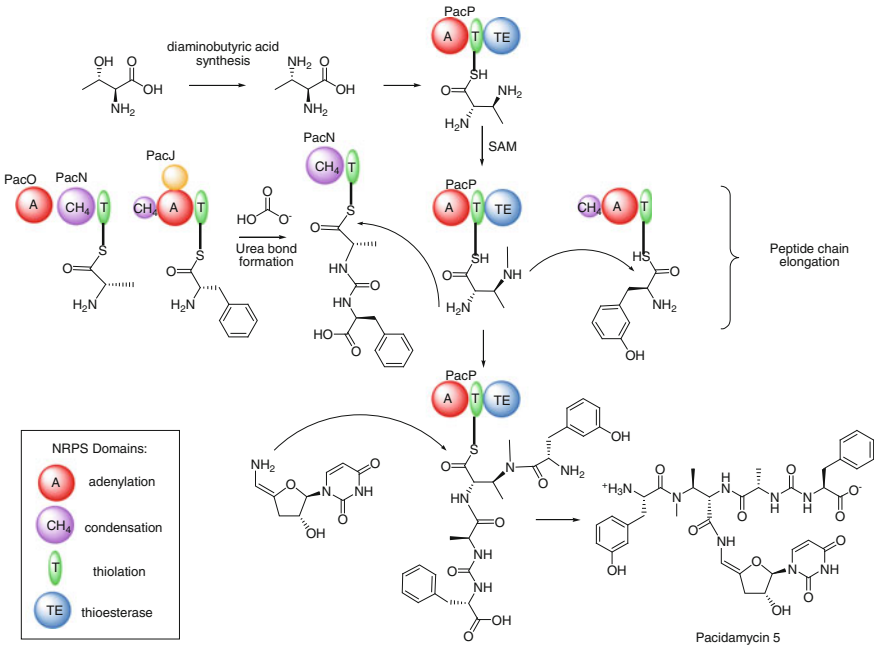


Fig. 9.4 Partial biosynthetic scheme for pacidamycin proposed by Zhang et al. (2010)

engineered halogenase (Deb Roy et al. 2010). This chlorination was achieved by introduction of prnA, the halogenase from pyrrolnitrin biosynthesis, into *S. coeruleorubidus* where it was functionally expressed. The resulting 7-chlorotryptophan analog of pacidamycin 4 (**3** in Fig. 9.3) was used as an intermediate in cross coupling reactions to create the extended aromatic analogs **4–7** in Fig. 9.3. The antibacterial properties of these new analogs have yet to be reported; however, it is clear that such processes enable the design and facile production of diversified analogs that will continue to define the structural requirements for activity.

Since the pacidamycins are naturally produced as suites of congeners differing in the N- and C-terminal residues, a simplified product profile would be desirable for the biosynthetic preparation of specific analogs. Goss and co-workers were able to accomplish this simplification by directing the metabolic flux into individual components utilizing engineered strains and mutasynthesis (Grüschow et al. 2011). Thus by disrupting the genes for the synthesis and incorporation of meta-tyrosine and supplementing cultures of the resultant strain with phenylalanine or tryptophan, only pacidamycin S or D respectively, was produced. Similar experiments were employed to produce single-component fermentations of pacidamycins 4 and 5 by disabling the adenylation domain of the N-terminal amino acid module. These results suggest that this pathway will be useful in the production of more highly divergent analogs through similar combinations of gene disruption and/or substitution and mutasynthesis.

9.3 Newer Compounds with Significantly Modified Structures

An active discovery program at the Daiichi Sankyo Company in Tokyo focused on inhibitors of bacterial translocase has yielded a number of interesting new UPA antibiotics (see Fig. 9.5). One rather subtle, yet significant, structural variation is found in antibiotics A-90289 A and B (Fujita et al. 2011). These homologs whose structural skeleton is apparently identical to the liposidomycins (the absolute stereochemistry was not defined) bear a sulfate group on the C-2′ position rather than on the C-2″ as found in all other liposidomycins. A-90289 A retains antibiotic potency and is an effective inhibitor of translocase I, indicating that the position of the sulfate moiety can be varied while retaining biological activity. A surprisingly divergent set of structural variations defines the unique phosphate ester-containing UPA, A-94964 (Fujita et al. 2008). The compound features an extended uronic acid moiety of eight carbons with oxygen substituents at the 6′ and 7′ positions. At the 6′ position, an undefined hexose unit branches off, whereas one arm of the phosphodiester links at position 7′. An aminohexose forms the other ester bond through its anomeric oxygen and is in turn linked through an amide bond to glycine that is capped by an α, β, γ, δ-unsaturated tri-methyl-branched C_{16} fatty acid. Despite these substantial structural changes A-94964 appears to function through inhibition of translocase I and shows modest potency against Gram-positive bacteria (Murakami et al. 2008). The structurally distinct capuramycin, A-102395, retains the nucleoside and glycosidic portions of the original compounds, but incorporates an unusual tripeptide for the caprolactam. The compound displayed potent inhibitory activity against translocase I, but was not active as an antibiotic. The authors speculate that this lack of activity may be due to lack of permeability into the bacterial membrane (Murakami et al. 2007).

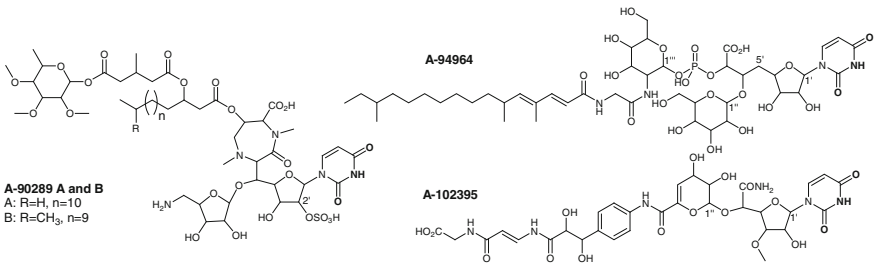


Fig. 9.5 Recently reported UPAs with significant structural modifications

9.4 Inhibition Mechanism and Structural Features

UPAs inhibit peptidoglycan synthesis by specifically inhibiting translocase I (MraY), an essential bacterial enzyme involved in cell wall biosynthesis (Isono and Inukai 1991; Inukai et al. 1993; Brandish et al. 1996). Developments since the earlier reports have been recently reviewed (Winn et al. 2010). The lack of a clear molecular basis for the inhibitory action of UPAs is in part due to the absence of detailed structural knowledge of the inhibitor-target complex; paucity of such data (Price and Momany 2005) hampers model-building for guiding inhibitor design and limits the full exploitation of this target.

It is well established that MraY interacts with the nucleotide-sugar moiety of its substrate, UDP-MurNAc pentapeptide, and catalyzes the transfer of phospho-MurNAc-pentapeptide to the membrane-bound C55 lipid, undecaprenylphosphate, to form a lipid intermediate (Brandish et al. 1996). The uracil and ribose moieties common to UPAs have been postulated as recognition elements that allow the UPAs to mimic the normal substrate to competitively inhibit MraY (Le Corre et al. 2007).

Mechanistic proposals have begun to emerge from studies of natural and synthetic MraY inhibitors. A recently proposed muraymycin-MraY binding model postulates that muraymycin's aminoribose amino group is positioned to interact with conserved Asp residues in cytoplasmic loop #2 (believed to assist with deprotonating the attacking undecaprenyl phosphate group) thereby competitively inhibiting the natural substrate's (UDP-MurNAc-pentapeptide) access to the active site and preventing transfer of MurNAc-pentapeptide to the membrane-bound lipid (Tanino et al. 2011).

Members of the muraymycin family were reported to inhibit MraY and show good antibacterial activity against a variety of Gram-positive organisms including *Staphylococcus aureus* and *Enterococci* (McDonald et al. 2002; Yamashita et al. 2003). The most active compound, muraymycin A1 (Fig. 9.6), was shown to protect mice against *S. aureus* infection at ED_{50} of 1.1 mg/kg. It was noted that the charged lipophilic side chain of muraymycin A1 conveyed excellent antibacterial activity, which could be correlated with the lipophilicity of the molecule (McDonald et al. 2002). A series of semi-synthetic muraymycin derivatives with modified amino groups demonstrated the importance of the aminoribose C-5″-NH2 group; derivatives substituted at this position (Fig. 9.6, R_2 is alkyl or acyl group) were not inhibitors of MraY while compounds substituted only at the secondary amino group (Fig. 9.6, R_1 is alkyl group) retained some activity, which appeared to correlate with lipophilicity (Lin et al. 2002).

Yamashita and co-workers (Yamashita et al. 2003) were the first to explore the SAR of the muraymycins via simplified synthetic analogs. Taking advantage of the SAR learned from modification of natural products (Lin et al. 2002), the group replaced the epi-capreomycidine with an arginine and removed the terminal aminoribose sugar (Fig. 9.6), which seem to only moderately affect translocase activity (Yamashita et al. 2003). The elegant total synthesis of muraymycin D2

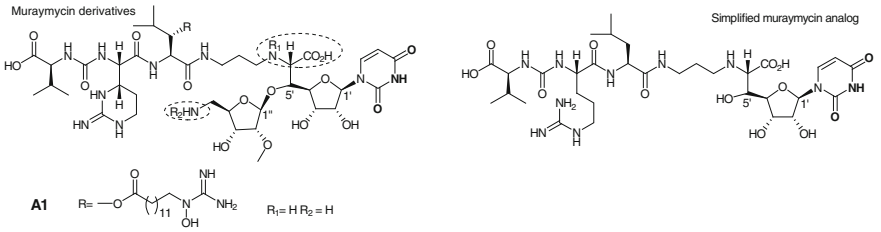


Fig. 9.6 Simplified muraymycin UPA analogs

and its epimer was achieved by Tanino and co-workers (Tanino et al. 2010b), and while the authors reported potent MraY activity for D2 and epi-D2, the compounds reportedly showed no antibacterial activity (Tanino et al. 2010a). The authors proceeded to define components that are needed for MraY activity. A truncated analog endowed with a lipophilic chain but lacking the epi-capreomycidine-urea-valine moiety was reported to be less active than the fully elaborated natural compound, confirming the importance of the urea dipeptide for activity (Tanino et al. 2010a). Also explored was the effect of the stereochemistry of the leucine moiety, revealing that the "natural" L configuration was more active against translocase I (Tanino et al. 2010a).

A number of muraymycin analogs were subsequently synthesized wherein the leucine was replaced by lipophilic substituents derived from key aldehyde intermediates (Tanino et al. 2010a). Though apparently not critical for MraY inhibition, the lipophilic moiety reportedly confers good membrane permeability resulting in improved antibacterial activity (Tanino et al. 2011). The synthetic lipophilic muraymycin analogs were less potent MraY inhibitors than the natural product muraymycin D2 (Tanino et al. 2010a). In other MraY inhibitor series such as the capuramycins, increased lipophilicity reportedly resulted in diminished MraY activity (Hotoda et al. 2003a).

Inhibition of MraY takes place at the inner face of the cytoplasmic membrane, requiring the inhibitor to cross the membrane to get to the active site (Anderson et al. 2000). Compound lipophilicity, known to facilitate passive membrane transport, has been reported to be an important property to optimize for antibacterial activity of UPA MraY inhibitors (Tanino et al. 2011; Ii et al. 2010; Lin et al. 2002; Dini et al. 2002). The inhibitors need to be lipophilic enough to cross the membrane and bind to the target without disrupting membrane integrity. It is likely that within a series of MraY inhibitors, logP values can guide the design of optimal compounds for cell membrane penetration (Li et al. 2011). Once in the cytoplasm, the optimized inhibitor should readily bind to the membrane-associated target.

Reports of lipophilic antibacterial agents designed to exploit MraY inhibition mechanism have highlighted a likely issue of detergent-like action contributing to antibacterial activity via membrane disruption, leading to possible nonspecific activity (Bogatcheva et al. 2011; Dubuisson et al. 2010; Nikonenko et al. 2009; Reddy et al. 2008). Reported correlation between lipophilic group chain length and

antibacterial activity, coupled with an inverse correlation between chain length and MraY inhibition appears to support an extra-target bactericidal mechanism (Ii et al. 2010).

9.5 Synthetic Approaches: Deconstruction-Construction of Natural Product Inhibitors of MraY

Studying the MraY inhibitory activity of natural and synthetic UPAs, including delineating the minimum structural fragments required for target inhibition, has provided not only mechanistic insights, but also a framework for designing novel MraY inhibitors. Simplified substituted phenyl capuramycin analogs having the structural features needed to inhibit MraY but showing ineffective antibacterial activity due in part to poor target access have been reported (Hotoda et al. 2003b). The caprolactam moiety has been shown to be nonessential for MraY inhibitory activity since synthetic capuramycin analogs lacking this moiety showed good MraY activity and these phenyl-containing analogs were reportedly equivalent to, or more potent than the natural product (Dubuisson et al. 2010). Since structural features necessary for MraY inhibition are present in these synthetic analogs and the closely related A-102395 (Murakami et al. 2007), the lack of antibacterial activity can be ascribed in part to poor bacterial membrane penetration (Hotoda et al. 2003b).

The synthesis of simplified oxazolidine-containing caprazamycin analogs by Ii and co-workers (Ii et al. 2010) further highlights the utility of a function-oriented synthetic approach where simple structural elements believed to be required for activity are installed. After establishing the importance of the uridine, arabinose, and lipophilic acyl moieties for activity and subsequently determining that the diazepinone ring was not essential, these workers made a series of derivatives wherein the oxazolidine served as a scaffold for holding the uracil, basic amino group and the fatty acyl chain in the correct orientation to confer antibacterial activity. Among the most active compounds (MIC's of 2–16 ug/mL; Fig. 9.7 compounds **19B** and **20B**) MraY inhibitory activity was significantly diminished relative to the nonacylated compound, suggesting an alternate mechanism for bactericidal activity (Ii et al. 2010).

Other examples of de-construction of natural MraY inhibitors to reveal core features, including stereochemical configuration, required for enzyme inhibition abound (Dini et al. 2000; Spork et al. 2011). In one study, the importance of a free C-5″ ribosyl NH2 group was demonstrated when its replacement with a hydroxyl group (See **RU64957** in Fig. 9.7) in a nonpeptidic riburamycin analog resulted in a near threefold reduction in MraY inhibition (Stachyra et al. 2004). Dini et al. (2000) reported that truncated synthetic liposidomycin analogs containing solely the nucleoside-amino sugar moiety (Fig. 9.7, $R_1 = OH$, $R_2 = NH_2$, $R_3 = H$) had moderate MraY inhibitory activity (i.e., the moiety is necessary but not sufficient

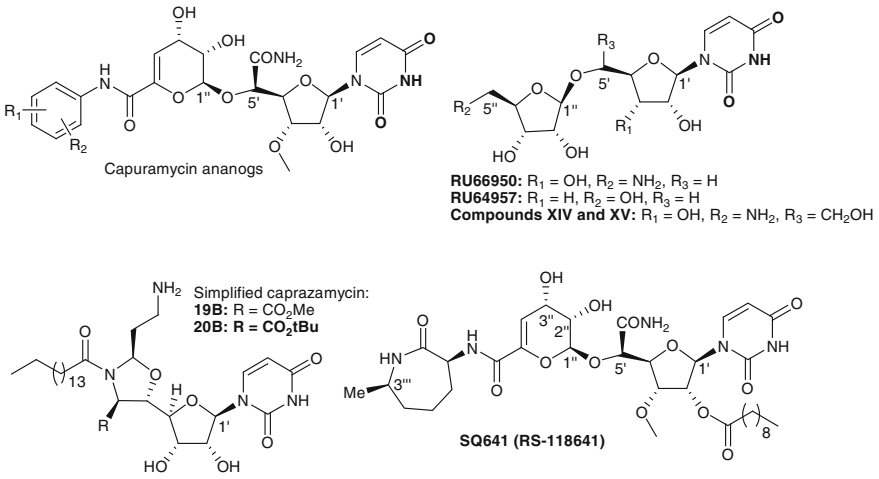


Fig. 9.7 Simplified and modified UPA analogs

for activity). The same study revealed that a compound with the natural 5′ S configuration (Fig. 9.7, $R_1 = OH$, $R_2 = NH_2$, $R_3 = CH_2OH$) showed 85-fold increased potency over its epimer, confirming the importance of the 5′ stereo-configuration (Dini et al. 2000). The more potent 5′ S isomer was still 76-fold less potent than mureidomycin B. From this deconstruction approach, low-molecular weight liposidomycin analogs with subnanomolar MraY activity but variable antibacterial activity were obtained (Dini et al. 2002). Cell membrane penetration can account for such variable activity.

In an excellent case study (Hotoda et al. 2003b; Hotoda et al. 2003a; Koga et al. 2004) of the de-construction-synthetic optimization approach, researchers at Daiichi-Sankyo created a library of capuramycin analogs from which SQ641, a C-2′-O-decanoyl, C-3‴-methyl analog (see Fig. 9.7), was identified as a good antimycobacterial agent (MIC = 1 ug/ml) that retained moderate MraY inhibition. While the C-2′-O-decanoyl substituent conferred sufficient lipophilicity for a compound to permeate through cell membrane, it also reportedly diminished the MraY inhibitory potency. The C-2′-acyl chain length appeared to inversely correlate with MraY inhibitory potency (i.e., the longer the chain length, the lower the potency), while positively correlating with antimycobacterial activity (Hotoda et al. 2003a).

While SQ641 showed modest MraY inhibition and good anti-*Mycobacterium tuberculosis* activity in vitro, its lipophilicity and low water solubility limited its cellular activity, requiring alternative delivery vehicle to improve in vivo activity (Nikonenko et al. 2009). Additional efforts to improve SQ641 activity spectrum (Bogatcheva et al. 2011) focused on acylation at the dihydropyran 2″- and 3″-O positions with either undecanoic acid or amino undecanoic acid. Esterification at both positions with undecanoic acid resulted in loss of antibacterial activity while

similar modification with amino undecanoic acid improved the antibacterial activity spectrum. These compounds, however, showed decreased MraY potency, supporting alternative mechanism(s) for antibacterial activity. Interestingly, SQ641 was reported to synergize with other antimycobacterial drugs against susceptible and resistant nontuberculous mycobacteria (Dubuisson et al. 2010) opening the possibility that membrane disruption by a UPA plays a role in the synergism.

9.6 Conclusions

There is mounting evidence that the UPAs require a degree of lipophilicity to enable them to cross the cell membrane and access the active site on the transmembrane target protein. It is not known whether the lipophilic moiety is cleaved to generate the pharmacophore once the UPA is at the active site. Because core MraY inhibitors (pharmacophores) are often more active than core plus long fatty chains in enzyme assays, it may be advantageous to design molecules with a dissociable lipophilic moiety. The muraymycins would be one example of a UPA with a cleavable side chain capable of giving rise to a potent MraY inhibitor.

Researchers have employed a number of approaches to identify antibacterial agents that act via inhibition of MraY. These include searching for new natural compounds, designing novel compounds, and evolving known inhibitors to exhibit better properties. Structure-based approaches focused on identifying the core structural elements required for retention of MraY inhibition. This can be followed by ADME (absorption, distribution, metabolism, and excretion)-driven installation of appropriate functionalities that confer physicochemical properties for target delivery and stability. It is likely that logP can be used to predict antibacterial potency within an MraY inhibitor compound class.

Approaches using antibacterial activity as the endpoint and sole guide for optimization have resulted in compounds that act by alternative mechanism(s). To exploit MraY's unique target-based selectivity and avoid nonspecific bactericidal action and detergent-like toxicity, it is suggested that MraY inhibitors should be designed and optimized with regard to both target enzyme inhibition and antibacterial activity. An attractive approach to new scaffolds and functionality is in the combination of biosynthetic methods coupled with synthetic derivatization. Taking advantage of selective bactericidal activity via MraY inhibition, it is important that the lipophilic fatty acid ("delivery") moiety of the UPA does not become the dominant active moiety. Optimization of a molecule with a balance of lipophilicity and hydrophilicity appears to be a reasonable approach to finding a good inhibitor of the unexploited target MraY.

The new insights gained into the biosynthetic processes involved in the assembly of the UPAs make it feasible to build new analogs through a combination of biosynthesis and chemical modifications. For example, the generation of truncated intermediates by fermentation for combinatorial synthesis with lipophilic

groups and/or amino acid residues is a strategy ripe for exploitation. And it seems clear that it is precisely this fine-tuning of the target activity and membrane permeability that is required to create a commercially viable agent.

References

Anderson MS, Eveland SS, Price NP (2000) Conserved cytoplasmic motifs that distinguish subgroups of the polyprenol phosphate:N-acetylhexosamine-1-phosphate transferase family. FEMS Microb Lett 191:169–175

Bogatcheva E, Dubuisson T, Protopopova M, Einck L, Nacy CA, Reddy VM (2011) Chemical modification of capuramycins to enhance antibacterial activity. J Antimicrob Chemother 66:578–587

Bouhss A, Trunkfield AE, Bugg TD, Mengin-Lecreulx D (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol Rev 32:208–233

Brandish PE, Kimura KI, Inukai M, Southgate R, Lonsdale JT, Bugg TD (1996) Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. Antimicrob Agents Chemother 40:1640–1644

Bugg TD, Braddick D, Dowson CG, Roper DI (2011) Bacterial cell wall assembly: still an attractive antibacterial target. Trends Biotechnol 29:167–173

Chatterjee S, Nadkarni SR, Vijayakumar EK, Patel MV, Ganguli BN, Fehlhaber HW, Vertesy L (1994) Napsamycins, new *Pseudomonas* active antibiotics of the mureidomycin family from *Streptomyces* sp. HIL Y-82,11372. J Antibiot (Tokyo) 47:595–598

Chen RH, Buko AM, Whittern DN, McAlpine JB (1989) Pacidamycins, a novel series of antibiotics with anti-*Pseudomonas aeruginosa* activity. II. Isolation and structural elucidation. J Antibiot 42:512–520

Deb Roy A, Gruschow S, Cairns N, Goss RJ (2010) Gene expression enabling synthetic diversification of natural products: chemogenetic generation of pacidamycin analogs. J Am Chem Soc 132:12243–12245

Dini C, Collette P, Drochon N, Guillot JC, Lemoine G, Mauvais P, Aszodi J (2000) Synthesis of the nucleoside moiety of liposidomycins: elucidation of the pharmacophore of this family of MraY inhibitors. Bioorg Med Chem Lett 10:1839–1843

Dini C, Didier-Laurent S, Drochon N, Feteanu S, Guillot JC, Monti F, Uridat E, Zhang J, Aszodi J (2002) Synthesis of sub-micromolar inhibitors of MraY by exploring the region originally occupied by the diazepanone ring in the liposidomycin structure. Bioorg Med Chem Lett 12:1209–1213

Dubuisson T, Bogatcheva E, Krishnan MY, Collins MT, Einck L, Nacy CA, Reddy VM (2010) In vitro antimicrobial activities of capuramycin analogues against non-tuberculous mycobacteria. J Antimicrob Chemother 65:2590–2597

Fujita Y, Kizuka M, Funabashi M, Ogawa Y, Ishikawa T, Nonaka K, Takatsu T (2011) A-90289 A and B, new inhibitors of bacterial translocase I, produced by *Streptomyces* sp. SANK 60405. J Antibiot 64:495–501

Fujita Y, Murakami R, Muramatsu Y, Miyakoshi S, Takatsu T (2008) A-94964, novel inhibitor of bacterial translocase I, produced by *Streptomyces* sp. SANK 60404. II. Physico-chemical properties and structure elucidation. J Antibiot 61:545–549

Gruschow S, Rackham EJ, Elkins B, Newill PL, Hill LM, Goss RJ (2009) New pacidamycin antibiotics through precursor-directed biosynthesis. ChemBioChem 10:355–360

Grüschow S, Rackham EJ, Goss RJM (2011) Diversity in natural product families is governed by more than enzyme promiscuity alone: establishing control of the pacidamycin portfolio. Chem Sci 2:2182–2186

Hotoda H, Daigo M, Furukawa M, Murayama K, Hasegawa CA, Kaneko M, Muramatsu Y, Ishii MM, Miyakoshi S, Takatsu T, Inukai M, Kakuta M, Abe T, Fukuoka T, Utsui Y, Ohya S (2003a) Synthesis and antimycobacterial activity of capuramycin analogs. Part 2: acylated derivatives of capuramycin-related compounds. Bioor Med Chem Lett 13:2833–2836

Hotoda H, Furukawa M, Daigo M, Murayama K, Kaneko M, Muramatsu Y, Ishii MM, Miyakoshi S, Takatsu T, Inukai M, Kakuta M, Abe T, Harasaki T, Fukuoka T, Utsui Y, Ohya S (2003b) Synthesis and antimycobacterial activity of capuramycin analogs. Part 1: substitution of the azepan-2-one moiety of capuramycin. Bioor Med Chem Lett 13:2829–2832

Ii K, Ichikawa S, Al-Dabbagh B, Bouhss A, Matsuda A (2010) Function-oriented synthesis of simplified saprazamycins: discovery of oxazolidine-containing uridine derivatives as antibacterial agents against drug-resistant bacteria. J Med Chem 53:3793–3813

Inukai M, Isono F, Takahashi S, Enokita R, Sakaida Y, Haneishi T (1989) Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. I. Taxonomy, fermentation, isolation and physico-chemical properties. J Antibiot 42:662–666

Inukai M, Isono F, Takatsuki A (1993) Selective inhibition of the bacterial translocase reaction in peptidoglycan synthesis by mureidomycins. Antimicrob Agents Chemother 37:980–983

Isono F, Inukai M (1991) Mureidomycin A, a new inhibitor of bacterial peptidoglycan synthesis. Antimicrob Agents Chemother 35:234–236

Isono K, Uramoto M, Kusakabe H, Kimura K, Isaki K, Nelson CC, McCloskey JA (1985) Liposidomycins: novel nucleoside antibiotics which inhibit bacterial peptidoglycan synthesis. J Antibiot 38:1617–1621

Kaysser L, Eitel K, Tanino T, Siebenberg S, Matsuda A, Ichikawa S, Gust B (2010a) A new arylsulfate sulfotransferase involved in liponucleoside antibiotic biosynthesis in streptomycetes. J Biol Chem 285:12684–12694

Kaysser L, Lutsch L, Siebenberg S, Wemakor E, Kammerer B, Gust B (2009) Identification and manipulation of the caprazamycin gene cluster lead to new simplified liponucleoside antibiotics and give insights into the biosynthetic pathway. J Biol Chem 284:14987–14996

Kaysser L, Tang X, Wemakor E, Sedding K, Hennig S, Siebenberg S, Gust B (2011) Identification of a napsamycin biosynthesis gene cluster by genome mining. ChemBioChem 12:477–487

Kaysser L, Wemakor E, Siebenberg S, Salas JA, Sohng JK, Kammerer B, Gust B (2010b) Formation and attachment of the deoxysugar moiety and assembly of the gene cluster for caprazamycin biosynthesis. Appl Environ Microbiol 76:4008–4018

Koga T, Fukuoka T, Doi N, Harasaki T, Inoue H, Hotoda H, Kakuta M, Muramatsu Y, Yamamura N, Hoshi M, Hirota T (2004) Activity of capuramycin analogues against *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium intracellulare* in vitro and in vivo. J Antimicrob Chemother 54:755–760

Le Corre L, Gravier-Pelletier C, Le Merrer Y (2007) Towards new MraY inhibitors: a serine template for uracil and 5-amino-5-deoxyribosyl scaffolding. Eur J Org Chemistry 32:5386–5394

Li Y-B, Xie Y-Y, Du N-N, Lu Y, Xu H-Z, Wang B, Yu Y, Liu Y-X, Song D-Q, Chen R-X (2011) Synthesis and in vitro antitubercular evaluation of novel sansanmycin derivatives. Bioorg Med Chem Lett 21:6804–6807

Lin YI, Li Z, Francisco GD, McDonald LA, Davis RA, Singh G, Yang Y, Mansour TS (2002) Muraymycins, novel peptidoglycan biosynthesis inhibitors: semisynthesis and SAR of their derivatives. Bioorg Med Chem Lett 12:2341–2344

McDonald LA, Barbieri LR, Carter GT, Kruppa G, Feng X, Lotvin JA, Siegel MM (2003) FTMS structure elucidation of natural products: application to muraymycin antibiotics using ESI multi-CHEF SORI-CID FTMS(n), the top-down/bottom-up approach, and HPLC ESI capillary-skimmer CID FTMS. Anal Chem 75:2730–2739

McDonald LA, Barbieri LR, Carter GT, Lenoy E, Lotvin J, Petersen PJ, Siegel MM, Singh G, Williamson RT (2002) Structures of the muraymycins, novel peptidoglycan biosynthesis inhibitors. J Am Chem Soc 124:10260–10261

Murakami R, Fujita Y, Kizuka M, Kagawa T, Muramatsu Y, Miyakoshi S, Takatsu T, Inukai M (2007) A-102395, a new inhibitor of bacterial translocase I, produced by *Amycolatopsis* sp. SANK 60206. J Antibiot (Tokyo) 60:690–695

Murakami R, Fujita Y, Kizuka M, Kagawa T, Muramatsu Y, Miyakoshi S, Takatsu T, Inukai M (2008) A-94964, a novel inhibitor of bacterial translocase I, produced by *Streptomyces* sp. SANK 60404. I. Taxonomy, isolation and biological activity. J Antibiot (Tokyo) 61:537–544

Nikonenko BV, Reddy VM, Protopopova M, Bogatcheva E, Einck L, Nacy CA (2009) Activity of SQ641, a capuramycin analog, in a murine model of tuberculosis. Antimicrob Agents Chemother 53:3138–3139

Price NP, Momany FA (2005) Modeling bacterial UDP-HexNAc: polyprenol-P HexNAc 1-P transferases. Glycobiology 15:29R–42R

Rackham EJ, Gruschow S, Ragab AE, Dickens S, Goss RJ (2010) Pacidamycin biosynthesis: identification and heterologous expression of the first uridyl peptide antibiotic gene cluster. ChemBioChem 11:1700–1709

Reddy VM, Einck L, Nacy CA (2008) In vitro antimycobacterial activities of capuramycin analogues. Antimicrob Agents Chemother 52:719–721

Siebenberg S, Kaysser L, Wemakor E, Heide L, Gust B, Kammerer B (2011) Identification and structural elucidation of new caprazamycins from *Streptomyces* sp. MK730-62F2 by liquid chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 25:495–502

Spork AP, Wiegmann D, Granitzka M, Stalke D, Ducho C (2011) Stereoselective synthesis of uridine-derived nucleosyl amino acids. J Org Chem 76:10083–10098

Stachyra T, Dini C, Ferrari P, Bouhss A, van Heijenoort J, Mengin-Lecreulx D, Blanot D, Biton J, Le Beller D (2004) Fluorescence detection-based functional assay for high-throughput screening for MraY. Antimicrob Agents Chemother 48:897–902

Tanino T, Al-Dabbagh B, Mengin-Lecreulx D, Bouhss A, Oyama H, Ichikawa S, Matsuda A (2011) Mechanistic analysis of muraymycin analogues: a guide to the design of MraY inhibitors. J Med Chem 54:8421–8439

Tanino T, Ichikawa S, Al-Dabbagh B, Bouhss A, Oyama H, Matsuda A (2010a) Synthesis and biological evaluation of muraymycin analogues active against anti-drug resistant bacteria. ACS Med Chem Lett 1:258–262

Tanino T, Ichikawa S, Shiro M, Matsuda A (2010b) Total synthesis of (-)-muraymycin D2 and its epimer. J Org Chem 75:1366–1377

Walsh CT, Zhang W (2011) Chemical logic and enzymatic machinery for biological assembly of peptidyl nucleoside antibiotics. ACS Chem Biol 6:1000–1007

Winn M, Goss RJ, Kimura K, Bugg TD (2010) Antimicrobial nucleoside antibiotics targeting cell wall assembly: recent advances in structure-function studies and nucleoside biosynthesis. Nat Prod Rep 27:279–304

Xie Y, Chen R, Si S, Sun C, Xu H (2007) A new nucleosidyl-peptide antibiotic, sansanmycin. J Antibiot 60:158–161

Yamaguchi H, Sato S, Yoshida S, Takada K, Itoh M, Seto H, Otake N (1986) Capuramycin, a new nucleoside antibiotic. Taxonomy, fermentation, isolation and characterization. J Antibiot (Tokyo) 39:1047–1053

Yamashita A, Norton E, Petersen PJ, Rasmussen BA, Singh G, Yang Y, Mansour TS, Ho DM (2003) Muraymycins, novel peptidoglycan biosynthesis inhibitors: synthesis and SAR of their analogues. Bioorg Med Chem Lett 13:3345–3350

Zhang W, Ntai I, Bolla ML, Malcolmson SJ, Kahne D, Kelleher NL, Walsh CT (2011a) Nine enzymes are required for assembly of the pacidamycin group of peptidyl nucleoside antibiotics. J Am Chem Soc 133:5240–5243

Zhang W, Ntai I, Neil LK, Christopher TW (2011b) tRNA-dependent peptide bond formation by the transferase PacB in biosynthesis of the pacidamycin group of pentapeptidyl nucleoside antibiotics. PNAS 108:12249–12253

Zhang W, Ostash B, Christopher TW (2010) Identification of the biosynthetic gene cluster for the pacidamycin group of peptidyl nucleoside antibiotics. PNAS 107:16828–16833

Chapter 10
Aminoglycoside Antibiotics

Herbert A. Kirst and Flavia Marinelli

Abstract Aminoglycosides were the second major antibiotic class to be discovered from fermentation of microorganisms. They are structurally characterized by an aminocyclitol that is substituted in several different patterns by amino- and/or neutral sugar moieties. They are potent, bactericidal, water-soluble compounds that are given by parenteral administration. They are especially useful for treatment of infections caused by Gram-negative bacteria, including *Pseudomonas aeruginosa.* Limitations of the class include the development of microbial resistance by a variety of mechanisms and patient toxicity, especially nephrotoxicity and ototoxicity. Several semi-synthetic derivatives have been developed that have improved features, especially increased activity against resistant strains of bacteria. The newest aminoglycoside, plazomicin, is a semi-synthetic derivative of sisomicin that is undergoing further evaluation in clinical trials.

10.1 Introduction

The antibiotic era is generally considered to have begun with Alexander Fleming's 1929 discovery of antibacterial activity produced by a *Penicillium* mold. The subsequent multi-year development and eventual therapeutic success of the first safe and efficacious penicillin antibiotics prompted other researchers to investigate

H. A. Kirst (✉)
7840 West 88th Street, Indianapolis, IN 46278, USA
e-mail: herbertkirst1@comcast.net

F. Marinelli
Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

F. Marinelli
The Protein Factory Research Center, Politecnico of Milano, ICRM CNR Milano and University of Insubria, Varese, Italy

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_10,

soil-derived microorganisms for their biosynthetic potential to create novel antimicrobial substances. The aminoglycosides, represented first by streptomycin, were the second major antibiotic class to be discovered from using this new research model involving microbial fermentations (Borders 2005). The discovery of streptomycin by Selman Waksman and his colleagues at Rutgers University reinforced the potential value of investigating fermentation products as a previously untapped and novel resource for finding useful new antibiotics (Waksman 1953).

The aminoglycoside antibiotics are structurally characterized by an aminocyclitol moiety to which is attached a variety of amino- and/or neutral sugars according to several different patterns of substitution. These structural features make the molecules strongly basic water-soluble entities. Although they exhibit strong bactericidal activity against many problematic pathogens, major problems encountered with this class are the development of microbial resistance and patient toxicity, especially ototoxicity and nephrotoxicity. Understanding and solving these limitations have been a major focus of research efforts over several decades. Just as observed for many other antibiotic classes, semi-synthetic aminoglycoside derivatives also now play a critical role in antimicrobial regimens. These derivatives enhance many of the therapeutic properties and expand the practical uses of the class. Several comprehensive reviews of the aminoglycosides have been published (Arya 2007; Dozzo and Moser 2010; Gilbert and Leggett 2009; Kirst and Allen 2007; Mitscher 2010).

10.2 Streptomycin

Pursuing this new strategy of investigating soil microbes, streptomycin was discovered as the fermentation product of *Streptomyces griseus* (Schatz et al. 1944). It has a pseudotrisaccharide structure composed of an aminocyclitol named streptidine that is coupled to an uncommon disaccharide moiety (Fig. 10.1) (Neidle et al. 1968). Removal of the two guanidine units from streptidine yields a smaller aminocyclitol called streptamine (Fig. 10.1).

In contrast to penicillin and many other earlier discovered agents which were active against only Gram-positive bacteria, streptomycin expanded the antimicrobial spectrum by inhibiting several important Gram-negative bacteria as well as *Mycobacterium tuberculosis*. However, the initial enthusiasm was dampened because the compound also readily provoked bacterial resistance which severely limited its use as a single agent. Thus, it is now used in antibiotic combinations in order to minimize development of resistance.

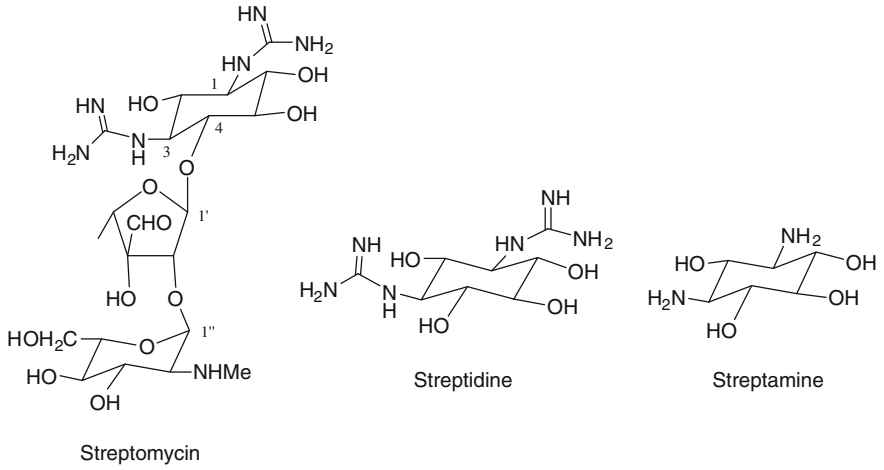
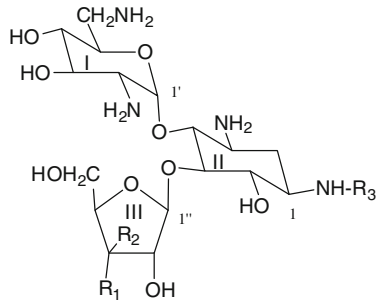


Fig. 10.1 Structures of streptomycin and components

10.3 4,5-Disubstituted-2-Deoxystreptamine Family

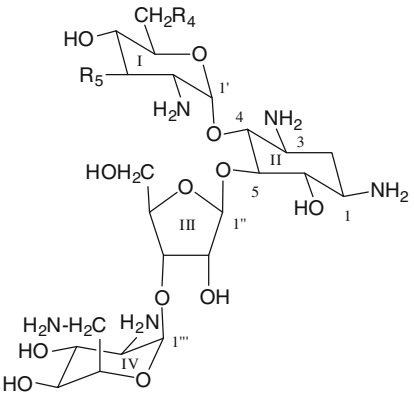
The majority of clinically useful aminoglycosides possess 2-deoxystreptamine (2-DOS) as their aminocyclitol, which is bis-substituted in either a 4,5- or 4,6-pattern. The former pattern was first found in the neomycin complex, which was another early discovery from the Waksman group (Waksman and Lechevalier 1949). Neomycin B is produced by *Streptomyces fradiae* and its structure is a pseudo-tetrasaccharide (Fig. 10.2) that possesses six amino groups (Hichens and Rinehart 1963). It also exhibits a broad spectrum of antimicrobial action, but its systemic toxicity was soon recognized and its use was consequently restricted to localized applications such as topical administrations.

Several other interesting members of the 4,5-disubstituted family were subsequently found by different research groups. The paromomycin and lividomycin groups differ from the neomycin complex in the pattern of their amino and hydroxyl groups and/or the stereochemistry of substituents at certain positions (Fig. 10.2). Ribostamycin is a pseudotrisaccharide that is missing the ring IV aminosugar that is present in the other groups. Lividomycin and ribostamycin have found some limited use as antibiotics in certain countries to treat Gram-negative infections. However, the most significant 4,5-aminoglycoside is probably butirosin, which is produced by *Bacillus circulans* (Woo et al. 1971). Its structure contains the 1-*N*-(4-amino-2(*S*)-hydroxybutyryl) (AHBA) moiety (Fig. 10.2) which provided the extremely valuable clue for developing a series of semi-synthetic derivatives having activity against many resistant organisms (see Sect. 10.7).



Ribostamycin: R_1 = OH, R_2 = R_3 = H
Butirosin B: R_1 = OH, R_2 = H
R_3 = 4-amino-2(*S*)-hydroxybutyryl

Xylostasin: R_2 = OH, R_1 = R_3 = H
Butirosin A: R_2 = OH, R_1 = H
R_3 = 4-amino-2(S)-hydroxybutyryl



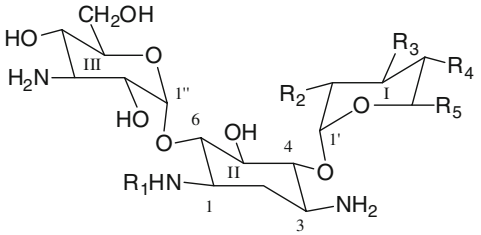
Neomycin B: R_4 = NH_2, R_5 = OH
Paromomycin I: R_4 = OH, R_5= OH
Lividomycin B: R_4 = OH, R_5 = H

Fig. 10.2 Representative 4,5-di-O-substituted-2-DOS-aminoglycosides

10.4 4,6-Disubstituted-2-Deoxystreptamine Family

The 4,6-disubstituted-2-DOS family has contributed the most important and widely used clinical aminoglycosides. They are generally divided into two groups based on the structures of their sugar substituents. Kanamycin A was reported in 1957 as the product of *Streptomyces kanamyceticus* (Umezawa et al. 1957) (Fig. 10.3). Kanamycin B contains a 2′-amino group instead of the 2′-hydroxyl group on ring I in kanamycin A, a change that increases activity about two-fold. Tobramycin is 3′-deoxy-kanamycin B and is produced from hydrolysis of 6″-O-carbamoyl-tobramycin (nebramycin factor 5′) that occurs during work-up of the fermentation broth (Koch et al. 1974).

While most early antibiotic research was focused on fermentation products obtained from *Streptomyces* species, scientists at Schering Corp. in the early 1960s directed their attention toward non-*Streptomyces*, especially *Micromonospora* species. This shift of focus yielded the gentamicin complexes and later, the sisomicin group of aminoglycosides (Figs. 10.4 and 10.5). The clinically used gentamicin complex is comprised predominantly of the three factors C_1, C_{1a}, and C_2 (Fig. 10.4) which differ from the kanamycins and tobramycin in their ring III aminosugar (Cooper 1971). In contrast, the sisomicin group contains an uncommon unsaturated sugar moiety at that position (Reimann et al. 1974). The three-component gentamicin complex pioneered the medical success of the aminoglycoside class and

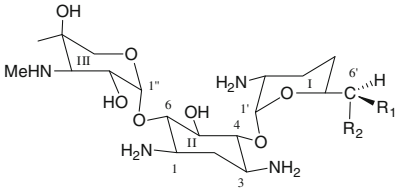


Kanamycin A: $R_1 = H$, $R_2 = R_3 = R_4 = OH$, $R_5 = NH_2$
Kanamycin B: $R_1 = H$, $R_2 = R_5 = NH_2$, $R_3 = R_4 = OH$
Kanamycin C: $R_1 = H$, $R_2 = NH_2$, $R_3 = R_4 = R_5 = OH$

Tobramycin : $R_1 = H$, $R_2 = R_5 = NH_2$, $R_3 = H$, $R_4 = OH$
Dibekacin: $R_1 = H$, $R_2 = R_5 = NH_2$, $R_3 = R_4 = H$

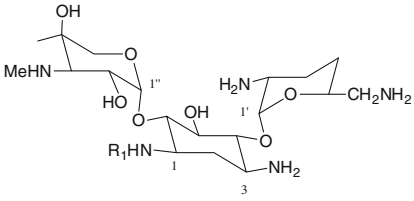
Amikacin: R_1 = 4-amino-2(*S*)-hydroxybutyryl , $R_2 = R_3 = R_4 = OH$, $R_5 = NH_2$
Habekacin: R_1 = 4-amino-2(*S*)-hydroxybutyryl , $R_2 = R_5 = NH_2$, $R_3 = R_4 = H$

Fig. 10.3 Important members of the kanamycin-tobramycin group



Gentamicin C_1: $R_1 = Me$, $R_2 = NHMe$
Gentamicin C_{1a}: $R_1 = H$, $R_2 = NH_2$

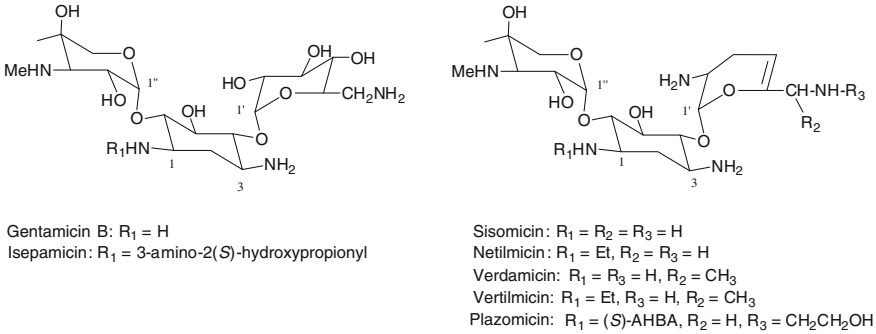
Gentamicin C_2: $R_1 = Me$, $R_2 = NH_2$
Gentamicin C_{2a}: $R_1 = NH_2$, $R_2 = Me$
Gentamicin C_{2b} (Micronomicin): $R_1 = H$, $R_2 = NHMe$



Gentamicin C_{1a}: $R_1 = H$
Etimicin: R_1 = ethyl

Fig. 10.4 Structures of gentamicin C compounds

established its clinical utility for treating Gram-negative bacteria and other susceptible pathogens. Tobramycin exhibited a similar spectrum of Gram-negative activity and may be preferred especially against *P. aeruginosa* where it is often considered more active (Singh 2001).



Gentamicin B: R_1 = H
Isepamicin: R_1 = 3-amino-2(*S*)-hydroxypropionyl

Sisomicin: $R_1 = R_2 = R_3$ = H
Netilmicin: R_1 = Et, $R_2 = R_3$ = H
Verdamicin: $R_1 = R_3$ = H, $R_2 = CH_3$
Vertilmicin: R_1 = Et, R_3 = H, $R_2 = CH_3$
Plazomicin: R_1 = (*S*)-AHBA, R_2 = H, $R_3 = CH_2CH_2OH$

Fig. 10.5 Semi-synthetic derivatives of gentamicin B-sisomicin group

10.5 Miscellaneous Natural Aminoglycosides

A few aminoglycosides have become useful products that have a structural pattern that differs from a disubstituted 2-DOS motif. Apramycin has a mono-substituted 2-DOS with an unusual bis-glycosyl moiety attached to the 4-hydroxyl group of 2-DOS (Fig. 10.6) (O'Connor et al. 1976). It is an important antibiotic for treatment and control of veterinary pathogens including certain Gram-negative bacteria (Mortensen et al. 1996). Spectinomycin has a 1,3-diamino-cyclitol embedded within an unusual tricyclic ring system in which the two cyclitol-amino groups are N-methylated (Cochran et al. 1972). It is still an important antibiotic for treating gonorrhea including infections by some resistant strains of gonococci (Lo et al. 2012). Hygromycin B is another pseudotrisaccharide in which the 1,3-diamino-cyclitol is N-methylated on only one amino group (Neuss et al. 1970). It is used as an anthelmintic agent to aid in the control of certain intestinal parasitic nematodes in poultry (Elanco Animal Health 2013). In contrast to all of these different 1,3-diaminocyclitols, fortimicin A possesses a 1,4-diaminocyclitol moiety (Fig. 10.6). It has antibacterial activity comparable to kanamycin that includes activity against certain resistant strains (Girolami and Stamm 1977). These compounds illustrate some of the wide diversity of structures that are achievable in the aminoglycoside class.

10.6 Mode of Action and Resistance

Aminoglycosides are one of the classical antibiotics that act by binding to the 16S rRNA subunit of the 30S bacterial ribosome, which inhibit protein synthesis by generating errors in translation. They interact with the ribosome decoding site deputed to the correct codon-anticodon recognition, notably targeting the A site for aminoacyl-tRNA binding. The various hydroxyl and amino substituents on the

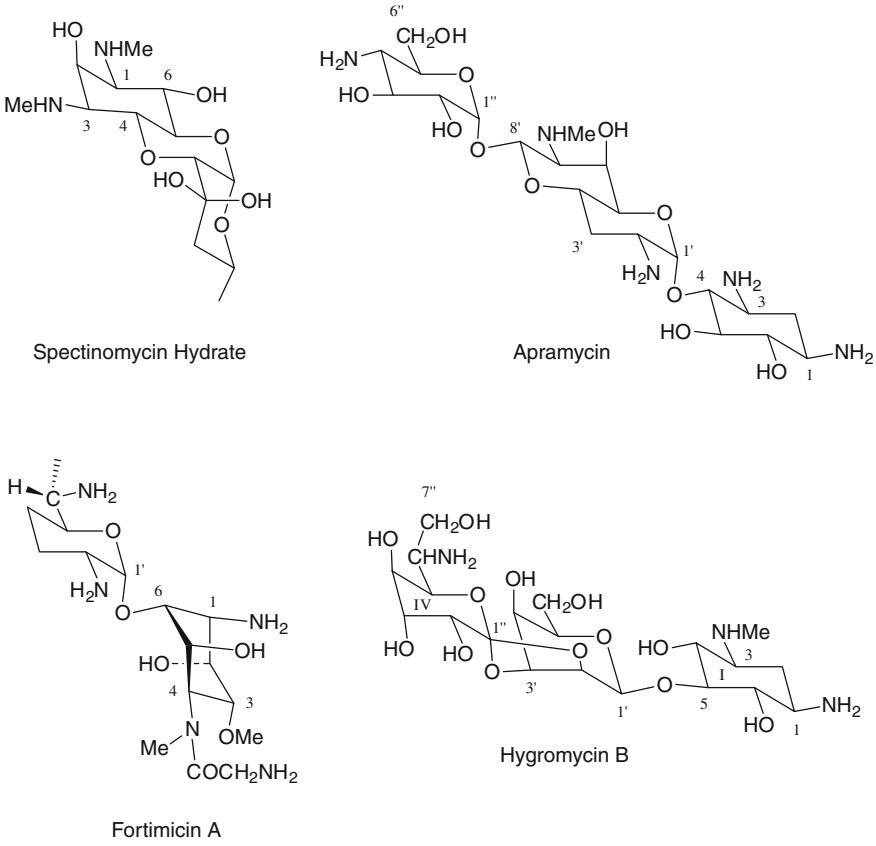


Fig. 10.6 Structures of miscellaneous aminoglycosides

cyclitol rings provide a high affinity hydrogen bonding network with regions of the 16S rRNA. X-ray and nuclear magnetic resonance imaging studies have pointed out specific ribosome interactions and diverse molecular mechanisms of action among structurally diverse aminoglycosides (Yonath 2005). While aminoglycosides are thought to be effective by inducing miscoding and hindering translocation, significant differences in the level of miscoding and effects on steps not directly linked to translocation have been recently observed (Tsai et al. 2013). Streptomycin and paromomycin increase the error rate of translation fixing the 30S subunit in its *ram* state which lowers the fidelity of tRNA recognition in the A site (Carter et al. 2000). Hygromycin B restricts the movement of helix H44 and hence, limits or inhibits the conformation changes crucial for the movement of this helix during translocation, and the antibiotic confiscates the tRNA in the A site (Brodersen et al. 2000). Spectinomycin inhibits elongation-factor-G catalyzed translocation of the peptidyl-tRNA from the A site to the P site (Carter et al. 2000). Recent studies show that apramycin mainly blocks translocation, whereas

gentamicin exhibits a more complex mode of action causing significant miscoding, blocking intersubunit rotation, and inhibiting translocation (Tsai et al. 2013). The observed variability in binding and action modes of structurally different aminoglycosides represent a promising starting point for designing novel aminoglycosides, using structural information.

The covalent modification of OH and NH_2 groups in the aminoglycosides by aminoglycoside-modifying enzymes (AMEs) interfere with the specific recognition of the 16S rRNA. Antibiotic deactivation by AMEs such as N-acetylation (AAC), O-adenylylation (ANT), and O-phosphorylation (APH), represent the major mechanism of bacterial resistance to aminoglycosides (see Paitan and Ron, this volume). Increasing resistance levels mediated by the first wave of plasmid-borne AMEs triggered the search for semi-synthetic aminoglycosides, in order to avoid enzyme deactivation, resulting in the creation and development of amikacin, arbekacin, netilmicin, and isepamicin (see below). AMEs have emerged over time that generate resistance to each of these semi-synthetic aminoglycosides (Wright 1999). Nevertheless, a thorough understanding of the existing AMEs is considered the foundation for the design of a new compound that is active in the presence of those AMEs that are, or will be, of clinical significance in the coming years (Zhanel et al. 2012).

Less common aminoglycoside resistance mechanisms are the regulation of intracellular concentration by overexpression of efflux pumps and/or changes in membrane permeability, and expression of ribosomal target modifying enzymes. Although the variety of mechanisms that lead to aminoglycoside resistance are numerous and the genetic sequences that encode them are even more so, no new mechanism for aminoglycoside modification has been identified in over 20 years (Tenover and Elvrum 1988).

A recent survey (Armstrong and Miller 2010), focusing on MDR pathogens isolated in 2007 have demonstrated that most common AMEs have not changed in recent years. The enzymes ANT(2″)-I, AAC(3)-II, and AAC(6′)-I (either alone or in combination) remain the key resistance determinants among the *Enterobacteriaceae*. AMEs, in particular APH(2″)-I and AAC(6′)-I, are considered relevant in the resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) to aminoglycosides (Tenover et al. 2011). Resistance in *P. aeruginosa* is commonly due to up-regulation of the MexXY efflux pump (Poole 2005). Blocking the uptake of aminoglycosides through changes in outer membrane permeability or porin down-regulation may occur in parallel and can also elevate aminoglycoside MICs (El'Garch et al. 2007). Two AMEs are also common in P. *aeruginosa*: ANT(2″)-I and AAC(6′)-II. Resistance among *Acinetobacter* spp. is often caused by combinations of AMEs in addition to permeability and efflux-based mechanisms (Marchand et al. 2004). Plasmid-mediated target modification is the least common means of generating aminoglycoside resistance, but it has recently surfaced in *Enterobacteriaceae* in the form of ribosomal methyltransferases (RMT) (Doi and Arakawa 2007). These enzymes methylate the ribosomal binding site and generate high MICs to all 4,6-linked aminoglycosides.

10.7 Semi-Synthetic Aminoglycosides

The activity of gentamicin, tobramycin, and the other natural-product aminoglycosides against Gram-negative bacteria established them as one of the major important antibiotic classes. However, several limitations of these agents were recognized early in their history, particularly the development of microbial resistance and the onset of patient toxicity, especially nephrotoxicity and ototoxicity. Consequently, efforts to alleviate these problems by the preparation and testing of semi-synthetic derivatives began soon after their discovery. Much of the early semi-synthetic work followed the results derived from structure–activity relationship (SAR) studies that were obtained from analyzing test results on the many natural-product aminoglycosides (Price et al. 1977).

Early observation from the SAR concerning the hydroxyl and amino groups in the kanamycin-tobramycin series suggested that optimum activity was attained with a 2′,6′-diamino-3′-deoxy substitution pattern in ring 1, which is characteristic of tobramycin. In the course of further investigating this interesting SAR during the synthesis of additional derivatives, 3′,4′-dideoxy-kanamycin B (Fig. 10.3) was prepared, which showed strong in vitro activity that included certain resistant strains (Umezawa et al. 1971). It was subsequently developed in Japan and other countries under the name of dibekacin.

However, the most significant early SAR direction learned from the natural products was the effect against resistant bacteria of the AHBA group that had been initially found in butirosin. In follow-up of this lead, many aminoglycoside substrates were converted to their AHBA derivatives and evaluated for their activity against batteries of resistant strains (Kawaguchi et al. 1972). The 1-*N*-AHBA derivative of kanamycin A was selected as the most broadly useful derivative and under the name of amikacin (Fig. 10.3), it became established as the most widely used semi-synthetic aminoglycoside antibiotic (Kawaguchi and Naito 1983).

The commercial and medical success of amikacin ensured that further research efforts at finding improved analogs would continue. However, although many compounds were prepared and evaluated, only a few became approved products. The 1-*N*-AHBA derivative of dibekacin was prepared and developed under the name of habekacin (Fig. 10.3) (Kondo et al. 1973). Derivatization of gentamicin factors other than members of the C complex was investigated, which produced the 1-*N*-(3-amino-2(*S*)-hydroxypropionyl) derivative (AHPA) of gentamicin B named isepamicin (Fig. 10.5) (Nagabhushan et al. 1978; Falagas et al. 2012).

In addition to *N*-acyl derivatives, a variety of *N*-alkyl derivatives were also prepared, especially 1-*N*-alkyl compounds. However, in this case, the 1-*N*-ethyl derivatives were selected as the optimum substitution. The first *N*-ethyl derivative to be developed was 1-*N*-ethyl-sisomicin, named netilmicin (Fig. 10.5). It was selected for its good potency, activity against some resistant bacteria, and lower chronic toxicity compared to sisomicin and gentamicin (Wright 1976). Other *N*-ethyl derivatives recently being studied especially in Asia are etimicin and

vertilmicin, which are the 1-*N*-ethyl derivatives of gentamicin C_{1a} and verdamicin, respectively (Fig. 10.5) (Chaudhary et al. 2012; Li et al. 2008).

Most recently, a new derivative has been prepared from sisomicin that has been modified to contain both a 1-*N*-AHBA group and a new 6′-*N*-hydroxyethyl substituent (Fig. 10.5). This new compound originated from Achaogen and is named plazomicin (formerly ACHN-490). It is currently being evaluated in clinical trials (Armstrong and Miller 2010, Zhanel et al. 2012). Since in vitro activity against resistant bacteria is easily measured and optimized, all of these semi-synthetic derivatives exhibit some degree of activity against some types of antibiotic-resistant bacteria and are thus potentially useful for treating infections by such strains if and when they occur. However, reduction of ototoxicity and nephrotoxicity is also very important, but they are more challenging concerns to consistently and accurately predict prior to clinical trials, so progress toward this goal has been much slower.

10.8 Microbiology and Pharmacology

As previously mentioned, the spectrum of antibacterial activity for the aminoglycosides covers many Gram-negative bacteria which are not covered by the more numerous classes of antibiotics active against Gram-positive pathogens (Becker and Cooper 2013; Gilbert and Leggett 2009; Kirst and Allen 2007; Magnet and Blanchard 2005; Mitscher 2010). This Gram-negative coverage importantly includes susceptible species of the *Enterobacteriaceae*, such as *E. coli* and species of *Klebsiella, Enterobacter, Proteus, Serratia*, etc., and the *Pasteurellaceae*, such as *Haemophilus influenzae* (see Paitan and Ron, this volume). In addition, a high degree of activity from some aminoglycosides against more resistant Gram-negative bacteria such as *P. aeruginosa* and, more recently, *Acinetobacter* species is critical because few other older antibiotics had such useful activity (Aggen 2010; Pagkalis et al. 2011). Aminoglycosides have activity against *Staphylococcus aureus* and are often used in combination with another antibiotic against that bacterium, including resistant strains. Some aminoglycosides are also used in combinations against *Mycobacterium tuberculosis* in order to minimize microbial resistance. Other specialty roles or niches for certain aminoglycosides have been noted within the chemistry sections above.

The aminoglycosides became less important starting in the late 1970s and onward when the newer more potent broad-spectrum fluoroquinolones (see Pucci and Wiles, this volume) and beta-lactam (see Leemans et al., this volume) antibiotics began to emerge that possessed stronger Gram-negative antibacterial activities along with much-reduced toxicity and side-effects. However, the now well-documented development and spread of bacterial resistance which arose during that same period has returned back to active service as many diverse classes of antibiotics as are available to use. Thus, the well-known aminoglycosides have come back at least partially in the mix of different antibiotics being used to

minimize the microbial resistance problems.The high amino-carbohydrate composition of aminoglycosides makes them highly water-soluble and strongly basic compounds. These agents are not orally absorbed to any significant extent and gastrointestinal applications are thus confined to localized events such as preoperative bowel cleansing or eradicating intestinal parasites. Topical applications include treatment of burn wounds where *P. aeruginosa* is a highly infective pathogen (Bracco et al. 2008). Similarly, a lung spray of an aminoglycoside, especially tobramycin, is inhaled to treat *P. aeruginosa* infections in cystic fibrosis patients (Prayle and Smyth 2010). Aminoglycosides are also used to treat certain eye infections (McCormick et al. 2008). For systemic infections, aminoglycosides must be parenterally administered, either by intravenous (i.v.) or intramuscular (i.m.) routes. Recent studies have indicated that fewer toxicity problems are generally encountered following a therapeutic regimen of once-daily dosing compared to a multiple-daily dosing schedule.

Most aminoglycosides are bactericidal antibiotics that exhibit concentration-dependent killing. They also display a prolonged post-antibiotic effect (PAE). These compounds are not protein bound to any significant extent. Their non-lipophilic highly polar nature hinders their passive movement across many biological barriers in the absence of any active-transport systems. They are largely non-metabolized within the body and are then excreted intact by glomerular filtration from the kidneys into the urine. Consequently, an adjustment to a decreased dose is required for patients with kidney disease or other renal impairments in order to prevent an unsafe accumulation of the drug and subsequent toxicity.

10.9 Toxicity

The principal and well-known problems that might be encountered with administration of aminoglycoside antibiotics are nephrotoxicity and ototoxicity (Pagkalis et al. 2011). Other toxicities may include vestibular problems (loss of balance or equilibrium), ocular and retinal effects, and neuromuscular blockade (Ariano et al. 2008; Penha et al. 2010; Pasquale and Tam 2005). The potential onset of nephrotoxicity can be monitored and it is usually reversible upon cessation of the drug, whereas ototoxicity is not as easily monitored, is generally irreversible, and may continue damage even after cessation of drug. Thus, those agents that show a greater potential for ototoxicity are avoided.

Aminoglycosides are not substantially metabolized and their primary route of excretion is through the kidneys. Consequently, the dosage must be appropriately lowered for patients that have kidney damage or problems that decrease excretion and thereby cause drug accumulation. Clinical pharmacodynamics experience demonstrated that an extended-interval dosage schedule of once/day maximized antibacterial activity and minimized nephrotoxicity compared to multiple-daily dosing. The long PAE of this class also contributes to a longer antibacterial effect between single daily doses.

Whole-body characterization of aminoglycoside-induced nephrotoxicity is similar in humans and laboratory animals (Quiros et al. 2011). Aminoglycosides concentrate in the renal cortex and damage the epithelial cells of the proximal renal tubules by a variety of possible effects. The molecular mechanisms of nephrotoxicity have been studied for a long period, but they are still not completely elucidated. The initial step appears to be binding of the strongly basic aminoglycoside molecule to acidic components of cell membranes such as phospholipids. This initial binding is followed by uptake of the aminoglycoside into the cells where a cascade of intracellular events ultimately leads to cell death, tubular necrosis, and, if damage continues, eventual destruction of organ function (Karasawa and Steyger 2011). Cytotoxicity through the intermediacy of reactive oxygen species (ROS) seems to be a currently popular explanation. A variety of agents have been shown to either augment or ameliorate nephrotoxicity (Ali et al. 2011).

Ototoxicity follows a relatively similar scheme (Xie et al. 2011; Warchol 2010; Selimoglu 2007). An aminoglycoside first binds to acidic components of cell membranes such as phospholipids and glycosaminoglycans and that event is followed by uptake into the cell and a subsequent cascade of destructive activities. Further work is still needed to provide a comprehensive description of the cytotoxic processes for both nephrotoxicity and ototoxicity.

The promise of creating highly active but less toxic aminoglycosides has always been a research goal that has not yet been achieved. The difficulties in toxicity research have been due to such problems as an unknown primary target(s) versus secondary follow-up effects, lack of an easily operated and predictable in vitro assay, relatively large amounts of drug needed for lengthy animal studies, and uncertain correlation of animal studies with human clinical results. It has also been long considered that antibacterial activity and toxicity most likely follow the same SAR, thereby making it almost impossible to separate the two effects within a single structure. However, these dogmas have recently been called into question. In the case of ototoxicity, some derivatives of apramycin were found whose antibiotic activity and NMDA-receptor activation were not correlated, suggesting possible dissociation of toxicity from activity (Harvey et al. 2000). More recently, detailed examination of a different potential mechanism of ototoxicity found that apramycin uniquely again showed dissociation of antibacterial activity from parameters of toxicity (Matt et al. 2012). Previous examination of the effects of certain aminoglycoside derivatives on inhibition of a membrane-embedded phospholipase had also suggested dissociation of antibacterial activity from nephrotoxicity might be possible (Kotretsou et al. 1995). It will be most interesting to see if any research group takes these results and finally creates a significantly less toxic aminoglycoside using these or related approaches.

10.10 Biosynthesis

Beside the successful examples of historical and recent chemical modification approaches, biological pathways can be exploited as attractive alternatives to create diverse novel aminoglycosides. These antibiotics are products of secondary carbohydrate metabolism. Producers are high-G + C Gram-positive microorganisms (*Actinobacteria*) with the exception of butirosin which is produced by a low-G + C bacterium belonging to the genus *Bacillus*. The first aminoglycoside biosynthetic cluster studied was the one for the synthesis of streptomycin in *S. griseus*. It contains 23 genes encoding for synthesis, regulation, transport, and self-resistance. The enzymatic steps catalyze reactions to prepare the three sugar moieties (streptidine-6-P, *TDP*-dihydrostreptose, and nucleoside diphospho-*N*-Me-L-glucosamine) and to couple them regio- and stereospecifically (for detailed enzymology see Piepersberg 1997; Walsh 2000; Wehmeier and Piepersberg 2009). The cytoplasmatic phase of streptomycin produces dihydrostreptomycin-6-P that is an inactive precursor which is specifically exported via an ATP-dependent pump and oxidized by an oxidase enzyme during trans-membrane passage. In the extracellular space streptomycin-6-P is transformed in active streptomycin by a phospholipase also encoded in the cluster. The streptomycin producer organisms provide self-resistance by accumulating only the inactive precursor inside the cell and activate it during and after secretion. In addition to that, these microbes can re-phosphorylate any streptomycin that comes back in the same C_6 position. A similar self-protective mechanism has been found in the kanamycin producers which inactivate the aminoglycoside by a 6′-*N*-acetyltransferase. Both these strategies suggest that self-resistance mechanisms in the producers anticipated those acquired by the clinical pathogens (Walsh 2000, Cundliffe and Demain 2010). In gentamicin producer, an additional level of self-resistance is due to the enzymatic N-methylation of the high affinity binding site in 16S rRNA to a lower affinity for aminoglycosides (Piepersberg 1997; Cundliffe and Demain 2010).

Regulation of the biosynthetic streptomycin cluster has been studied in detail since it is one of the first examples of quorum sensing. In *S. griseus* the butanolide A factor is responsible for a pleiotropic regulation, which controls, through the A factor receptor and StrR repression, the biosynthesis of streptomycin (Ohnishi et al. 1999).

The recent analyses of the gene clusters for almost all known aminoglycosides reveal that the logic of their biosynthetic pathways is similar in terms of enzymatic modification of NDP-sugars for deoxygenation and reductive amination and for aminocyclitol generation and glycosylation couplings (Wehmeier and Piepersberg 2009). The biosynthetic pathways of the 4,5-disubstituted-2-deoxystreptamine family have been well understood for butirosin and neomycin by heterologous expression of involved enzymes (Kudo and Eguchi 2009). The biochemistry of the biosynthetic pathways leading to 4,6-disubstituted-2-deoxystreptamine family is still a developing field (Wehmeier and Piepersberg 2009). However, the recent discovery of the complete kanamycin pathway and the engineered biosynthesis of

a novel kanamycin analog that is active against some kanamycin and amikacin-resistant Gram-negative bacteria demonstrate the potential of biological approaches for production of more robust aminoglycosides (Park et al. 2011). Recent reviews cover the enzymology of aminoglycoside biosynthesis and the potential of biological approaches for the heterologous production of the existing natural and semi-synthetic aminoglycosides and the creation of new ones by chemo-enzymatic synthesis (Kudo and Eguchi 2009; Park et al. 2013; Wehmeier and Piepersberg 2009).

10.11 Conclusions

Though the aminoglycosides are one of the oldest and better-studied classes, ongoing research in several fields may discover new agents with improved features that will further expand the future uses and importance of this class. A thorough understanding of their interaction with the ribosome, their biosynthesis, and mechanisms of resistance may provide novel useful derivatives. Today clinical uses of aminoglycosides are generally directed toward treatment of severe infections caused by Gram-negative bacteria, including some resistant strains, or uses in certain combinations with other antibiotics, such as Gram-positive cell-wall inhibitors with which they are synergistic. In addition to the microbial resistance issues governing susceptibility, the limiting concern is still potential toxicity to the patient. Changes in dosing schedules, such as once-daily administrations, and monitoring of drug concentrations and effects have improved the safety of aminoglycosides. Recent developments in the comprehension of toxicity effects, may lead to eventually realizing the long-sought goal of significantly less toxic aminoglycoside antibiotics.

References

Aggen JB et al (2010) Synthesis and spectrum of the neoglycoside ACHN-490. Antimicrob Agents Chemother 54:4636–4642

Ali BH, Al Za'abi M, Blunden G, Nemmar A (2011) Experimental gentamicin nephrotoxicity and agents that modify it: a mini-review of recent research. Basic Clin Pharmacol Toxicol 109:225–232

Ariano RE, Zelenitsky SA, Kassum DA (2008) Aminoglycoside-induced vestibular injury: maintaining a sense of balance. Ann Pharmacother 42:1282–1289

Armstrong ES, Miller GH (2010) Combating evolution with intelligent design: the neoglycoside ACHN-490. Curr Opin Microbiol 13:565–573

Arya DP (2007) Aminoglycoside antibiotics: from chemical biology to drug discovery. John Wiley & Sons, Hoboken

Becker B, Cooper MA (2013) Aminoglycoside antibiotics in the 21st century. ACS Chem Biol 8:105–115

Borders DB (2005) Antibiotics, survey. In: Seidel A (ed) Kirk-Othmer Encyclopedia of Chemical Technology, 5th edition. John Wiley & Sons, New York

Bracco D et al (2008) Pharmacokinetic variability of extended interval tobramycin in burn patients. Burns 34:791–796

Brodersen DE, Clemons WM Jr, Carter AP, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V (2000) The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. Cell 103:1143–1154

Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407:340–348

Chaudhary M, Naidu GK, Kumar S, Payaasi A (2012) Comparative antibacterial activity of a novel semisynthetic antibiotic: etimicin sulphate and other aminoglycosides. World J Microbiol Biotechnol 28:3365–3371

Cochran TG, Abraham DJ, Martin LL (1972) Stereochemistry and absolute configuration of the antibiotic spectinomycin: an X-ray diffraction study. J Chem Soc, Chem Commun 1972:494–495

Cooper DJ (1971) Comparative chemistry of some aminoglycoside antibiotics. Pure Appl Chem 28:455–467

Cundliffe E, Demain AL (2010) Avoidance of suicide in antibiotic-producing microbes. J Ind Microbiol Biotechnol 37:643–672

Doi Y, Arakawa Y (2007) 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin Infect Dis 45:88–94

Dozzo P, Moser HE (2010) New aminoglycoside antibiotics. Expert Opin Ther Patents 20:1321–1341

Elanco Animal Health (2013) http://www.elanco.us./product-services

El'Garch F, Jeannot K, Hocquet D, Llanes-Barakat C, Plésiat P (2007) Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob Agents Chemother 51:1016–1021

Falagas ME, Karageorgopoulos DE, Georgantzi GG, Sun C, Wang R, Rafailidis PI (2012) Susceptibility of Gram-negative bacteria to isepamicin: a systemic review. Expert Rev Anti-Infect Ther 10:207–218

Gilbert DN, Leggett JE (2009) Aminoglycosides. In: Mandell GL, Bennett JE, Dolin R (eds) Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases, 7th edn, chapter 26. Churchill Livingstone, New York

Girolami RL, Stamm JM (1977) Fortimicins A and B, new aminoglycoside antibiotics. IV. J Antibiot 30:564–570

Harvey SC, Li X, Skolnick P, Kirst HA (2000) The antibacterial and NMDA receptor activating properties of aminoglycosides are dissociable. Eur J Pharmacol 387:1–7

Hichens M, Rinehart KL Jr (1963) Chemistry of the neomycins. XII. J Am Chem Soc 85:1547–1548

Karasawa T, Steyger PS (2011) Intracellular mechanisms of aminoglycoside-induced cytotoxicity. Integr Biol 3:879–886

Kawaguchi H, Naito T (1983) Amikacin. In: Bindra JS, Lednicer D (eds) Chronicles of drug discovery, vol 2, Chapter 10. Wiley, New York

Kawaguchi H, Naito T, Nakagawa S, Fujisawa K (1972) BB-K 8, a new semisynthetic aminoglycoside antibiotic. J Antibiot 25:695–708

Kirst HA, Allen NE (2007) Aminoglycoside antibiotics. In: Triggle DJ, Taylor JB (eds) Comprehensive medicinal chemistry II, vol 7. Elsevier, Oxford

Koch KF, Rhoades JA, Hagaman EW, Wenkert E (1974) Carbon-13 nuclear magnetic resonance spectral analysis of tobramycin and related antibiotics. J Am Chem Soc 96:3300–3305

Kondo S, Iinuma K, Yamamoto H, Maeda K, Umezawa H (1973) Syntheses of 1-n-(S)-4-amino-2-hydroxybutyryl)-kanamycin B and-3′,4′-dideoxykanamycin B active against kanamycin-resistant bacteria. J Antibiot 26:412–415

Kotretsou S, Mingeot-Leclercq MP, Constantinou-Kokotou V et al (1995) Synthesis and antimicrobial and toxicological studies of amino acid and peptide derivatives of kanamycin A and netilmicin. J Med Chem 38:4710–4719

Kudo F, Eguchi T (2009) Biosynthetic enzymes for the aminolgycosides butirosin and neomycin. Methods Enzymol 459:493–519

Li C-R, Yang X-Y, Lou R-H et al (2008) In vitro antibacterial activity of vertilmicin and its susceptibility to modifications by the recombinant AAC(6′)-APH(2′) enzyme. Antimicrob Agents Chemother 52:3875–3882

Lo JY, Ho KM, Lo AC (2012) Surveillance of gonococcal antimicrobial susceptibility resulting in early detection of emerging resistance. J Antimicrob Chemother 67:1422–1426

Magnet S, Blanchard JS (2005) Molecular insights into aminoglycoside action and resistance. Chem Rev 105:477–497

Marchand I, Damier-Piolle L, Courvalin P, Lambert T (2004) Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrob Agents Chemother 48:3298–3304

Matt T, Ng CL, Lang K et al (2012) Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. Proc Nat Acad Sci US 109:10984–10989

McCormick C et al (2008) Effectiveness of a new tobramycin (0.3%) and dexamethasone (0.05%) formulation in the treatment of experimental *Pseudomonas* keratitis. Curr Med Res Opin 24:1569–1575

Mitscher LA (2010) Tetracycline, aminoglycoside, macrolide, and miscellaneous antibiotics. In: Abraham DJ, Rotella DP (eds) Burger's medicinal chemistry, drug discovery, and development, 7th edn. Wiley, New York

Mortensen JE, Nanavaty J, Veenhuizen MF, Shryock TR (1996) Reviewing apramycin's properties and use in controlling colibacillosis in pigs. Vet Med 91:473–477

Nagabhushan TL, Cooper AB, Tsai H, Daniels PJL, Miller GH (1978) The syntheses and biological properties of 1-N-(S-4-amino-2-hydroxybutyryl)-gentamicin B and 1-N-(S-3-amino-2-hydroxypropionyl)-gentamicin B. J Antibiot 31:681–687

Neidle S, Rogers D, Hursthouse MB (1968) The crystal and molecular structure of streptomycin oximeselenate. Tetrahedron Lett 9:4725–4728

Neuss N, Koch KF, Molloy BB, Day W, Huckstep LL, Dorman DE, Roberts JD (1970) Structure of hygromycin B. Helv Chim Acta 53:2314–2319

O'Connor S, Lam LKT, Jones ND, Chaney MO (1976) Apramycin, a unique aminocyclitol antibiotic. J Org Chem 41:2087–2092

Ohnishi Y, Kameyama S, Onaka H, Horinouchi S (1999) The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. Mol Microbiol 34:102–111

Pagkalis S, Mantadakis E, Mavros MN, Ammari C, Falagas ME (2011) Pharmacological considerations for the proper use of aminoglycosides. Drugs 71:2277–2294

Park JW, Park SR, Nepal KK, Han AR, Ban YH, Yoo YJ, Kim EJ, Kim EM, Kim D, Sohng JK, Yoon YJ (2011) Discovery of parallel pathways of kanamycin biosynthesis allows antibiotic manipulation. Nat Chem Biol 7:843–852

Park SR, Park JW, Ban YH, Sohng JK, Yoon YJ (2013) 2-Deoxystreptamine-containing aminoglycoside antibiotics: recent advances in the characterization and manipulation of their biosynthetic pathways. Nat Prod Rep 30:11–20

Pasquale TR, Tam JS (2005) Non antimicrobial effects of antibacterial agents. Clin Infect Dis 40:127–135

Penha FM, Rodrigues EB, Maia M et al (2010) Retinal and ocular toxicity in ocular application of drugs and chemicals–part II. Ophthalmic Res 44:205–224

Piepersberg W (1997) Molecular biology, biochemistry and fermentation of aminoglycoside antibiotics. In: Strohl WR (ed) Biotechnology of antibiotics, 2nd edn, Marcel Dekker Inc., New York

Poole K (2005) Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49:479–487

Prayle A, Smyth AR (2010) Aminoglycoside use in cystic fibrosis: therapeutic strategies and toxicity. Curr Opin Pulm Med 16:604–610

Price KE, Godfrey JC, Kawaguchi H (1977) Effect of structural modifications on the biological properties of aminoglycoside antibiotics containing 2-deoxystreptamine and supplement. In: Perlman D (ed) Structure-activity relationships among the semisynthetic antibiotics. Academic Press, New York

Quiros Y et al (2011) An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. Toxicol Sci 119:245–256

Reimann H, Cooper DJ, Mallams AK et al (1974) The structure of sisomicin, a novel unsaturated aminocyclitol antibiotic from *Micromonospora inyoensis*. J Org Chem 39:1451–1457

Schatz A, Bugie E, Waksman SA (1944) Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. Proc Soc Exp Biol Med 55:66–69

Selimoglu E (2007) Aminoglycoside-induced ototoxicity. Curr Pharm Design 13:119–126

Singh MP (2001) Tobramycin. Curr Opin Investig Drugs 2:755–765

Tsai A, Uemura S, Johansson M, Puglisi EV, Marshall RA, Aitken CE, Korlach J, Ehrenberg M, Puglisi JD (2013) The impact of aminoglycosides on the dynamics of translation elongation. Cell Rep. 3:497–508

Tenover FC, Elvrum PM (1988) Detection of two different kanamycin resistance genes in naturally occurring isolates of *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob Agents Chemother 32:1170–1173

Tenover FC, Tickler I, Armstrong ES, Kubo A, Lopez S, Persing DH, Miller GH (2011) Activity of ACHN-490 against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients in US hospitals. Int J Antimicrob Agents 38:352–354

Umezawa H, Ueda M et al (1957) Production and isolation of a new antibiotic: kanamycin. J Antibiot 10:181–188

Umezawa H, Umezawa S, Tsuchiya T, Okazaki Y (1971) 3′,4′-dideoxy-kanamycin B active against kanamycin-resistant *Escherichia coli* and *Pseudomonas aeruginosa*. J Antibiot 24:485–487

Waksman SA (1953) Streptomycin: background, isolation, properties, and utilization. Science 118:259–266

Waksman SA, Lechevalier HA (1949) Neomycin, a new antibiotic active against streptomycin-resistant bacteria, including tuberculosis organisms. Science 109:305–307

Walsh C (2000) Antibiotics. Actions, origins, resistance. ASM Press, Washington, DC

Warchol ME (2010) Cellular mechanisms of aminoglycoside ototoxicity. Curr Opin Otolaryngol Head Neck Surg 18:454–458

Wehmeier UF, Piepersberg W (2009) Enzymology of aminoglycosides biosynthesis-deduction from gene clusters. Methods Enzymol 459:459–491

Woo PWK, Dion HW, Bartz QR (1971) Butirosins A and B, aminoglycoside antibiotics. III. Structures. Tetrahedron Lett 12:2625–2628

Wright JJ (1976) Synthesis of 1-N-ethylsisomicin: a broad-spectrum semisynthetic aminoglycoside antibiotic. J Chem Soc, Chem Commun 1976:206–208

Wright GD (1999) Aminoglycoside-modifying enzymes. Curr Opin Microbiol 2:499–503

Yonath A (2005) Antibiotics targeting ribosomes: resistance, selectivity, synergism and cellular regulation. Annu Rev Biochem 74:649–679

Xie J, Talaska AE, Schacht J (2011) New developments in aminoglycoside therapy and ototoxicity. Hearing Res 281:28–37

Zhanel GG, Lawson CD, Zelenitsky S (2012) Comparison of the next-generation aminoglycoside plazomicin to gentamicin, tobramycin and amikacin. Expert Rev Anti-Infect Ther 10:459–473

Chapter 11
Macrolide Antibiotics

Herbert A. Kirst

Abstract Macrolide antibiotics are an important class that are used to treat respiratory tract, skin and skin-structure, sexually transmitted, and various other infections. They exert their antimicrobial activity by inhibiting ribosomal protein biosynthesis. Resistance to antibiotics arises when antibiotic binding at its target site is disrupted, efflux pumps remove antibiotic from cells, or antibiotic is converted to an inactive metabolite. Following the isolation of erythromycin and many other macrolides from fermentation broths of soil microbes, three generations of semi-synthetic 14-, 15-, and 16-membered derivatives have been prepared and tested. Two second generation derivatives, clarithromycin and azithromycin, are the more utilized macrolides at this time. Ketolides are third generation derivatives of erythromycin that possess activity against many macrolide-resistant bacteria. Use of the first approved ketolide, telithromycin, has been restricted due to side effects, but some other ketolides have entered into development studies and clinical trials.

11.1 Introduction

The development and spread of resistance to antibiotics have been a continual problem since the discovery of antibiotics (Davies and Davies 2010). In the late 1980s, the appearance of resistance to vancomycin in Gram-positive bacteria was especially disturbing (see Chap. 2). This event energized a prolonged search for new agents having activity against resistant bacteria, both Gram-positive and (more recently) Gram-negative species. Many antibiotics (see Chaps. 10, 12–15) inhibit protein synthesis as their mechanism of action (MOA), making it one of the

H. A. Kirst (✉)
7840 West 88th Street, Indianapolis, IN 46278, USA
e-mail: herbertkirst1@comcast.net

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_11, © Springer-Verlag Berlin Heidelberg 2014

most common and important antibacterial mechanisms (Lange et al. 2007). Despite an increasing prevalence of pathogens that show multiple patterns of resistance, this MOA remains as important today as it has been for the past several decades of antibiotic usage. Furthermore, new antimicrobial agents that inhibit some part of protein synthesis continue to be developed.

Macrolide antibiotics are one of the foremost classes that exert their antibacterial activity through this MOA (Hermann 2005; McCoy et al. 2011; Wilson 2004; Yonath 2005). The parent macrolides are produced by fermentation of soil microorganisms and some of these older macrolides are still useful therapeutic agents today (Demain 2009; Demain and Sanchez 2009; Omura 2011). They also represent an invaluable resource of chemical starting materials that have spawned many important semi-synthetic derivatives which possess various improved features responsible for increased efficacy and safety. This chapter will summarize the macrolide antibiotics, both older agents that are still important and newer agents that are in some stage of the preclinical or clinical development pipeline.

11.2 Erythromycin and Its Semi-synthetic Derivatives

Macrolide antibiotics constitute a large class that is organized by the size and substitution patterns of their highly substituted macrolactones to which particular saccharide moieties are attached (Kaneko et al. 2007; Kirst 2005; Mitscher 2010). Fermentation-derived macrolide antibiotics have a 14- or 16-membered lactone, while 15-membered macrolides are created by chemical ring expansion of a 14-membered ring. Many other macrolide compounds are known whose macrolactones have different substitution patterns or have fewer than 14 or more than 16 members, but the antibacterial activity of those compounds is generally too weak and/or too limited for useful clinical applications (Shiomi and Ōmura 2002).

Erythromycin A is the prototype of 14-membered macrolide antibiotics (Fig. 11.1). It is the major component of a complex produced by fermentation of a soil actinomycete now classified as *Saccharopolyspora erythraea*. First generation derivatives of erythromycin were synthesized soon after its discovery that included many acid-addition salts, esters, and salt-ester combinations designed to increase stability under acidic conditions (e.g., stomach) and to improve oral bioavailability. Acid-addition salts also improved water solubility for intravenous administration, but intramuscular administration was too painful upon injection to be used. All of these derivatives revert to erythromycin free base, which is the active entity of these first-generation derivatives. Early studies were conducted to learn the cause for the acid instability of erythromycin. These studies discovered a facile intramolecular cyclization by the C-6 hydroxyl group with the C-9 ketone to form a 6,9-hemiketal followed by 8,9-dehydration to initially yield the 8,9-anhydro-6,9-hemiketal intermediate (Fig. 11.1), which then underwent further degradation (Kurath et al. 1971). This insight provided a mechanistic rationale for

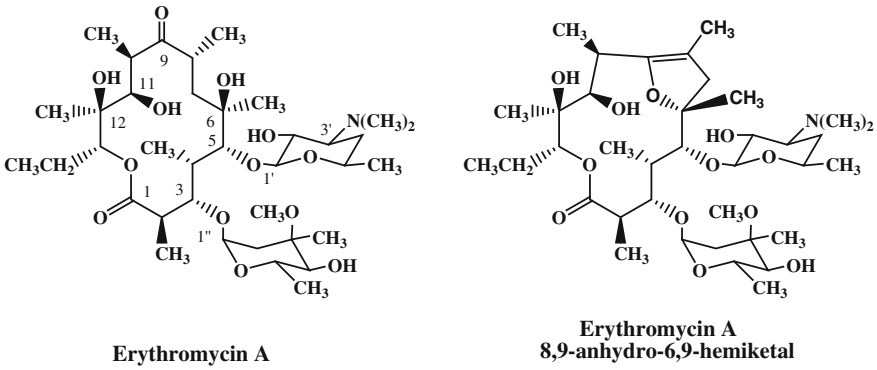


Fig. 11.1 Structures of erythromycin A and its intramolecular cyclization product

structural modifications of erythromycin that later produced the desired greater stability and oral bioavailability.

Second generation semi-synthetic derivatives of erythromycin were prepared by chemical transformations that modified certain of those functional groups that contribute to the intramolecular cyclization of erythromycin. Early members of this group were erythromycin-11,12-cyclic carbonate, 9-(*S*)-erythromycylamine, and roxithromycin (Fig. 11.2). The presence of either the exocyclic 5-membered ring in the 11,12-cyclic carbonate or the C-9 oxime in roxithromycin made these derivatives less prone than erythromycin to undergo the irreversible intramolecular cyclization sequence. In erythromycylamine, the C-9 ketone was replaced by an amino group which rendered the derivative incapable of forming the 6,9-hemiketal. Erythromycylamine was later re-examined as the active component in the pro-drug, dirithromycin. However, all of these earlier derivatives were superseded by clarithromycin and azithromycin, both of which became the more widely used second generation macrolides (Fig. 11.2) (Sivapalasingam and Steigbigel 2010; Zuckerman et al. 2011).

Clarithromycin is the 6-O-methyl ether of erythromycin, in which the 6-hydroxyl group is substituted and can no longer engage in intramolecular cyclization. Azithromycin is a ring-expanded 15-membered derivative in which the C-9 ketone is replaced via Beckmann rearrangement and N-methylation with a ring-embedded N-methylamino-methylene unit, a change that eliminates the C-9 ketone from participation in intramolecular cyclization. The collective group of compounds having an amino group incorporated within the macrolactone framework has been named azalides. Dirithromycin and flurithromycin were later entries into second generation derivatives (Fig. 11.2). Dirithromycin is an oxazine pro-drug of 9-(*S*)-erythromycylamine. Flurithromycin contains an 8-fluorosubstituent that prevents irreversible dehydration of the 6,9-hemiketal. Each of these diverse modifications provided a unique approach to circumventing the propensity of erythromycin for intramolecular cyclization and thereby achieved greater stability in each individual way.

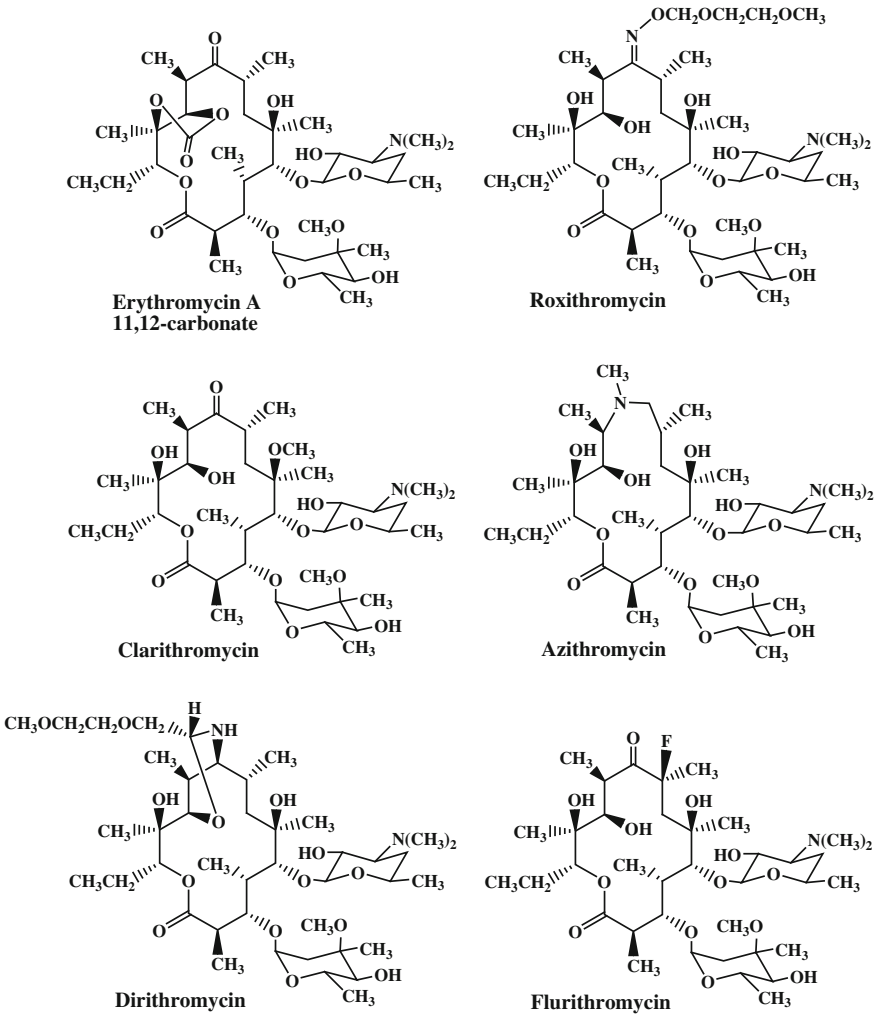


Fig. 11.2 Structures of second generation derivatives of erythromycin

Ketolides constitute the third generation, so-named due to their 3-keto functionality that replaces the 3-O-cladinosyl moiety of erythromycin, as exemplified by the first commercial ketolide, telithromycin (Ketek®) (Fig. 11.3) (Bryskier and Denis 2002; Sivapalasingam and Steigbigel 2010; Van Bambeke et al. 2008; Zhanel and Neuhauser 2005; Zuckerman et al. 2011). More recent ketolides that have entered the antibiotic pipeline include cethromycin, modithromycin, and solithromycin (Fig. 11.3) (Butler and Cooper 2011; Donadio et al. 2010; Kirst 2010). Cethromycin (Restanza™) originated from the antibiotic discovery programs at Abbott Laboratories (ABT-773) (Hammerschlag and Sharma 2008;

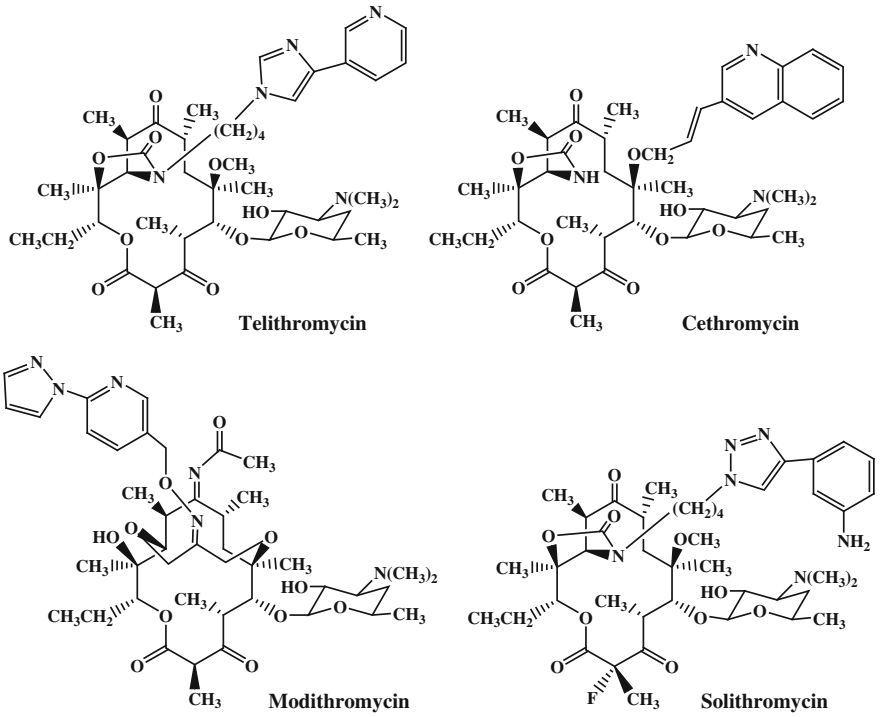


Fig. 11.3 Structures of representative ketolides

Rafie et al. 2010). It was in late stage development for treating community acquired bacterial pneumonia (CABP) and was also being investigated for biodefense applications. However, its future development is currently uncertain because Advanced Life Sciences suspended operations in May, 2011 (Advanced Life Sciences 2012; Bush and Pucci 2011; Sutcliffe 2011).

Several series of macrolides have been prepared at Enanta Pharmaceuticals that contain additional rings created by different bridging linkages between various hydroxyl groups of erythromycin. These series have thus been given the general names of bicyclolides, tricyclolides, etc. Bicyclolides are being investigated for both oral and intravenous administration against bacterial pathogens in hospital and community settings and are also being tested for biodefense applications against several pathogenic bacteria (Enanta 2012). The initial clinical candidate, modithromycin (formerly EDP-420 and S-013420) (Fig. 11.3), contains an additional internal ring that bridges the C-6 and C-11 hydroxyl groups. It has been jointly investigated in clinical trials with Shionogi & Company (Furuie et al. 2010; Jiang et al. 2009).

Solithromycin (formerly CEM-101) is the first 2-fluoro-ketolide in clinical development. Its structure also has a four-carbon linker between the 11,12-carbamate and a bis-heterocyclic side chain composed of a relatively stable 1,2,3-triazole

and a 2-aminophenyl group (Fig. 11.3) (Pereira and Fernandes 2011). It was licensed by Cempra Pharmaceuticals from Optimer Pharmaceuticals (Cempra 2012). Solithromycin has completed a phase 2 clinical trial for CABP by oral administration while an intravenous formulation is in a phase 1 trial (Cempra 2012; Fernandes et al. 2011; Sutcliffe 2011).

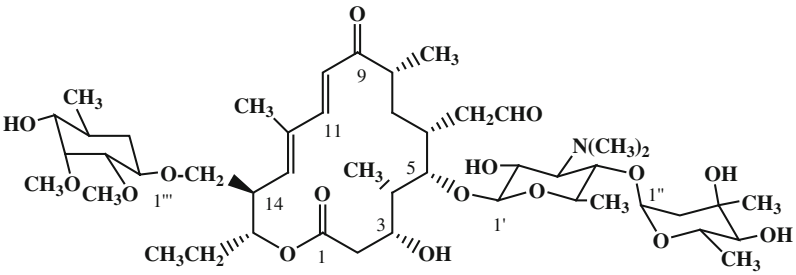
Research is still very actively in progress to discover new derivatives of erythromycin (Kirst 2010; Ma and Ma 2011; Ying and Tang 2010). In addition to new ketolides, other modifications around the 2,3-position of the core macrolactone include the so-called acylides, alkylides and anhydrolides. Several additional modifications are also being investigated around the 11,12-position (Kirst 2010). BAL19403 possesses a heterocyclic substituent linked to a 11,12-lactone rather than a 11,12-cyclic carbamate. It demonstrated good activity against resistant propionibacteria (Heller et al. 2007). Changes in the two saccharide moieties are also being explored with the synthesis of 3′-N- or 4″-O-modified derivatives of erythromycin. These efforts indicate that the search will continue for additional new derivatives of erythromycin having improved clinical efficacy and activity against resistant pathogens.

Macrolide antibiotics also have important applications in veterinary medicine. Two of the more recent azalides, tulathromycin (Draxxin®) and gamithromycin (Zactran®) are used exclusively for veterinary purposes such as treatment of respiratory infections in animals (Forbes et al. 2011; Shryock and Richwine 2010).

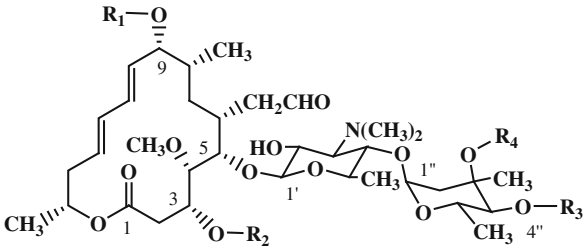
11.3 16-Membered macrolide antibiotics

16-Membered macrolide antibiotics are divided into two large families, tylosin and leucomycin-spiramycin, based on different substitution patterns of their macrolactones (Fig. 11.4). Tylosin is produced by fermentation of *Streptomyces fradiae* and is its family prototype. It is an important veterinary antibiotic, but it has not been developed for use in human medicine (Elanco 2012). A few other members of the tylosin family have also been developed exclusively for applications in veterinary medicine, including two semi-synthetic derivatives of tylosin, tilmicosin (Micotil®, Pulmotil®) and tildipirosin (Zuprevo®), which are being used to treat respiratory infections in animals (Buret 2010; Menge et al. 2012). Some clinical investigations have occurred in the past with a few members of the tylosin family, but none of these compounds appear to have yet been successfully developed for human medicine.

The leucomycin family is more numerous and more complicated because many members have been obtained from fermentation of different microorganisms by different research groups and given different names or corporate code numbers (Kirst 2005). Leucomycin was initially isolated as a complex of ten components from culture broths of *Streptomyces kitasatoensis* (Fig. 11.4) (Omura 2011). Midecamycin and spiramycin were also isolated as multi-component complexes from culture broths of *Streptomyces mycarofaciens* and *Streptomyces*



Tylosin



Leucomycin A_3 (Josamycin): R_1 = R_4 = H; R_2 = acetyl; R_3 = isovaleryl
Spiramycin I: R_1 = β-D-forosaminyl; R_2 = R_4 = H; R_3 = α-L-mycarosyl

Leucomycin A_5: R_1 = R_2 = R_4 =H; R_3 = *n*-butyryl
Rokitamycin: R_1 = R_2 = H; R_3 = *n*-butyryl; R_4 = propionyl

Midecamycin A_1: R_1 = R_4 = H; R_2 = R_3 = propionyl
Miokamycin: R_1 = R_4 = acetyl; R_2 = R_3 = propionyl

Fig. 11.4 Structures of representative 16-membered macrolides

ambofaciens, respectively. Some members of this family are used in human medicine, such as josamycin (leucomycin A_3), midecamycin, and spiramycin. Although none of these 16-membered macrolides have been registered for the U.S. market, spiramycin is used to treat certain infections caused by *Toxoplasma gondii* in pregnant women (Montoya and Remington 2008).

The most important semi-synthetic derivatives in the leucomycin family are miokamycin and rokitamycin (Alvarez-Elcoro and Yao 2002). Chemical acylation of leucomycin-type macrolides, especially of their 3″-hydroxyl group, increased the half-life of antibiotic activity while retaining good in vitro potency. This discovery was applied to prepare the semi-synthetic derivatives miokamycin (9,3″-di-O-acetyl derivative of midecamycin A_1) and rokitamycin (3″-O-propionyl derivative of leucomycin A_5) (Fig. 11.4). As mentioned above for the 16-membered parent macrolides, neither of these two derivatives has been registered for the U.S. market.

As with the 14-membered family, new research on 16-membered macrolides also continues in the effort to discover new antibiotics (Cui and Ma 2011; Przybylski 2010). However, the amount of effort has been significantly less than that devoted to 14- and 15-membered agents. One reason may be that 16-membered ketolides synthesized thus far have not demonstrated activity comparable to 14-membered ketolides (Creemer et al. 2002; Mutak et al. 2004; Terui et al. 2006). Analogous to 14-membered ketolides, the attachment of additional substituents to the 16-membered ring may be required to achieve the necessary stronger ribosomal binding and greater activity.

11.4 Antimicrobial Features

Macrolide antibiotics possess a moderately wide range of antimicrobial activity in which they inhibit susceptible strains of many Gram-positive bacteria, certain Gram-negative bacteria, and a variety of other pathogenic organisms (Dang et al. 2007; Roberts 2008; Sivapalasingam and Steigbigel 2010; Zuckerman et al. 2011). They penetrate well into many cells and tissues and exhibit activity against many microbes that dwell in an intracellular environment (Mulazimoglu et al. 2005). However, they generally lack useful activity against enterococci and most enteric and coliform Gram-negative bacteria. Interestingly, a recent study reported that in vitro activity of macrolides against *Pseudomonas aeruginosa* was highly dependent on the type of growth medium being used for the MIC test. MIC values were much lower when eukaryotic cell growth media were used compared to higher MICs when cation-adjusted Mueller–Hinton broth was employed (Buyck et al. 2011). This MIC differential may further help to explain the positive clinical effects of macrolides that are observed in cases involving *P. aeruginosa*, such as diffuse panbronchiolitis and cystic fibrosis (Crosbie and Woodhead 2009; Friedlander and Albert 2010).

The activity of macrolides may be bactericidal or bacteriostatic, depending on the particular microorganism, antibiotic concentration, contact time, and other experimental conditions. Most macrolides contain an amino group in their structures and thus they are basic substances that form acid-addition salts with increased water solubility. However, the un-ionized free base is the active form, so microbial penetration and antimicrobial activity is increased at higher pH values.

Although second generation macrolides had been more focused toward solving the earlier problems involving stability and oral bioavailability, some of those derivatives also showed greater potency against certain microorganisms that partially expanded the antimicrobial spectrum compared to erythromycin and 16-membered macrolides (Ali et al. 2002; Blondeau et al. 2002; Sivapalasingam and Steigbigel 2010; Zuckerman et al. 2011). Clarithromycin and azithromycin emerged as the more widely used macrolides due to some favorable clinical features, including somewhat broadened spectrum of activity and greater efficacy,

improved pharmacokinetics, less frequent dosing schedule, and better gastrointestinal tolerance. Among some prominent traits of this group, azithromycin was more effective in lowering MIC values against many Gram-negative bacteria while clarithromycin was more active against Gram-positive bacteria. The efficacy of clarithromycin against *Haemophilus influenzae* was aided by its in vivo conversion to its more active 14-hydroxy metabolite whereas azithromycin had a lower MIC against *H. influenzae*. The enhanced activity of these macrolides has been widely useful against pathogens that are responsible for many respiratory tract infections, skin and soft tissue infections, and sexually transmitted diseases. Among other applications, they are used to treat gastrointestinal (GI) problems caused by *Helicobacter pylori*. They exhibit activity against many non-tuberculous mycobacteria, especially against the *Mycobacterium avium* complex (MAC) that has aided treatment of MAC infections in AIDS patients (Young and Bermudez 2002).

With problems of stability and oral bioavailability substantially addressed by several second generation derivatives, the alarming rise in microbial resistance to antibiotics started to draw more attention, especially during the late 1980s. In response to this disturbing development, macrolide research began to shift in order to search for new agents that would combat this dangerous trend. The third generation of macrolides was thus intended to address the growing problems of microbial resistance to antibiotics, resulting in the emergence of the first ketolides in the mid-1990s (Bryskier and Denis 2002; Van Bambeke et al. 2008).

11.5 Mechanism of Action

The bacterial ribosome is a large and complex structure composed predominantly of RNA and protein that performs the vital task of bacterial protein biosynthesis. Thus, disruption of ribosome function by antibiotics causes serious deleterious effects to the microorganism, including death. The highly complex nature of protein biosynthesis on the ribosome makes for multiple ways in which the overall process can be disrupted. In addition, a second MOA involving inhibition of ribosome assembly by macrolides has been proposed (Champney 2006; Siibak et al. 2009).

Detailed knowledge has been rapidly expanding about the ribosome's structure, its mechanisms for functioning, and its interactions with antibiotic substances (Allen 2002; Blanchard et al. 2010; Bogdanov et al. 2010; Dunkle et al. 2010; Garrett et al. 2000; Kannan and Mankin 2011; Mankin 2008; McCoy et al. 2011; Wilson 2011). To briefly summarize, the programmed sequential addition of individual amino acids onto a growing peptide chain occurs at the peptidyl transferase center (PTC) located in the large (50S) subunit of the ribosome. The PTC catalyzes the sequential formation of the growing peptide's amide bonds. As the peptide chain becomes extended upon the addition of each new amino acid, the lengthening peptide moves outward through the exit tunnel of the ribosome. Macrolide antibiotics bind in the region of the exit tunnel near the PTC where their

presence either completely blocks or partially hinders progression of the nascent peptide out through this tunnel. Different macrolides may bind in different arrangements, but the overall result is inhibition of protein synthesis by preventing the proper elongation of the peptide. Depending on the macrolide, various types of prematurely terminated peptides may be released. Some macrolides such as 16-membered ones containing the 5-O-mycaminosyl-mycarosyl disaccharide have sufficient length to reach the PTC and disrupt formation of amide bonds.

X-ray crystallographic studies using co-crystals of macrolides bound in the large ribosomal subunit have now been performed using many different macrolides and ribosomes from several different microbes (Wilson 2011). Those results are consistent with the overall MOA and provide valuable visual evidence of how different macrolides bind to particular ribosomes in each individual manner. Although ribosomes are generally considered to have conserved structures, it is now recognized that antibiotic-ribosome interaction and binding may differ between ribosomes from different microbial species, so overly generalized interpretations of results may not be valid (Kannan and Mankin 2011; Wilson 2011). Additional studies are likely forthcoming that will greatly expand our detailed knowledge about this MOA. They will also suggest specific ways by which macrolide binding might be strengthened and thus will guide medicinal chemistry research in synthesizing new more potent derivatives (Sutcliffe 2005; Wimberly 2009). The importance of this technology and its significant impact on new drug discovery was celebrated by the award of the 2009 Nobel Prize in Chemistry to Profs. Ramakrishnan, Steitz, and Yonath for their pioneering work on the structure and function of ribosomes.

11.6 Microbial Resistance to Macrolides

The isolation of erythromycin from fermentation cultures and the first reports of clinical studies were both published in 1952 (Haight and Finland 1952; Heilman et al. 1952; McGuire et al. 1952). Unfortunately, microbial resistance to erythromycin was also observed soon after its clinical appearance (Leclercq and Courvalin 1991a). In addition, it was early recognized that the level of resistance could be correlated with the amount of antibiotic usage which had placed selective pressures on the microbial population and thereby selected resistant strains (Westh 1996). The clinical significance of resistance to macrolides was initially considered as low, but as years of antibiotic usage increased, so also did serious concerns steadily increase about the continuous rise in resistance to not just macrolides, but to all antibiotics (Boucher et al. 2009; Leclercq and Courvalin 1991b; Mulazimoglu et al. 2005).

The more common mechanism of resistance to macrolide antibiotics is modification of the target site responsible for activity, which is the ribosome. Other resistance mechanisms include antibiotic efflux systems, decreased uptake or permeability into the cell, various mutations to ribosomal RNA and proteins, and

modification of the antibiotic structure by inactivating enzymes (Dang et al. 2007; Douthwaite and Vester 2000; Mlynarczyk et al. 2010; Roberts 2008; Sutcliffe and Leclercq 2002).

Target site modification in bacteria disrupts macrolide ribosomal binding which thereby prevents or hinders the antibiotic from accomplishing its objective of inhibiting protein synthesis. In one common manifestation, the ribosomal binding sites overlap between the macrolide, lincosaminide, and streptogramin B antibiotics (see Chap. 14) resulting in cross-resistance between these three structurally unrelated classes and producing a phenotype named MLS_B resistance (Leclercq and Courvalin 1991a; Weisblum 1995a). MLS_B resistance is caused by enzymatic N^6-methylation of an adenine residue located in the overlapping binding region of ribosomal RNA. That N-methylation produces a conformational change in the ribosome that significantly weakens bonding by the antibiotic. This enzymatic methylation is genetically controlled by numerous readily transferable *erm* (erythromycin ribosome methylase) genes that are now found in a wide host of bacteria (Roberts 2008, 2011). MLS_B resistance can be either inducible or constitutive and 16-membered macrolides are generally non-inducers (Allen 1977, 1995; Weisblum 1995b). Two old fermentation-derived 3-keto-14-membered macrolides (pikromycin and narbomycin) were also shown to be non-inducers that were nevertheless active against macrolide-inducibly-resistant staphylococci (Allen 1977). Ketolides show a similar pattern in their response to inducibility (Bonnefoy et al. 1997). However, like other macrolides, they are not active against constitutively resistant strains (Sivapalasingam and Steigbigel 2010; Van Bambeke et al. 2008).

Ketolides possess several important structural changes compared to traditional derivatives of erythromycin that lead to significant advantages in antimicrobial activity and resistance patterns. These structural changes include replacement of the 3-O-mycarosyl substituent with a 3-keto group, addition of a rigid ring system across either the 11,12- or 6,11-positions, and attachment of a bis-heterocyclic moiety via a short carbon linker to various positions within the C-6 to C-12 region (Fig. 11.3). Among additional changes, modithromycin also contains a C-9 acylimine in place of the C-9 ketone. As a result from these structural changes, ketolides acquired a second ribosomal binding site to accommodate the heterocyclic chain in addition to the single ribosomal binding site used by older macrolides (Dang et al. 2007; Wilson 2011; Zhanel and Neuhauser 2005). X-ray structures of telithromycin-ribosomal complexes depict the binding patterns in these ribosomes (Dunkle et al. 2010; Tu et al. 2005). The second binding site strengthens ketolide-ribosomal binding affinity which increases antimicrobial potency. For macrolide-resistant strains, extension of ketolide binding into a second domain provides a new mechanism to overcome or circumvent the ribosomal N-methylation resistance mechanism and thus gives rise to activity against those resistant bacteria (Zhanel and Neuhauser 2005; Zuckerman et al. 2011).

Analysis of a crystal study of *Escherichia coli* ribosomes complexed with solithromycin proposed the presence of three binding sites with the third site coming from the positioning of the 2-fluoro substituent (Fernandes et al. 2011;

Llano-Sotelo et al. 2010; Sutcliffe 2011). Such a result could further strengthen ketolide-ribosomal binding and increase potency relative to non-fluorinated analogs. However, the exact mechanism and in vitro activity resulting from a 2-fluoro substituent may depend on the specific ketolide structure rather than follow a generalized SAR rule for all ketolides (Hwang et al. 2008; Keyes et al. 2003; Llano-Sotelo et al. 2010). Hydrogen bonding from the 2-aminophenyl group also contributes to overall ribosomal binding of solithromycin. The proposal of three binding sites for a single ketolide structure would provide a valuable new mechanism for overcoming the N-methylation and other macrolide-resistance mechanisms in macrolide resistant strains (McGhee et al. 2010). It will be interesting to watch the results of future SAR studies focused in this direction.

The extended and stronger binding that results from the structural changes in the C-6 to C-12 region of ketolides more than compensates for the reduction in activity that occurs upon removal of the 3-O-cladinosyl subunit from erythromycin, an absence that does have the positive effect of removing inducibility of resistance (Allen 1977). Consequently, ketolides tend to show greater activity compared to erythromycin and second generation derivatives against both susceptible and resistant staphylococci, streptococci, and other important pathogens. 2-Fluoro-ketolides appear to increase that activity differential even further although the universality of that trend is still unproven and needs to be more fully investigated. Another caution is that binding of macrolides to ribosomes from different species may yield different results so over-generalizations should be avoided (Kannan and Mankin 2011; Wilson 2011).

Detailed analyses of comparative potencies or resistance patterns among ketolides and older macrolides are beyond the scope and available space of this review and such surveys have been published by many others (Dang et al. 2007; Rafie et al. 2010; Sivapalasingam and Steigbigel 2010; Sutcliffe 2011; Van Bambeke et al. 2008; Zhanel and Neuhauser 2005; Zuckerman et al. 2011). Driven by the medical needs and therapeutic potential that is still available from the ketolide template, it is likely that the creation of novel ketolide structures has not yet reached any limits and additional innovative structures should be revealed in due course.

11.7 Pharmacology

The two semi-synthetic derivatives clarithromycin and azithromycin are the dominant macrolide antibiotics currently being used in clinical practice. They are prescribed to treat upper and lower respiratory tract infections caused by a range of pathogens, skin and skin structure infections, several sexually transmitted diseases, and a wide spectrum of other infections caused by various bacteria and other pathogenic organisms (Van Bambeke et al. 2008; Sivapalasingam and Steigbigel 2010; Zuckerman et al. 2011). Among the latter uses is treatment of MAC infections in AIDS patients and eradication of gastrointestinal *H. pylori* often by

means of combination therapy. Macrolides also play an important clinical role as an alternative to β-lactam antibiotics for patients who are allergic to the latter agents.

In addition to their overtly bacteriostatic or bactericidal activities against pathogens, macrolides have been long known to display a variety of anti-inflammatory (AIF) and immunomodulatory (IMM) properties in the host that make some significant contributions to the overall efficacy of these agents. Numerous studies, analyses, and reviews of these systems have been made over several decades by many investigators (Altenburg et al. 2011; Buret 2010; Harvey et al. 2009; Kovaleva et al. 2012; Zarogoulidis et al. 2012). However, the complexities of the numerous AIF and IMM networks cause difficulties in separating the component parts and in dissecting primary causes from many secondary effects. The situation is further complicated because different macrolides may show opposite effects, thereby making generalities difficult to establish. Consequently, many of the basic mechanisms by which these effects occur still remain incompletely understood. Some attempts have also been made to create derivatives that dissociate the direct antimicrobial activity from non-antibiotic effects, but this objective has thus far only met with very limited success. The most successful separation of activities has been found with compounds derived from intramolecular cyclization of erythromycin, first as motilin agonists in the GI tract and more recently as lead structures for AIF or IMM applications (Sugawara et al. 2011).

As described above, successive generations of derivatives have steadily improved many clinical attributes of this class, allowing it to remain an important contributor to the therapeutic armamentarium for nearly 60 years. In this therapeutic role, macrolides are generally regarded as among the safest antibiotics, with the majority of side effects involving various disturbances of the GI tract. One advantage of several second generation derivatives was a lower incidence and reduced severity of GI effects compared to erythromycin (Periti et al. 1993).

Telithromycin is the most recent commercial macrolide and is currently the only ketolide that has received regulatory approval, which occurred in Europe and some Latin countries in 2001 and the U.S. in 2004. However, during its more extensive use following the clinical trials and approvals, serious problems were reported which included incidents of severe hepatotoxicity, certain visual side effects, and exacerbation of myasthenia gravis. In response to these safety concerns, stronger labeling warnings were written and in 2007, the U.S. FDA restricted use of telithromycin to the treatment of CABP (Van Bambeke et al. 2008; Sivapalasingam and Steigbigel 2010; Zuckerman et al. 2011). One recent study proposed that certain nicotinic acetylcholine receptors that may be associated with those side effects are located in the liver, eye, and muscle. These receptors may be inhibited by telithromycin and may be responsible for these undesirable effects (Bertrand et al. 2010; Fernandes et al. 2011; Sutcliffe 2011). Furthermore, the pyridine component in telithromycin has been suspected of involvement in this activity. The older macrolides, clarithromycin and azithromycin, and the newer ketolide, solithromycin, did not show the same level of

inhibition as telithromycin, suggesting that this test could perhaps be used to predict the possibility of these side effects. These developments are encouraging that the side effects of telithromycin may be more structure-specific and not shared by all ketolides.

11.8 Biosynthesis

Early studies of the biosynthesis of erythromycin and other macrolides revealed the formation of their aglycones by sequential coupling of small organic acids (acetate, propionate, etc.) (Corcoran 1964). Following the addition of each acid, the newly formed subunit was then appropriately modified to give the desired stereospecific sub-structure using the processes of ketone reduction, dehydration, and enoyl reduction as appropriate to produce the final product (Kwan and Schulz 2011). Lastly, cyclization of the resultant 14- or 16-membered acyclic chains yielded the aglycones (Corcoran 1981; Omura and Tanaka 1984).

Later studies discovered a strongly programmed process that assembled the aglycones via large and highly organized modular structures called a polyketide synthase (PKS) (Cortes et al. 1990; Donadio et al. 1991). Following cyclization that cleaves the polyketide chain from the PKS, the resultant aglycone is converted to the macrolide antibiotic by appropriate post-PKS transformations, such as hydroxylation, O-methylation, O-glycosylation, etc. (Rix et al. 2002; Zhao and Liu 2010). Investigations by numerous researchers have revealed many further details about the general biosynthetic pathways and PKS-controlled processes and confirmed the generality of this biosynthetic mechanism for the construction of numerous polyketide structures (Cane 2010; Hertweck 2009; Khosla 2009; McDaniel et al. 2005; van Lanen and Shen 2008). This greatly detailed knowledge about the biochemistry and genetics of biosynthesis now allows more rationale and control for genetic engineering of biosynthetic pathways in microorganisms, including applications for combinatorial biosynthesis to create new molecules and for improvements in the fermentative production of known compounds (Baltz 2006; Khosla et al. 2007). All of these biosynthetic possibilities open additional routes to new structural diversification and nicely complement the chemical synthetic routes to produce new antibiotic structures.

11.9 Conclusions

Macrolide antibiotics continue to be an important class for treatment of many infectious diseases. Their 2009 sales in the U.S. were $4.8 billion, making them the fourth largest class in sales (after cephalosporins, broad spectrum penicillins, and fluoroquinolones) (Hamad 2010). Approximately 60 years have passed since erythromycin and many other macrolides were discovered and isolated from

culture broths of soil microorganisms. During that period, an extremely large number of semi-synthetic 14-, 15-, and 16-membered macrolides have been prepared and evaluated, which can be divided conveniently into three generations of derivatives. Two second generation derivatives of erythromycin, clarithromycin and azithromycin, are currently the more utilized macrolides. Ketolides have emerged as third generation derivatives of erythromycin that show useful activity against many macrolide-resistant bacteria. Even though the first approved ketolide, telithromycin, has encountered some serious problems with side effects, other ketolides are being synthesized and some have entered the clinical development pipeline. Based on both the undeveloped potential still remaining for this class and the medical need for new agents, research efforts within the macrolide class will undoubtedly continue. From these continuing efforts, new members possessing important and useful improvements in antimicrobial spectrum, efficacy, and safety should be discovered and developed. Such future discoveries will ensure that the macrolide antibiotic class will remain an important contributor to the global anti-infective armamentarium.

References

Advanced Life Sciences (2012) http://www.advancedlifesciences.com/phoenix.zhtml?c=190126&p=irol=news&nyo=0

Ali AB, Goldstein FW, Acar JF (2002) In vitro activity of macrolides against traditional susceptible bacteria. In: Schönfeld W, Kirst HA (eds) Macrolide antibiotics. Birkhäuser, Basel

Allen NE (1977) Macrolide resistance in *Staphylococcus aureus*: inducers of macrolide resistance. Antimicrob Agents Chemother 11:669–674

Allen NE (1995) Biochemical mechanisms of resistance to non-cell wall antibacterial agents. Prog Med Chem 32:157–238

Allen NE (2002) Effects of macrolide antibiotics on ribosome function. In: Schönfeld W, Kirst HA (eds) Macrolide antibiotics. Birkhäuser, Basel

Altenburg J, de Graaff CS, van der Werf TS, Boersma WG (2011) Immunomodulatory effects of macrolide antibiotics. Respiration 81:67–87

Alvarez-Elcoro S, Yao JDC (2002) Antimicrobial macrolides in clinical practice. In: Ōmura S (ed) Macrolide antibiotics: chemistry, biology, and practice, 2nd edn. Academic Press, San Diego

Baltz RH (2006) Molecular engineering approaches to peptide, polyketide and other antibiotics. Nat Biotech 24:1533–1540

Bertrand D, Bertrand S, Neveu E, Fernandes P (2010) Molecular characterization of off-target activities of telithromycin: a potential role for nicotinic acetylcholine receptors. Antimicrob Agents Chemother 54:5399–5402

Blanchard SC, Cooperman BS, Wilson DN (2010) Probing translation with small-molecule inhibitors. Chem Biol 17:633–645

Blondeau JM, DeCarolis E, Metzler KL, Hansen GT (2002) The macrolides. Expert Opin Investig Drugs 11:189–215

Bogdanov AA, Sumbatyan NV, Shishkina AV, Karpenko VV, Korshunova GA (2010) Ribosomal tunnel and translation regulation. Biochem (Moscow) 75:1501–1516

Bonnefoy A, Girard AM, Agouridas C, Chantot JF (1997) Ketolides lack inducibility properties of MLS_B resistance phenotype. J Antimicrob Chemother 40:85–90

Boucher HW, Talbot GH, Bradley JS et al (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Inf Dis 48:1–12
Bryskier A, Denis A (2002) Ketolides: novel antibacterial agents designed to overcome resistance to erythromycin A within gram-positive cocci. In: Schönfeld W, Kirst HA (eds) Macrolide antibiotics. Birkhäuser, Basel
Buret AG (2010) Immuno-modulation and anti-inflammatory benefits of antibiotics: the example of tilmicosin. Can J Vet Res 74:1–10
Bush K, Pucci MJ (2011) New antimicrobial agents on the horizon. Biochem Pharmacol 82:1528–1539
Butler MS, Cooper MA (2011) Antibiotics in the clinical pipeline. J Antibiot 64:413–425
Buyck J, Tulkens PM, Van Bambeke F (2011) Increased susceptibility of *Pseudomonas aeruginosa* to macrolides in biologically-relevant media by modulation of outer membrane permeability and of efflux pump expression. 51st Interscience conference on antimicrobial agents and chemotherapy, Chicago, 17–20 Sep, IL; E-122
Cempra Pharmaceuticals (2012) http://www.cempra.com
Cane DE (2010) Programming of erythromycin biosynthesis by a modular polyketide synthase. J Biol Chem 285:27517–27523
Champney WS (2006) The other target for ribosomal antibiotics: inhibition of bacterial ribosomal subunit formation. Infect Disorders-Drug Targets 6:377–390
Corcoran JW (1964) The biosynthesis of erythromycin. Lloydia 27:1–14
Corcoran JW (1981) Biochemical mechanisms in the biosynthesis of the erythromycins. In: Corcoran JW (ed) Antibiotics, vol 4: Biosynthesis. Springer, Berlin
Cortes J, Haydock SF, Roberts GA, Bevitt DJ, Leadlay PF (1990) An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. Nature 348:176–178
Creemer LC, Toth JE, Kirst HA (2002) Synthesis and in vitro antimicrobial activity of 3-keto 16-membered macrolides derived from tylosin. J Antibiot 55:427–436
Crosbie PA, Woodhead MA (2009) Long-term macrolide therapy in chronic inflammatory airway diseases. Eur Respir J 33:171–181
Cui W, Ma S (2011) Recent advances in the field of 16-membered macrolide antibiotics. Mini-Rev Med Chem 11:1009–1018
Dang V, Nanda N, Cooper TW, Greenfield RA, Bronze MS (2007) Macrolides, azalides, ketolides, lincoamides, and streptogramins. J Okla State Med Assoc 100:75–81
Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433
Demain AL (2009) Antibiotics: natural products essential to human health. Med Res Rev 29:821–842
Demain AL, Sanchez S (2009) Microbial drug discovery: 80 years of progress. J Antibiot 62:5–16
Donadio S, Staver MJ, McAlpine JB, Swanson SJ, Katz L (1991) Modular organization of genes required for complex polyketide biosynthesis. Science 252:675–679
Donadio S, Maffioli S, Monciardini P, Sosio M, Jabes D (2010) Antibiotic discovery in the twenty-first century: current trends and future perspectives. J Antibiot 63:423–430
Douthwaite S, Vester B (2000) Macrolide resistance conferred by alterations in the ribosome target site. In: Garrett RA, Douthwaite SR, Liljas A, Matheson AT, Moore PB, Noller HF (eds) The ribosome: structure, function, antibiotics, and cellular interactions. ASM Press, Washington, DC
Dunkle JA, Xiong L, Mankin AS, Cate JHD (2010) Structures of the *Escherichia coli* ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. Proc Natl Acad Sci U S A 107:17152–17157
Elanco Animal Health (2012) http://www.elanco.com.au/product_range/index.html
Enanta Pharmaceuticals (2012) http://www.enanta.com
Fernandes P, Pereira D, Jamieson B, Keedy K (2011) Solithromycin. Drugs Future 36:751–758

Forbes AB, Ramage C, Sales J, Baggott D, Donachie W (2011) Determination of the duration of antibacterial efficacy following administration of gamithromycin using a bovine *Mannheimia haemolytica* challenge model. Antimicrob Agents Chemother 55:831–835

Friedlander AL, Albert RK (2010) Chronic macrolide therapy in inflammatory airways diseases. Chest 138:1202–1212

Furuie H, Saisho Y, Yoshikawa T, Shimada J (2010) Intrapulmonary pharmacokinetics of S-013420, a novel bicyclolide antibacterial, in healthy Japanese subjects. Antimicrob Agents Chemother 54:866–870

Garrett RA, Douthwaite SR, Liljas A, Matheson AT, Moore PB, Noller HF (eds) (2000) The ribosome: structure, function, antibiotics, and cellular interactions. ASM Press, Washington, DC

Haight TH, Finland M (1952) Laboratory and clinical studies on erythromycin. N Eng J Med 247:227–232

Hamad B (2010) The antibiotics market. Nat Rev Drug Discov 9:675–676

Hammerschlag MR, Sharma R (2008) Use of cethromycin, a new ketolide, for treatment of community-acquired respiratory infections. Expert Opin Investig Drugs 17:387–400

Harvey RJ, Wallwork BD, Lund VJ (2009) Anti-inflammatory effects of macrolides: applications in chronic rhinosinusitis. Immunol Allergy Clin N Am 29:689–703

Heilman FR, Herrell WE, Wellman WE, Geraci JE (1952) Some laboratory and clinical observations on a new antibiotic, erythromycin (ilotycin). Proc Staff Meet Mayo Clin 27:285–304

Heller S, Kellenberger L, Shapiro S (2007) Antipropionibacterial activity of BAL19403, a novel macrolide antibiotic. Antimicrob Agents Chemother 51:1956–1961

Hermann T (2005) Drugs targeting the ribosome. Curr Opin Struct Biol 15:355–366

Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angew Chem Int Ed 48:4688–4716

Hwang C-K, Duffield J, Chiu Y-H, Liang C-H, Yao S, Roberets N, Babakhani F, Sears P, Shue Y-K, Ichikawa Y, Fernandes P, Pereira D, Romero A (2008) SAR of 11,12-carbamate macrolides/ketolides linked with 1,4-substituted-[1,2,3]-triazoles. 48th Interscience conference on antimicrobial agents and chemotherapy, Washington, 25–28 Oct, DC; F1-3973

Jiang L, Wang M, Or YS (2009) Pharmacokinetics of EDP-420 after ascending single oral doses in healthy adult volunteers. Antimicrob Agents Chemother 53:1786–1792

Kaneko T, Dougherty TJ, Magee TV (2007) Macrolide antibiotics. In: Triggle DJ, Taylor JB (eds) Comprehensive medicinal chemistry II, vol 7. Elsevier, Oxford

Kannan K, Mankin AS (2011) Macrolide antibiotics in the ribosome exit tunnel: species-specific binding and action. Ann N Y Acad Sci 1241:33–47

Keyes RF, Carter JJ, Englund EE, Daly MM, Stone GG, Nilius AM, Ma Z (2003) Synthesis and antibacterial activity of 6-O-arylbutynyl ketolides with improved activity against some key erythromycin-resistant pathogens. J Med Chem 46: 1795–1798

Khosla C (2009) Structures and mechanisms of polyketide synthases. J Org Chem 74:6416–6420

Khosla C, Tang Y, Chen AY, Schnarr NA, Cane DE (2007) Structure and mechanism of the 6-deoxyerythronolide B synthase. Annu Rev Biochem 76:195–221

Kirst HA (2005) Macrolide antibiotics. In: Seidel A (ed) Kirk-Othmer encyclopedia of chemical technology, 5th edn. Wiley, New York

Kirst HA (2010) New macrolide, lincosaminide and streptogramin B antibiotics. Expert Opin Ther Patents 20:1343–1357

Kovaleva A, Remmelts HH, Rijkers GT, Hoepelman AI, Biesma DH, Oosterheert JJ (2012) Immunomodulatory effects of macrolides during community-acquired pneumonia: a literature review. J Antimicrob Chemother 67:530–540

Kurath P, Jones PH, Egan RS, Perun TJ (1971) Acid degradation of erythromycin A and erythromycin B. Experientia 27:362

Kwan DH, Schulz F (2011) The stereochemistry of complex polyketide biosynthesis by modular polyketide synthases. Molecules 16:6092–6115

Lange RP, Locher HH, Wyss PC, Then RL (2007) The targets of currently used antibacterial agents: lessons for drug discovery. Curr Pharm Design 13:3140–3154
Leclercq R, Courvalin P (1991a) Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob Agents Chemother 35:1267–1272
Leclercq R, Courvalin P (1991b) Intrinsic and unusual resistance to macrolide, lincosamide, and streptogramin antibiotics in bacteria. Antimicrob Agents Chemother 35:1273–1276
Llano-Sotelo B, Dunkle J, Klepacki D, Zhang W, Fernandes P, Cate JHD, Mankin AS (2010) Binding and action of CEM-101, a new fluoroketolide antibiotic that inhibits protein synthesis. Antimicrob Agents Chemother 54:4961–4970
Ma X, Ma S (2011) Significant breakthroughs in search for anti-infectious agents derived from erythromycin A. Curr Med Chem 18:1993–2015
Mankin AS (2008) Macrolide myths. Curr Opin Microbiol 11:414–421
McCoy LS, Xie Y, Tor Y (2011) Antibiotics that target protein synthesis. WIREs RNA 2:209–232
McDaniel R, Welch M, Hutchinson CR (2005) Genetic approaches to polyketide antibiotics. Chem Rev 105:543–558
McGhee P, Clark C, Kosowska-Shick KM, Nagai K, Dewasse B, Beachel L, Appelbaum PC (2010) *In vitro* activity of CEM-101 against *Streptococcus pneumoniae* and *Streptococcus pyogenes* with defined macrolide resistance mechanisms. Antimicrob Agents Chemother 54:230–238
McGuire JM, Bunch RL, Anderson RC, Boaz HE, Flynn EH, Powell HM, Smith JW (1952) Ilotycin, a new antibiotic. Antibiot Chemother 2:281–283
Menge M, Rose M, Bohland C, Zschiesche E, Kilp S, Metz W, Allan M, Ropke R, Nurnberger M (2012) Pharmacokinetics of tildipirosin in bovine plasma, lung tissue, and bronchial fluid (from live, nonanesthetized cattle). J Vet Pharmacol Ther 35:550–559
Mitscher LA (2010) Tetracycline, aminoglycoside, macrolide, and miscellaneous antibiotics. In: Abraham DJ, Rotella DP (eds) Burger's medicinal chemistry, drug discovery, and development, 7th edn. Wiley, New York
Mlynarczyk B, Mlynarczyk A, Kmera-Muszynska, Majewski S, Mlynarczyk G (2010) Mechanisms of resistance to antimicrobial drugs in pathogenic Gram-positive cocci. Mini-Rev Med Chem 10:928–937
Montoya JG, Remington JS (2008) Management of *Toxoplasma gondii* infection during pregnancy. Clin Infect Dis 47:554–566
Mulazimoglu L, Tulkens PM, Van Bambeke F (2005) Macrolides. In: Yu VL, Edwards G, McKinnon PS, Peloquin C, Morse GD (eds) Antimicrobial therapy and vaccines, vol 2. E-Sun Technologies, Pittsburgh
Mutak S, Marsic N, Kramaric MD, Pavlovic D (2004) Semisynthetic macrolide antibacterials derived from tylosin. Synthesis and structure-activity relationships of novel desmycosin analogues. J Med Chem 47:411–431
Omura S (2011) Microbial metabolites: 45 years of wandering, wondering and discovering. Tetrahedron 67:6420–6459
Omura S, Tanaka Y (1984) Biochemistry, regulation, and genetics of macrolide production. In: Ōmura S (ed) Macrolide antibiotics: chemistry, biology, and practice, 1st edn. Academic Press, Orlando
Pereira D, Fernandes P (2011) Synthesis and antibacterial activity of novel 4-aryl-[1,2,3]-triazole containing macrolides. Bioorg Med Chem Lett 21:510–513
Periti P, Mazzei T, Mini E, Novelli A (1993) Adverse effects of macrolide antibacterials. Drug Saf 9:346–364
Przybylski P (2010) Modifications and biological activity of natural and semisynthetic 16-membered macrolide antibiotics. Curr Med Chem 18:1993–2015
Rafie S, MacDougall C, James CL (2010) Cethromycin: a promising new ketolide antibiotic for respiratory infections. Pharmacother 30:290–303
Rix U, Fischer C, Remsing LL, Rohr J (2002) Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat Prod Rep 19:542–580

Roberts MC (2008) Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. FEMS Microbiol Lett 282:147–159
Roberts MC (2011) Environmental macrolide-lincosamide-streptogramin and tetracycline resistant bacteria. Frontiers Microbiol 2:40
Shiomi K, Ōmura S (2002) Discovery of new macrolides. In: Ōmura S (ed) Macrolide antibiotics: chemistry, biology, and practice, 2nd edn. Academic Press, San Diego
Shryock TR, Richwine A (2010) The interface between veterinary and human antibiotic use. Ann N Y Acad Sci 1213:92–105
Siibak T, Peil L, Xiong L, Mankin A, Remme J, Tenson T (2009) Erythromycin- and chloramphenicol-induced ribosomal assembly defects are secondary effects of protein synthesis inhibition. Antimicrob Agents Chemother 53:563–571
Sivapalasingam S, Steigbigel NH (2010) Macrolides, clindamycin, and ketolides. In: Mandell GL, Bennett JE, Dolin R (eds) Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 7th edn. Churchill Livingstone, Philadelphia
Sugawara A, Sueki A, Hirose T, Nagai K, Gouda H, Shima H, Akagawa KS, Omura S, Sunazuka T (2011) Novel 12-membered non-antibiotic macrolides from erythromycin A: EM900 series as novel leads for anti-inflammatory and/or immunomodulatory agents. Bioorg Med Chem Lett 21:3373–3376
Sutcliffe JA (2005) Improving on nature: antibiotics that target the ribosome. Curr Opin Microbiol 8:534–542
Sutcliffe JA (2011) Antibiotics in development targeting protein synthesis. Ann N Y Acad Sci 1241:122–152
Sutcliffe JA, Leclercq R (2002) Mechanisms of resistance to macrolides, lincosamides, and ketolides. In: Schönfeld W, Kirst HA (eds) Macrolide antibiotics. Birkhäuser, Basel
Terui Y, Kinoshita K, Kaneda Y, Akashi T, Hamaguchi T, Kawashima A (2006) Synthesis of 2-methyl 16-membered macrolide derived from tylosin. J Antibiot 59:98–104
Tu D, Blaha G, Moore PB, Steitz TA (2005) Structures of MLS_BK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell 121:257–270
Van Bambeke F, Harms JM, Van Laethem Y, Tulkens PM (2008) Ketolides: pharmacological profile and rational positioning in the treatment of respiratory tract infections. Expert Opin Pharmacother 9:267–283
Van Lanen SG, Shen B (2008) Advances in polyketide synthase structure and function. Curr Opin Drug Discov Devel 11:186–195
Weisblum B (1995a) Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother 39:577–585
Weisblum B (1995b) Insights into erythromycin action from studies of its activity as inducer of resistance. Antimicrob Agents Chemother 39:797–805
Westh H (1996) Erythromycin-resistant *Staphylococcus aureus* in Denmark. APMIS Supplement 57:1–37
Wilson DN (2004) Antibiotics and the inhibition of ribosome function. In: Nierhaus KH, Wilson DN (eds) Protein synthesis and ribosome function. Wiley-VCH, Weinheim
Wilson DN (2011) On the specificity of antibiotics targeting the large ribosomal subunit. Ann N Y Acad Sci 1241:1–16
Wimberly BT (2009) The use of ribosomal crystal structures in antibiotic drug design. Curr Opin Investig Drugs 10:750–765
Ying L, Tang D (2010) Recent advances in the medicinal chemistry of novel erythromycin-derivatized antibiotics. Curr Topics Med Chem 10:1441–1469
Yonath A (2005) Antibiotics targeting ribosomes: resistance, selectivity, synergism, and cellular regulation. Annu Rev Biochem 74:649–679
Young LS, Bermudez LE (2002) Activity of macrolides against mycobacteria. In: Schönfeld W, Kirst HA (eds) Macrolide antibiotics. Birkhäuser, Basel
Zarogoulidis P, Papanas N, Kioumis I, Chatzaki E, Maltezos E, Zarogoulidis K (2012) Macrolides: from in vitro anti-inflammatory and immunomodulatory properties to clinical practice in respiratory diseases. Eur J Clin Pharmacol 68:479–503

Zhanel GG, Neuhauser MM (2005) Ketolides (telithromycin, cethromycin). In: Yu VL, Edwards G, McKinnon PS, Peloquin C, Morse GD (eds) Antimicrobial therapy and vaccines, vol 2. E-Sun Technologies, Pittsburgh

Zhao L, Liu H-W (2010) Pathway and enzyme engineering and applications for glycodiversification. In: Yeh W-K, Yang H-C, McCarthy JR (eds) Enzyme technologies: Metagenomics, evolution, biocatalysis, and biosynthesis. Wiley, Hoboken

Zuckerman JM, Qamar F, Bono BR (2011) Review of macrolides (azithromycin, clarithromycin), ketolides (telithromycin) and glycylcyclines (tigecycline). Med Clin N Am 95:761–791

Chapter 12
Tetracycline Antibiotics and Novel Analogs

Olga Genilloud and Francisca Vicente

Abstract Tetracyclines are a class of broad spectrum, orally available antibiotics with activity against a wide range of Gram-positive and Gram-negative pathogens and protozoan parasites. They have been used extensively since their discovery in the late 1940s for human and animal infections given the absence of major side-effects. Tetracyclines are bacteriostatic and inhibit bacterial growth by interfering with protein synthesis. The emergence and wide-spread of microbial resistance, especially due to highly efficient efflux transporters and ribosomal protection mechanisms, have limited their application. Understanding the molecular interaction of tetracyclines with their targets and the resistance mechanisms has clarified their mode of action and set the foundation for the development of the latest third generation of tetracyclines, such as the glycylcyclines and totally synthetic analogs.

12.1 Introduction

Tetracyclines are broad-spectrum agents that exhibit activity against a wide range of Gram-positive and Gram-negative bacteria, including the intracellular pathogens *Chlamydiae*, *Mycoplasma*, and *Rickettsiae*, as well as eukaryote protozoan parasites (Roberts 2003). These antibiotics show good oral absorption and low toxicity, and since their discovery in the 1940s, they have been used extensively in both humans and animals. Besides their antibiotic activities, these compounds have also been described with applications in other therapeutic areas related to inflammatory processes, angiogenesis and anti-apoptotic agents (Griffin et al. 2010).

O. Genilloud (✉) · F. Vicente
Parque Tecnológico Ciencias de la Salud, Fundación MEDINA, Avda Conocimiento 3
18016 Granada, Spain
e-mail: olga.genilloud@medinaandalucia.es

F. Vicente
e-mail: francisca.vicente@medinaandalucia.es

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_12,

The discovery of chlortetracycline (Aureomycin, Lederle), the first described tetracycline compound from a strain of *Streptomyces aureofaciens* (Duggar 1948), was rapidly followed by the isolation of oxytetracycline (Terramycin, Pfizer) as a product of *Streptomyces rimosus* (Finlay et al. 1950). These were soon followed by other natural tetracyclines effectively produced by fermentation, such as tetracycline (Achromycin) produced by strains of *S. aureofaciens, S. rimosus*, and *S. viridofaciens* and demethylchlortetracycline from *S. aureofaciens,* which were extensively used in human and veterinary therapy, animal growth promotion, and aquaculture (Chopra and Roberts 2001; Nelson and Levy 2011) (Table 12.1, Fig. 12.1).

A second generation of semi-synthetic-related compounds, modifications of naturally occurring tetracyclines, and novel synthesis compounds, were rapidly developed to improve spectrum profile and solubility. Over the past few decades, the use of tetracyclines for the treatment of common infections has declined and has been replaced by the broad spectrum fluoroquinolones (see Pucci and Wiles, this volume), but the former are now regaining status after the introduction of new and more active compounds. In addition the emergence of microbial resistances mostly derived from extensive use limited their effectiveness. The increased rate of bacterial resistance to these compounds has fostered recent advances in understanding tetracycline resistance mechanisms and structure–activity relationships, providing new opportunities for the development of a third-generation of new compounds to overcome these resistances.

12.2 Chemical Structure and Structure Activity Relationships of the Class of Tetracyclines

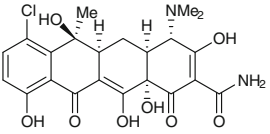
Tetracyclines are polyketide antibiotics which present a highly modified chemical scaffold with a linear tetracyclic core (rings A to D) of complex stereochemistry with contiguous chiral centers that allow interaction with a variety of cellular targets (Fig. 12.2). The presence of several chiral centers and reactive substituents derived from their complex microbial biosynthetic route represented a challenge for the *de novo* synthesis of tetracyclines that was first reported in 1968 (Muxfeldt et al. 1968), followed later by different synthetic approaches (Charest et al. 2005). In all cases, the very poor yields of these synthetic routes determined the industrial fermentation production of these antibiotics and the precursors for semi-synthetic derivatives.

Tetracyclines are produced naturally by many *Streptomyces* species following a programmed synthesis determined by Type II polyketide synthases that generate the core scaffold and the tailoring reactions ensuring the addition of functional groups with a specific configuration. The biosynthesis of chlortetracycline and oxytetracycline was initially studied through classical approaches involving blocked mutant analysis, substrate feeding, and precursor analysis (Miller et al. 1964; Mc Cormick

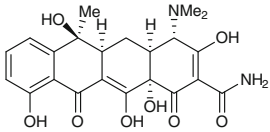
Table 12.1 Natural, semi-synthetic and synthetic tetracyclines

Generic name	Chemical name	Commercial name	Discovery	Origin
First generation				
Chlortetracycline	7-chlortetracycline	Aureomycin	1948	Natural
Oxytetracycline	5-hydroxytetracycline	Terramycin	1949	Natural
Tetracycline	Tetracycline	Achromycin	1953	Natural
Demethylchlortetracycline	6-demethyl-7-chlortetracycline	Declomycin	1957	Natural
Second generation				
Rolitetracycline	2-N-pyrrolidinomethyltetracycline	Reverin	1958	Semi-synthetic
Limecycline	2-N-lysinomethyltetracycline	Tetralysal	1961	Semi-synthetic
Clomocycline	N-methylol-7-chlortetracycline	Megaclor	1963	Semi-synthetic
Methacycline	6-methylene-5-hydroxytetracycline	Rondomycin	1965	Semi-synthetic
Doxycycline	6-deoxy-5-hydroxytetracycline	Vibramycin	1967	Semi-synthetic
Minocycline	7-dimethylamino-6-demethyl-6-deoxytetracycline	Minocin	1972	Semi-synthetic
Third generation				
Tigecycline	9-(t-butylglycylamido)-minocycline	Tigacyl	1993	Semi-synthetic
Omadacycline (MK-2764/PTK0796)	Aminomethylcycline	Phase III	2003	Synthetic
Eravacycline (TP-434)	Fluorocycline	Phase II	2010	Synthetic

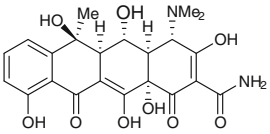
Modified from Chopra and Roberts (2001)



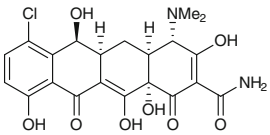
chlortetracycline (aureomycin)



tetracycline (achromycin)



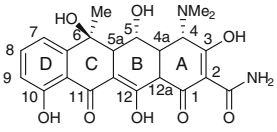
oxytetracycline (terramycin)



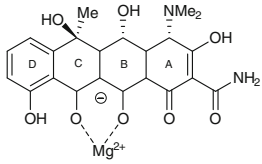
demethylchlortetracycline

Fig. 12.1 Natural tetracyclines

Fig. 12.2 Nomenclature and cation ion complex



Nomenclature



tetracycline complex with Mg^{2+}

et al. 1965), but it is only recently that the biosynthetic clusters involved have been sequenced providing new insights into their biosynthesis (Nakano et al. 2004; Zhang et al. 2007) (Fig. 12.3).

The development of a second generation of semi-synthetic tetracyclines was focused on improving their potency and circumventing emerging resistance mechanisms by modifying antibiotic scaffolds (Table 12.1). These approaches generated more lipophilic compounds such as doxycycline and minocycline, with better absorption and pharmacokinetics (Fig. 12.4) (Agwuh and MacGowan 2006). The need to increase water solubility to improve oral absorption and ensure parenteral administration resulted in the development of other series of semi-synthetic compounds such as rolitetracycline and lymecycline (Table 12.1). The chemical features that determine antibacterial activity were established from structure–activity studies that quickly revealed the key role of the dimethylamino group on the carbon C4 for antimicrobial activity, 6-deoxy-6-demethyltetracycline being the simplest tetracycline to maintain activity. In fact, whereas all dedimethylamino

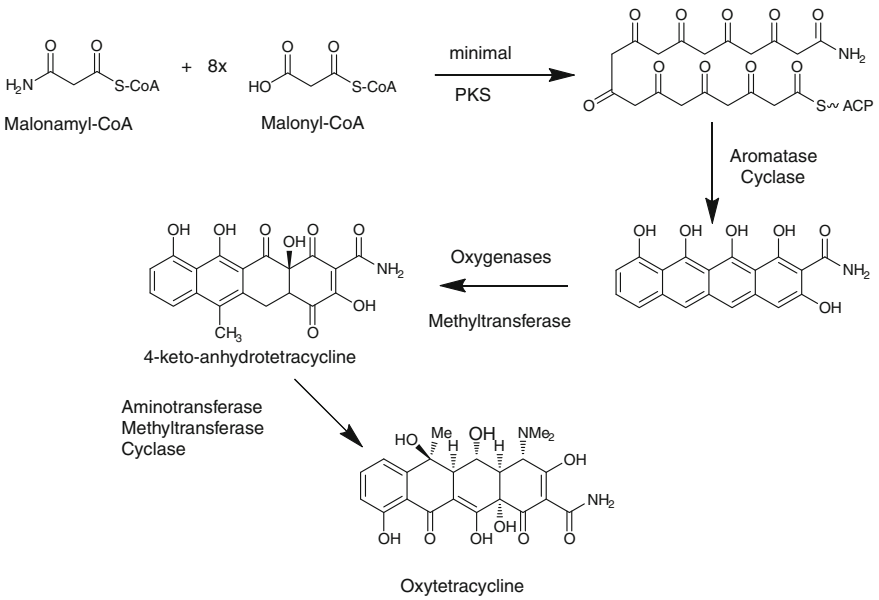


Fig. 12.3 Biosynthesis of tetracyclines—model of oxytetracycline biosynthesis proposed by Zhang et al. (2006)

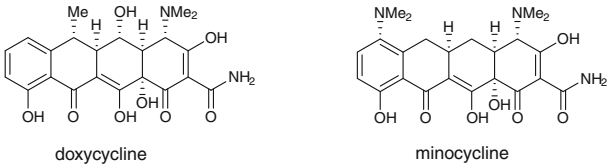


Fig. 12.4 Second generation semi-synthetic tetracyclines

tetracyclines are devoid of antimicrobial activity, they maintain the ability to bind metalloproteinases with different therapeutic applications. Additional features found to be essential for antimicrobial activity include the maintenance of the fused tetracycle, the natural occurrence of the α stereochemical configurations of the C4a, C12a, and C4 positions, and the presence of the keto-enol functional groups in positions C11, C12, and C12a (Fig. 12.2). One of the main characteristics is the presence of keto-enol functional groups on one face of the scaffold that favor the chelation of divalent ions and, thus, the antimicrobial and pharmacokinetic properties of the molecule. In addition, the existing region-specific functional groups allow the hydrogen bond interaction of the compound with biological targets. A replacement of the C2 carboxyamide with other groups results in less antibacterial activity (Mitscher 1978). The addition of a substituent to the amide nitrogen determined an increase in water solubility as in the case of rolitetracycline

and lymecycline, but all changes in positions C1, C3, C4a, C10, C11, and C12 had a negative impact on the antimicrobial activity (Chopra and Roberts 2001).

Whereas the tetracycline structure–activity studies revealed that each of the rings had to be six-membered and carbocyclic to retain antimicrobial activity, thiatetracyclines, and other analogs known as atypical tetracyclines (anhydrotetracyclines, 4-epi-anhydroteracycline, and chelocardin) have shown a different structure–activity relationship. These compounds are bactericidal and they act at the level of the cytoplasmic membrane (Chopra 1994).

The third generation of tetracyclines includes the semi-synthetic glycylcyclines and aminomethylcyclines. Glycylcyclines are 9-glycylamido analogs, such as tigecycline or GAR-936, the 9-*t*-butylglycylamido derivative of minocycline that has now been on the market since 2005 (Peterson 2008). As will be discussed in a later section, this structural modification ensures a more efficient interaction with the ribosome and avoids classic tetracycline resistance mechanisms including both efflux of first- and second-generation tetracyclines and ribosomal protection (Table 12.1) (Bauer et al. 2004; Zhanel et al. 2004).

12.3 Mode of Action of Tetracyclines

Tetracyclines can be divided into two groups according to their mode of action, bacteriostatic tetracyclines and bactericidal atypical tetracyclines.

The classical tetracyclines (tetracycline, chlortetracycline, doxycycline, and minocycline) are bacteriostatic compounds associated with the reversible inhibition of protein synthesis. They bind to the bacterial ribosome and prevent the association of aminoacyl-tRNA with the ribosomal acceptor (A) site of the bacterial ribosome (Chopra et al. 1992; Hash et al. 1964; Semenkov et al. 1982) therefore halting protein synthesis.

All tetracyclines need to cross membrane systems to interact with their target. In Gram-negative bacteria they are known to cross the outer membrane through the OmpF and OmpC porin channels in the form of coordinated complexes with positively charged ions. They accumulate in the periplasm, releasing a lipophilic molecule that can diffuse through the cytoplasmic membrane via an energy-dependent mechanism. The same diffusion mechanism is also used to penetrate membranes of Gram-positive bacteria. Once internalized in the cytoplasm, where internal pH and divalent metal concentrations are higher, tetracyclines can become chelated to Mg^{2+} and bind the ribosome in a chelated complex form (Chopra et al. 1992; Nikaido and Thanassi 1993; Schnappinger and Hillen 1996). The ribosomal protein S7 is a component of the high-affinity binding site (Goldman et al. 1983), where the proteins S3, S8, S14, and S19 also play an important role in the binding. Chemical footprinting has shown the proximity of tetracycline to the 16S rRNA (Moazed and Noller 1987) and the weakened ribosome-tRNA interaction upon tetracycline binding (Epe et al. 1987). The detailed crystal structure of tetracycline in complex with the 30S ribosomal subunit of *Thermus thermophilus* has shown

one strong binding site for tetracycline on the ribosomal 30S subunit (Brodersen et al. 2000) confirming a previous hypothesis on the direct involvement of the binding site near the A site in the mode of action (Schnappinger and Hillen 1996) and five additional binding sites on both subunits (Pioletti et al. 2001). Once in the binding site, the chelated tetracycline complex interacts with the 16S RNA component of the ribosome and acts as a physical barrier preventing binding of tRNA to the A site (Zarivach et al. 2002). The remaining low affinity binding sites have been proposed to act synergistically to ensure the inhibitory effect of tetracyclines (Pioletti et al. 2001). This is consistent with a model where the hydrophilic side of the molecule would be responsible for the chemical interactions with the 16S rRNA, leaving an open space in the hydrophobic side, prone to chemical substitutions well tolerated in natural tetracyclines and semi-synthetic analogs (Nelson 2002; Zacheri and Wright 2008).

The bactericidal tetracyclines (chelocardin, 6-thiatetracycline, and anhydrotetracycline), also known as atypical tetracyclines, are poor inhibitors of protein synthesis as they do not bind to the conventional tetracycline binding site on the 30 s ribosomal unit. They have been shown to prevent the incorporation of nucleic acids into DNA and RNA and to disrupt the cytoplasmic membrane (Rasmussen et al. 1991; Chopra 1994; Schnappinger and Hillen 1996). The preferred lipophilic form of these tetracyclines and their planar structure favor their entry into the cytoplasmic membrane, causing membrane damage and lysis. Although these compounds are potent antimicrobials with low MICs, their adverse side effects have prevented their development in the clinic and they have very little therapeutic value (Chopra 1994).

12.4 Mechanisms of Resistance to Tetracyclines

Tetracycline resistance determinants are wide spread among bacteria, with more than 46 tetracycline resistance determinants described so far, and often located in mobile genetic elements (Chopra and Roberts 2001; Nelson and Levy 2011). They can also be found in the antibiotic producing bacteria, frequently linked to genes encoded by the tetracycline biosynthetic pathways. Among these determinants, four different mechanisms have been identified for bacterial tetracycline resistance. The active tetracycline efflux from the cell and the disruption of ribosomal interaction by ribosomal protection proteins are those most frequently found among pathogens and environment bacteria. The Tet family of cytoplasmic membrane-bound efflux proteins is one of the most efficient resistance mechanisms to ensure the reduction of the tetracycline intracellular concentration. Most of these *Tet* determinants are of Gram-negative origin (TetA-E, G, and H) and are encoded in large conjugative plasmids, whereas TetK, L, P, and OtrB occur in Gram-positive bacteria (Chopra and Roberts 2001; Levy and McMurry 1974; McMurry et al. 1980; see Paitan and Ron, this volume). The expression of the efflux proteins in Gram-negatives is regulated by a divergently expressed repressor of the TetR family that binds as a homodimer with

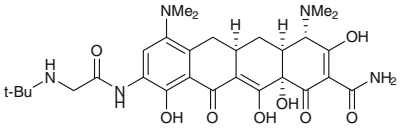
the tetracycline Mg^{2+}complex with 1000-fold higher affinity than the ribosome, avoiding protein synthesis inhibition. This binding to TetR triggers the efficient expression of the efflux protein among which TetA is one of best studied drug-specific efflux proteins. TetA determines a high-level resistance to tetracycline, efficiently pumping the antibiotic out of the cell in an energy-dependent mechanism (Lomovskaya and Watkins 2001). The tetracycline transporters in Gram-positive bacteria are also inducible but this regulation is not mediated by a transcriptional repressor, but rather by a mechanism of translational attenuation (Schnappinger and Hillen 1996).

Ribosomal protection proteins such as Tet(O) and Tet(M) are soluble cytoplasmic proteins with GTPase activity. They bind to the ribosome and induce the dissociation of the tetracycline from the high affinity binding site, but only provide resistance at low level concentrations of tetracycline (Connell et al. 2003; Taylor and Chau 1996).

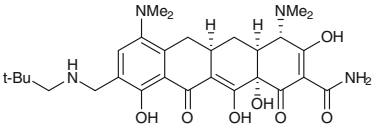
The drug enzymatic inactivation and 16S rRNA mutations altering the target site are alternative resistance mechanisms. TetX is the first reported tetracycline inactivating enzyme that hydroxylates tetracycline at position C11a, rendering an unstable product and disrupting the tetracycline Mg^{2+} complex (Yang et al. 2004), but they have not been reported to be of clinical relevance (Ball et al. 1980; Burdett 1991; Schnappinger and Hillen 1996; Ross et al. 1998).

12.5 Pharmacological and Clinical Issues of Semi-Synthetic Tetracyclines

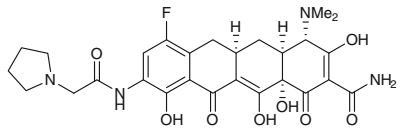
Tetracyclines are orally available although some compounds such as doxycycline are administered as parenteral formulations, and others such as rolitetracycline were only developed for parenteral administration (Williams 1998). The most commonly used semi-synthetic tetracyclines in the clinic are doxycycline and minocycline which are well-tolerated and safe. As a broad spectrum antibiotic, doxycycline is indicated for a variety of infections, including anthrax, community acquired pneumonia, Lyme disease, cholera, syphilis or periodontal infections, whereas minocycline, with the same indications as doxycycline, is also used in the treatment of severe acne. Orally administered tetracyclines are absorbed at the stomach level and proximal small intestine, and they form chelates with calcium and other divalent cations present in food that limit their absorption. Serum levels reach 2–5 μg/ml at normal doses and the maintenance of therapeutic levels requires four daily dosages of first-generation tetracyclines. The longer half-life of the second-generation compounds doxycline and minocycline has permitted reducing the dosage to once or twice a day. Thus, doxycline is currently considered as an essential drug by the World Health Organization (WHO 2011).



tigecycline



omadacycline



eravacycline

Fig. 12.5 Third generation of tetracyclines

12.6 Third Generation of Tetracyclines

12.6.1 Tigecycline

In an attempt to restore the potential of tetracyclines as broad-spectrum antibiotics, systematic searches for tetracycline analogs with activity against both tetracycline-susceptible and tetracycline-resistant organisms were performed in the early 1990s (Chopra 2001). These efforts led to the identification of glycylcyclines and tigecycline, the first representative of the glycylcycline class of antibacterial agents to be marketed for clinical use (Petersen et al. 1999) (Fig. 12.5). Tigecycline (GAR-936, Wyeth), the 9-*tert*-butyl-glycylamido derivative of minocycline, is the most-recently developed glycylcycline.

As previous tetracyclines, tigecycline enters bacterial cells through energy dependent pathways or passive diffusion and reversibly binds to the 30S ribosomal subunit by blocking the incorporation of amino-acyl tRNA molecules into the A site of the ribosome, thus inhibiting protein synthesis (Chopra 2001; Garrison et al. 2005). Compared to other tetracyclines, tigecycline binds to corresponding ribosomal sites with greater affinity and irrespective of the presence of mutations that confer resistance to tetracyclines (Bauer et al. 2004; Zhanel et al. 2004).

Tigecycline is not affected by either of the main tetracycline-resistance mechanisms of ribosomal protection and active drug efflux, probably due to the steric hindrance due of the large 9-*t*-butyl-glycylamido side chain (Fig. 12.5). It has been shown to exhibit in vitro and in vivo activity (generally bacteriostatic) against a broad spectrum of bacterial pathogens, and no cross-resistance with other antibiotics has been observed (Chopra 2001).

The above-stated properties of tigecycline confer in vitro activity against a wide range of bacterial pathogens, including Gram-positive and Gram-negative aerobic and anaerobic species (Pankey 2005) with the exception of *Pseudomonas aeruginosa* (Doan et al. 2006). Tigecycline is highly active in vitro against both

nosocomial and community-acquired methicillin-susceptible or -resistant *S. aureus* strains, vancomycin-susceptible, or -resistant *E. faecium* and *E. faecalis*, and penicillin-susceptible or -resistant strains of *S. pneumoniae* (Noskin 2005; see Rossolini et al., this volume).

Tigecycline has also shown adequate activity against *Acinetobacter* species of potential clinical significance other than *A. baumannii*, such as *Acinetobacter junii, Acinetobacter anitratus*, *Acinetobacter calcoaceticus,* and *Acinetobacter lwoffi* (Karageorgopoulos et al. 2008; see Paitan and Ron, this volume). Tigecycline therefore has great value as a new therapeutic agent against important pathogens.

Tigecycline presents a good safety profile at the liver and kidney metabolism levels. Tigecycline does not affect the cytochrome P450 (CYP450) enzyme family, including the isoforms CYP1A2, CYP2C8, CYP2C19, CYP2D6, and CYP3A4, nor is tigecycline expected to alter the metabolism of drugs metabolized by CYP450 enzymes, based upon in vitro studies. Tigecycline itself is not metabolized by CYP450 (Stein and Craig 2006).

No dose adjustment is needed in patients with renal insufficiency or mild-to-moderate hepatic impairment, or in the elderly population, although in patients with severe hepatic impairment, the initial dose should be 100 mg followed by a reduced maintenance dose of 25 mg every 12 h (Kasbekar 2006). Thus, tigecycline might offer benefits over other antibiotics, such as vancomycin, which has restricted use in specific populations, such as patients with renal insufficiency.

Tigecycline is only available as an injectable formulation for clinical use, unlike currently marketed tetracyclines which are available in oral dosage forms. Tigecycline has a higher steady-state apparent volume of distribution (7–10 L/kg) than the other tetracyclines (range of 0.14–1.6 L/kg), indicating extensive distribution and concentration in human tissue outside of the plasma volume. Protein binding is approximately 68 %. Tigecycline has a half-life of 36 h in humans, less than 15 % of tigecycline is excreted unchanged in the urine. On the basis of available data, it does not appear that the pharmacokinetics of tigecycline is markedly influenced by patient's gender or age. Tigecycline is efficacious, can be safely used and is well-tolerated with adverse effects typical of the tetracyclines (i.e., nausea, vomiting, and headache) (Zhanel et al. 2004). Tigecycline has been on the market since 2005.

12.6.2 Omadacycline

Omadacycline (MK-2764/PTK0796, Paratek Pharmaceuticals) is an aminomethyl-substituted derivative of minocycline with a similar in vitro antibacterial spectrum as tigecycline (Fig. 12.5). Like tigecycline, potent activity was observed in vitro against Gram-positive and Gram-negative bacteria causing acute bacterial skin and skin structure infections (ABSSSI) and CABP (Sutcliffe 2011). The MIC_{90} values for MRSA are below 0.5 μg/ml and against most *Enterobacteriaceae* were higher than 2 μg/ml, whereas the MIC_{90} values for *E. coli*, *Klebsiella pneumoniae*, and

Proteus mirabilis/vulgaris were 2, 8, 16 μg/ml, respectively. Like tigecycline, activity against *Pseudomonas aeruginosa* has not been reported.

In vivo PK/PD studies against *S. pneumoniae* in a murine pneumonia model demonstrated a linear PK profile over a range of 0.5–10 mg/kg doses and potent killing activity at 10 mg/kg (Tessier et al. 2006). A separate PK/PD study against a variety of Gram-negative and Gram-positive pathogens showed potency that was generally superior to that of tigecycline (Andes et al. 2006). The IV efficacy (ED_{50}) in neutropenic murine lung infection models challenged with *S. pneumoniae* ranged from 3.53 to 11 mg/kg, whereas ED_{50} values were lower in systemic murine models (0.09–0.14 mg/kg) challenged with pneumococci. In murine sepsis caused by susceptible or MDR staphylococci, omadacycline was more effective than vancomycin or linezolid when the challenge organism was MSSA, quinolone-resistant *S. aureus*, or MR *Staphylococcus epidermidis*. In systemic infection murine models with *E. faecalis*, omadacycline is superior to linezolid with an ED_{50} of 4.5 mg/kg. The efficacy of omadacycline is equivalent to minocycline in a tetracycline-susceptible *E. coli* urinary tract infection (UTI) model (ED_{50} 4.3 mg/kg) (Sutcliffe 2011).

Omadacycline has completed Phase I and Phase II clinical trials, both as intravenous (IV) and as oral therapy (http://clinicaltrials.gov). In Phase II clinical trial studies, omadacycline was shown to be well-tolerated, efficacious, and non-inferior to linezolid for the treatment of cSSSI caused by MRSA (Arbeit et al. 2008). From the two Phase III clinical trials that were in place for studying its safety and efficacy in patients with cSSSI, one has been withdrawn. Omadacycline has been designated by the FDA in 2013 as a qualified infectious disease product (QIDP) for both IV and oral formulations in the treatment of acute bacterial skin and skin structure infections (ABSSSI) and community-acquired bacterial pneumonia (CABP).

12.6.3 Eravacycline

Eravacycline (TP-434, Tetraphase Pharmaceuticals) is a new member of the synthetic class of 7-fluoro-6-demethyl-6-deoxytetracyclines, also termed 'fluorocyclines', with potent broad in vitro and in vivo activity against a number of MDR Gram-negative and Gram-positive aerobic and anaerobic bacteria (Fig. 12.5). These include MRSA, *A. baumannii* (MIC_{90}, 2 μg/ml), and clinically important species of *Enterobacteriaceae* including *E. coli* (MIC_{90}, 0.5 μg/ml) or *K. pneumoniae* isolates (MIC_{90}, 1 μg/ml) that produce extended spectrum β-lactamases (ESBL) and/or are carbapenem resistant but is not active against *P. aeruginosa* (Xiao et al. 2012; Sutcliffe 2011). As many of the compounds of this class, TP-434 is active against strains carrying resistance determinants encoding tetracycline-specific efflux pumps (including *mepA*), ribosomal protection mechanisms and tetracycline inactivating enzymes. TP-434 shows low spontaneous resistance ($<1 \times 10^{-9}$ in Gram-positive and 10^{-7}–10^{-9} in Gram-negative pathogens). The compound is efficacious in IV

murine models of infection including mouse septicemia model and murine model of pneumonia (Sutcliffe 2011). TP-434 is not metabolized in the liver and CYP P450 mediated drug–drug interactions are unlikely. The PK was determined in different animal models in oral and IV administration that suggested biliary excretion as the major route of elimination. Safety and tolerability tests identified no clinically significant safety issues with these compounds. TP-434's oral bioavailability provides a dosing advantage. A fully synthetic approach to generate novel fluorocyclines has expanded significantly the chemical space at the C-9 position and has identified compounds with improved oral efficacy and pharmacokinetics in animal models (Clark et al. 2012). A Phase 2 clinical trial with an IV formulation of TP-434 has been completed in 2012 for complicated intra-abdominal infections (cIAI). Eravacycline can be dosed both orally and by IV infusion and has recently completed a Phase II clinical trial for use in complicated intra-abdominal infection showing a convenient dosing regimen to be administered once or twice a day as a monotherapy (Clinicaltrials.gov 2012). Eravacycline is currently undergoing Phase I trials for potential use as oral therapy.

12.7 Non-antibiotic Applications of Tetracyclines

Several non-antibiotic properties of tetracyclines have been well described. Tetracyclines are known to inhibit members of the matrix metalloproteinases (MMPs), a family of zinc-dependent proteases involved in many physiological processes and in tumor invasion, inflammation, and tissue remodeling (Griffin et al. 2010). Inhibition of MMPs can be applied to pathologies related with MMP-mediated proteolysis of the extracellular matrix. At present the only MMP inhibitor clinically available is doxycycline which is used for the treatment of periodontitis. The mechanism of inhibition is mediated by a reversible interaction between the tetracycline and the metal ion in the metalloproteinase. Another characteristic is the ability of tetracyclines to scavenge reactive oxygen species (ROS) produced under many pathological conditions. The presence of a multiple substituted phenol ring reacts with a free radical and stabilizes as a non-reactive phenolic radical (Kraus et al. 2005). Tetracyclines, in particular minocycline and doxycycline, have been shown to have a neuroprotective effect and possess anti-apoptotic properties that were associated with a reduction in caspase-1 and caspase-3 expression and mitochondrial stabilization. This class of compounds also exerts anti-inflammatory effects and doxycycline has been successfully used in the treatment of inflammatory skin conditions such as acne and rosacea (Griffin et al. 2010; Nelson and Levy 2011).

12.8 Conclusions

Whereas there is still a role for less toxic tetracyclines that can bypass third-generation efflux mechanisms, it is expected that the synthesis of new tetracycline analogs from totally synthetic compounds will deliver novel potent compounds as resistance determinants are well understood and new molecules can be designed on a molecular level to avoid the most common resistance mechanisms. Novel generations will be required to be used against resistant bacteria in the treatment of infectious diseases, as the number of useful antibiotics in the clinic will continue to decrease as tetracycline-resistant pathogens continue to emerge.

References

Agwuh KN, MacGowan A (2006) Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. J Antimicrob Chemother 58:256–265

Andes DR, Craig W, Odinecs A, In vivo pharmacodynamics of MX-2764/PTK-0796 against various gram-positive and gram-negative bacteria in the thighs of neutropenic and normal mice. In: 46th interscience conference on antimicrobial agents and chemotherapy (ICAAC), San Francisco, 27–30 September 2006, abstract F-1974

Arbeit RD, Roberts JA, Forsythe AR et al (2008) Safety and efficacy of PTK 0796: results of the phase 2 study in complicated skin and skin structure infections following IV and oral step down therapy. In 48th interscience conference on antimicrobial agents and chemotherapy (ICAAC), Washington, DC, 24–28 October 2008, abstract L-1515b

Ball PR, Shales SW, Chopra I (1980) Plasmid-mediated tetracycline resistance in *Eschrichia coli* involves increased efflux of the antibiotic. Biochem Biophys Res Commun 93:74–81

Bauer G, Berens C, Projan SJ, Hillen W (2004) Comparison of tetracycline and tigecycline binding to ribosomes mapped by dimethylsulphate and drug-directed Fe2þ cleavage of 16S rRNA. J Antimicrob Chemother 53:592–599

Brodersen DE, Clemons WM, Carter AP, Morgan-warren RJ, Wimberly BT, Ramakrishnan V (2000) The structural basis for the action of the antibiotic tetracycline, pactamycin and hygromycin b on the 30S ribosomal subunit. Cell 103:1143–1154

Burdett V (1991) Purification and characterization of Tet(M), a protein that renders ribosomes resistant to tetracycline. J Biol Chem 266:2872–2877

Charest MG, Lerner DR, Meyers AG (2005) Synthesis of 8-9-tetracycline. J Am Chem Soc 127:8292–9293

Chopra I, Hawkey PM, Hinton M (1992) Tetracyclines, molecular and clinical aspects. J Antimicrob Chemother 29:245–277

Chopra I (1994) Tetracycline analogs whose primary target is not the bacterial ribosome. Antimicrob Agents Chemother 38:637–640

Chopra I (2001) Glycylcyclines: third-generation tetracycline antibiotics. Curr Opin Pharmacol 1:464–469

Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65:232–260

Clark RB, Hunt DK, He M, Achorn C, Chen CL, Deng Y, Fyfe C, Grossman TH, Hogan PC, O'Brien WJ, Plamondon L, Rönn M, Sutcliffe JA, Zhu Z, Xiao XY (2012) Fluorocyclines. 2. Optimization of the C-9 side-chain for antibacterial activity and oral efficacy. J Med Chem 55:606–622

ClinicalTrials.gov. (2012) Study to compare TP-434 and Ertapenem in CA complicated intra-abdominal infections. NCT01265784

Connell SR, Tracz DM, Nierhaus KH, Taylor DE (2003) Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother 47:3675–3681

Doan TL, Fung HB, Mehta D, Riska PF (2006) Tigecycline: a glycylcycline antimicrobial agent. Clin Ther 28:1079–1106

Duggar BM (1948) Aureomycin: a product of the continuing search for new antibiotics. Ann N Y Acad Sci 51:177–181

Epe B, Woolley P, Hornig H (1987) Competition between tetracycline and tRNA at both P and A sites of the ribosome of *Escherichia coli*. FEBS Lett 213:443–447

Finlay AC, Hobby GL et al (1950) Terramycin, a new antibiotic. Science 111(2874):85

Garrison MW, Neumiller JJ, Setter SM (2005) Tigecycline: an investigational glycylcycline antimicrobial with activity against resistant Gram-positive organisms. Clin Ther 27:12–22

Goldman RA, Hasan T, Hall CC, Strycharz WA, Cooperman BS (1983) Photoincorporation of tetracycline into *Escherichia coli* ribosomes. Identification of the major proteins photolabeled by native tetracycline and tetracycline photoproducts and implications for the inhibitory action of tetracycline on protein synthesis. Biochemistry 22:359–368

Griffin MO, Fricovsky E, Ceballos G, Villarreal F (2010) Tetracyclines: a pleitropic family of compounds with promising therapeutic properties. Review of the literature. Am J Physiol Cell Physiol 299:C539–C548

Hash JH, Wishnick M, Miller PA (1964) On the mode of action of the tetracycline antibiotics in *Staphylococcus aureus*. J Biol Chem 239:2070–2078

Karageorgopoulos DE, Kelesidis T, Kelesidis I, Falagas ME (2008) Tigecycline for the treatment of multidrug-resistant (including carbapenem-resistant) *Acinetobacter* infections: a review of the scientific evidence. J Antimicrob Chemother 62:45–55

Kasbekar N (2006) Tigecycline: a new glycylcycline antimicrobial agent. Am J Health Syst Pharm 63:1235–1243

Kraus RL, Pasieczny R, Lariosa-Willingham K, Turner MS, Jiang A, Trauger JW (2005) Antioxidant properties of minocycline: neuroprotection in an oxidative stress assay and direct radical-scavenging activity. J Neurochem 94:819–827

Levy SB, McMurry L (1974) Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance. Biochem Biophys Res Commun 56:1060–1068

Lomovskaya O, Watkins WJ (2001) Efflux pumps: their role in antibacterial drug discovery. Curr Med Chem 8:1699–1711

McCormick JR, Joachim UH, Jensen ER, Johnson S, Sjolander NO (1965) Biosynthesis of the tetracyclines. VII. 4-hydroxy-6-methylpretetramid, an intermediate accumulated by a blocked mutant of *Streptomyces aureofacien*s. J Am Chem Soc 87:1793–1794

McMurry L, Petrucci R, Levy SB (1980) Active efflux of tetracycline encoded by four genetically different resistant determinants in *E. coli*. Proc Natl Acad Sci U S A 77:3974–3977

Miller PA, Saturnelli A, Martin JH, Itscher LA, Bohonos N (1964) A new family of tetracycline precursors. N-demethylanhydrotetracyclines. Biochem Biophys Res Commun 16:285–291

Mitscher LA (1978) The chemistry of the tetracycline antibiotics. Marcel Dekker Inc, New York

Moazed D, Noller HF (1987) Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature 327:389–394

Muxfeldt H, Hardtmann G, Kathawala F, Vedejs E, Mooberry JB (1968) Tetracyclines. VII. Total synthesis of dl-terramycin. J Am Chem Soc 90:6534–6536

Nakano T, Miyake K, Endo H, Dairi T, Mizukami T, Katsumata R (2004) Identification and cloning of the gene involved in the final step of chlortetracycline biosynthesis in *Streptomyces aureofaciens*. Biosci Biotechnol Biochem 68:1345–1352

Nelson ML (2002) The chemistry and biology of the tetracyclines. In: Annual reports in medicinal chemistry, vol 37. Academic Press, New York

Nelson ML, Levy SB (2011) The history of the tetracyclines. Ann N Y Acad Sci 1241:17–32

Nikaido H, Thanassi DG (1993) Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. Antimicrob Agents Chemother 37:1393–1399

Noskin GA (2005) Tigecycline: a new glycylcycline for treatment of serious infections. Clin Infect Dis 41:s303–s314

Pankey GA (2005) Tigecycline. J Antimicrob Chemother 56:470–480

Petersen PJ, Jacobus NV, Weiss WJ, Sum PE, Testa RT (1999) In vitro and in vivo antibacterial activities of a novel glycylcycline, the 9-*t*-butylglycylamido derivative of minocycline (GAR-936). Antimicrob Agents Chemother 43:738–744

Peterson LR (2008) A review of tigecycline the first glycylcycline. Int J Antimicrob Agents 32:S215–S222

Pioletti M, Schlunzen F, Harms J, Zarivach R, Gluhmann M, Avila H et al (2001) Crystal structure of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. EMBO J 20:1829–1839

Rasmussen B, Noller HF, Daubresse G, Oliva B, Misulovin Z, Rothstein DM, Ellestad GA, Gluzman Y, Tally FP, Chopra I (1991) Molecular basis of tetracycline action: identification of analogs whose primary target is not the bacterial ribosome. Antimicrob Agents Chemother 35:2306–2311

Roberts MC (2003) Tetracycline therapy: update. Clin Infect Dis 36:462–467

Ross JI, Eady EA, Cove JH, Cunliffe WJ (1998) 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. Antimicrob Agents Chemother 42:1702–1705

Schnappinger D, Hillen W (1996) Tetracyclines: antibiotic action, uptake and resistance mechanisms. Arch Microbiol 165:359–369

Semenkov YuP, Makarov EM, Makhno VI, Kirillov SV (1982) Kinetic aspects of tetracycline action on the acceptor (A) site of *Escherichia coli* ribosomes. FEBS Lett 144:125–129

Stein GE, Craig WE (2006) Tigecycline: a critical analysis. Clin Infect Dis 43:518–524

Sutcliffe JA (2011) Antibiotics in development targeting protein synthesis. Ann NY Acad Sci 1:122–152

Taylor DE, Chau A (1996) Tetracycline resistance mediated by ribosomal protection. Antimicrob Agents Chemother 40:1–5

Tessier PR, Fan HW, Tanaka SK, Nicolau DP (2006) In: 46th interscience conference on antimicrobial agents and chemotherapy (ICAAC), San Francisco, 27–30 September 2006, abstract F-1973

World Health Organization (2011) WHO model list of essential medicines. 17th list (http://whqlibdoc.who.int/hq/2011/a95053_eng.pdf)

Williams DN (1998) Tetracyclines. In: Gorbach SL, Bartlett JG, Blacklow NR (eds) Infectious diseases, 2nd edn. WB Saunders, Philadelphia

Xiao XY, Hunt DK, Zhou J et al (2012) Fluorocyclines. 1. 7-Fluoro-9-pyrrolidinoacetamido-6-demethyl-6-deoxytetracycline: a potent, broad spectrum antibacterial agent. J Med Chem 55:597–605

Yang W, Moore IF, Koteva KP et al (2004) TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. J Biol Chem 279:52346–52352

Zacheri B, Wright GD (2008) Chemical biology of tetracycline antibiotics. Biochem Cell Biol 86:124–136

Zarivach R, Bashan A, Schluenzen F, Harms J, Pioletti M, Franceschi F, Yonath A (2002) Initiation and inhibition of protein biosynthesis studies at high resolution. Curr Protein Pept Sci 3:55–65

Zhanel GG, Homenuik K, Nichol K, Noreddin A, Vercaigne L, Embil J, Gin A, Karlowsky JA, Hoban DJ (2004) The glycylcyclines: a comparative review with the tetracyclines. Drugs 64:63–88

Zhang W, Ames BD, Tsai SC, Tang Y (2006) Engineered biosynthesis of a novel amidated polyketide, using the malonamyl-specific initiation module from the oxytetracycline polyketide synthase. Appl Environ Microbiol 72:2573–2580

Zhang W, Watanabe K, Wang CC, Tang Y (2007) Investigation of early tailoring reactions in the oxytetracycline biosynthetic pathway. J Biol Chem 282:25717–25725

Chapter 13
Oxazolidin-2-Ones: Antibacterial Activity and Chemistry

Giovanni Zappia, Cinzia Ingallina, Francesca Ghirga and Bruno Botta

Abstract Since the introduction in the pharmaceutical market of linezolid, an oxazolidin-2-one based new class of antibacterial agents with a unique mechanism of action, this cyclic carbamate bought a wider popularity and interest for the scientific community. In fact the 1,3-oxazolidin-2-one nucleus is a popular heterocycle framework in synthetic organic chemistry for its use as chiral auxiliary in stereoselective transformations. This chapter describes the antibacterial activity of linezolid and the status of the activities of other oxazolidin-2-ones based antibacterial agents under active clinical investigation. Linezolid and other oxazolidin-2-one antibacterial agents are currently prepared by chemical synthesis via diverse synthetic routes that have been extensively studied in academic and industrial labs. Biological active oxazolidin-2-one derivatives are quite rare among natural products.

13.1 Introduction

The role of antimicrobial chemotherapy as a primary cause for the increase in average life expectancy in the twentieth century is a statement widely accepted all over the world. However, disease-causing microbes that have become resistant to

G. Zappia (✉)
Dipartimento di Scienze Biomolecolari, Università di Urbino "Carlo Bo",
Piazza Rinascimento 6, I-61029 Urbino, Italy
e-mail: giovanni.zappia@uniurb.it

C. Ingallina · F. Ghirga · B. Botta
Dipartimento di Chimica e Tecnologie del Farmaco Università di Roma "Sapienza",
00185 Rome, Italy

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_13,

antibiotic drug therapy represent an increasing public health problem. In this context, wound infections, gonorrhea, tuberculosis, pneumonia, septicemia, etc., are just a few of the infections that have become hard to treat with antibiotics. The problem has many aspects, the major being that microbes that cause infections are remarkably resilient and can develop new and somewhat unpredictable resistance mechanisms to antibiotics. Another part of the problem is due to increasing use of existing antibiotics in human and veterinary medicine and in agriculture. Thus, unrestricted and often inappropriate use of most antibiotics is the main reason for the rapid loss of antibiotics efficacy.

As a consequence, almost 70 % of the bacteria that cause infections in hospitals are resistant to at least one of the drugs usually employed for their treatment. Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs. An alarming increase in resistance of bacteria that cause community acquired infections has also been documented, especially among staphylococci and pneumococci, which represent the prevalent causes of infection disease and mortality.

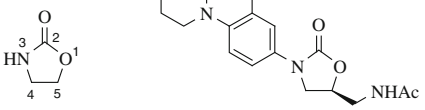
The speed by which bacteria develop resistance to antibiotics, in contrast with the slow development of new drugs, has led the experts to warn of a "post-antibiotic era" (Falagas and Bliziotis 2007). This statement is substantiated by the observation that in the last 35 years, only four new novel antibacterial structural classes have been approved by the US Food and Drug Administration (FDA) and the class of the oxazolidinone compounds is considered one of the most important among them. Currently, linezolid is the only oxazolidinone authorized by the European Medicines Agency (EMA) and the FDA (Brickner et al. 2008; Dryden 2011; Zappia et al. 2007a).

The present contribution focus on the biological activity of linezolid and its antibacterial derivatives currently in different phases of clinical development. In addition, a brief recognition of the chemical routes for the synthesis of oxazolidin-2-one derivatives and the occurrence of biological active natural products is reported.

13.2 Oxazolidinones as Antibacterial Agents: Linezolid and Related Compounds

The 1,3-oxazolidin-2-one ring is a cyclic carbamate skeleton quite rare in natural product chemistry but very popular in medicinal chemistry since the introduction in the pharmaceutical market of linezolid, an oxazolidin-2-one based antibacterial drug (Fig. 13.1).

The oxazolidin-2-one antibacterial class of compounds is considered as one of the most important new class of antibacterials to emerge in the last 30 years, with potent activity against multidrug-resistant Gram-positive bacteria.



Linezolid

Fig. 13.1 Structures of the 1,3-oxazolidin-2-one ring and of the linezolid

Oxazolidinones also have a large application in synthetic organic chemistry, since the Evans' report in 1981 (Evans et al. 1981) on the use of enantiomerically pure 4-substituted oxazolidin-2-ones as chiral auxiliaries in asymmetric synthesis. In addition, their use as protective groups for the 1,2 amino-alcohol system have attracted the interest of the scientists as documented in current literature.

Linezolid, was approved for use in 2000 and in the same year introduced in the market by Pfizer under the tradename Zyvox®. Zyvox is approved for use against serious Gram-positive infections, including those caused by *Streptococcus pneumoniae*, and the challenging methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* strains (see Rossolini, this volume). It is commercialized as 600 mg film-coated tablets, 100 mg/5 mL granules for oral suspension or 2 mg/mL solution for infusion.

Linezolid is generally considered to be well tolerated (French 2003), and the most common adverse events documented in the Zyvox package insert are: diarrhea, nausea, and headache (Pfizer 2010). Linezolid is a weak, reversible, and nonselective monoamine oxidase (MAO) inhibitor (Humphrey et al. 2001).

Reversible myelosuppression has been observed in patients on long-term linezolid therapy, with effects that can include anemia, thrombocytopenia, leucopenia, or pancytopenia, when used for greater than 2 weeks, in patients with preexisting myelosuppression, or in patients receiving concomitant administration of drugs that induce bone marrow suppression (Gorchynski and Rose 2008).

13.2.1 Linezolid Discovery and Structure-Activity Relationship

A large number of publications, reviews, and patents document the interest of the various research groups, from industry and academia, in the oxazolidinone class of antibacterials. Furazolidone can be considered as the first active member of the oxazolidinone class reported in the literature (Rogers et al. 1956) and it was probably the initial candidate responsible for the genesis of further work on oxazolidinone antibacterials.

In the late 1970s E.I.du Pont de Nemours & Company labs reported in some patents on antimicrobial oxazolidinones, the compounds S-6123 (Fig. 13.2) that showed modest in vitro activity against *S. aureus* and *S. pneumoniae*, but good oral

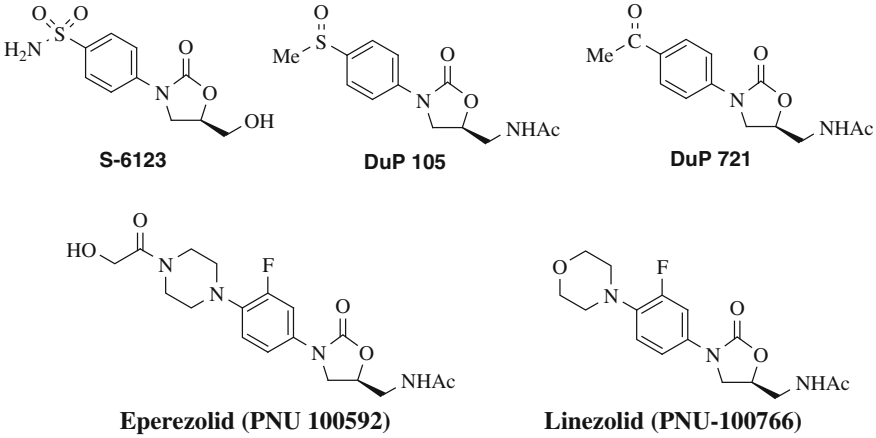


Fig. 13.2 Structures of S-6123, DuP 105 and DuP 721, and eperezolid compared to linezolid

efficacy (ED50 = 17.1 mg/kg) against *S. aureus* in a mouse lethal infection model (Gregory 1984).

Further investigation led in the mid-1980s to the identification of DuP 105 and DuP 721 (Fig. 13.2) (Slee et al. 1987) endowed with an interesting activity and pharmacokinetic (PK) performance profiles. Both compounds proved unsuitable for further development due to their toxic nature.

DuPont laboratories can be considered as the first to take extensive SAR studies in this area. These efforts were accompanied since 1987 by the results from The Upjohn labs. On the basis of their results some key features emerged and are summarized in Fig. 13.3.

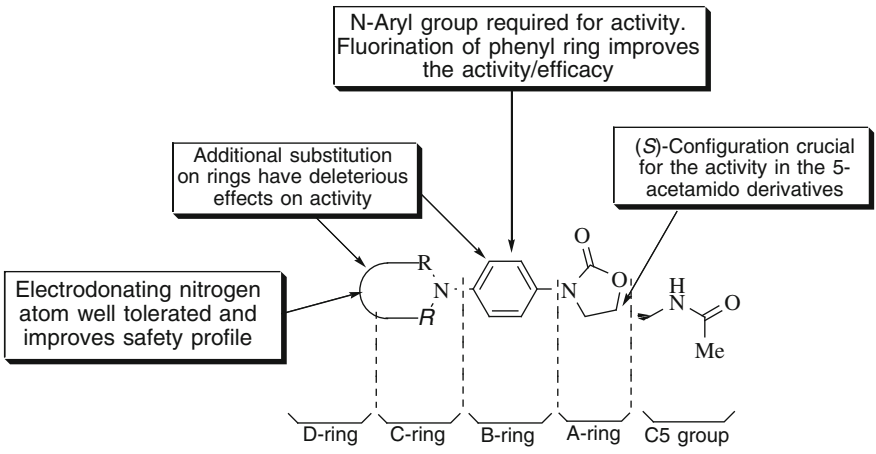


Fig. 13.3 Key features emerging on SAR studies on linezolid and its derivatives

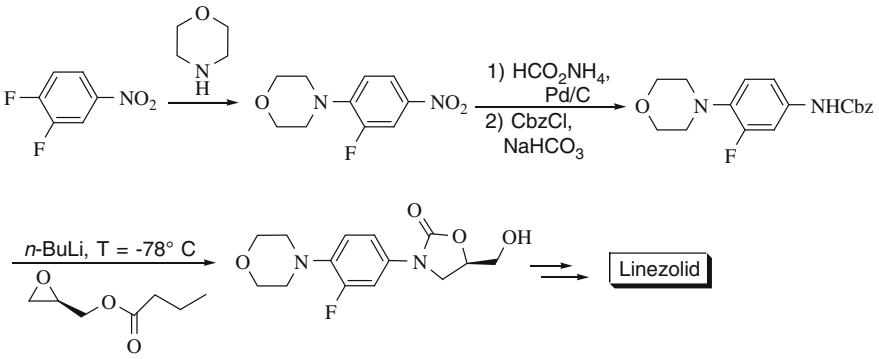


Fig. 13.4 Stereoselective synthesis of linezolid (Brickner et al. 2008)

Intensive work at Upjohn led to the identification first of the analog PNU-100592 (U-100592/eperezolid) and subsequently to the PNU-100766 (U-100766/linezolid).

Eperezolid and linezolid were almost identical in their overall preclinical profiles, including solubility, antimicrobial potency and spectrum of activity, efficacy in murine infection models, optimal clearance, PK in dog, and overall safety profile in 30-day rat and dog multiple dose toxicity studies (Brickner et al. 1996). Linezolid was selected after a phase I based on its superior PK profile, that suggested twice daily dosing would be achievable (Zurenko et al. 1997).

An optimized stereoselective synthesis (Brickner et al. 2008) of linezolid is outlined in Fig. 13.4.

13.2.2 Pharmacology

The bioavailability of linezolid is 95–100 %, so there is no required dosage adjustment for conversion from intravenous (IV) to oral administration, with a maximum plasma concentrations (Cmax) within 1–2 h (MacGowan 2003; Dryden 2011). When a high-fat meal is given with linezolid, the mean time to reach Cmax is delayed 1.5–2.2 h, Cmax is decreased by 15–20 %, but AUC values are the same (Welshman et al. 1998; Sisson et al. 1999). Absorption of the oral suspension is similar to that of the film-coated tablets.

The level of plasma protein binding of linezolid is 31 %, it is concentration independent and the volume of distribution approximates to the total body water content of 40–50 L.

According to different studies (Slatter et al. 2001; Stalker and Jungbluth 2003), linezolid is excreted primarily intact and undergoes hepatic oxidation (Fig. 13.5) to give two main inactive metabolites, an aminoethoxyacetic acid and a hydroxyethyl glycine derivative.

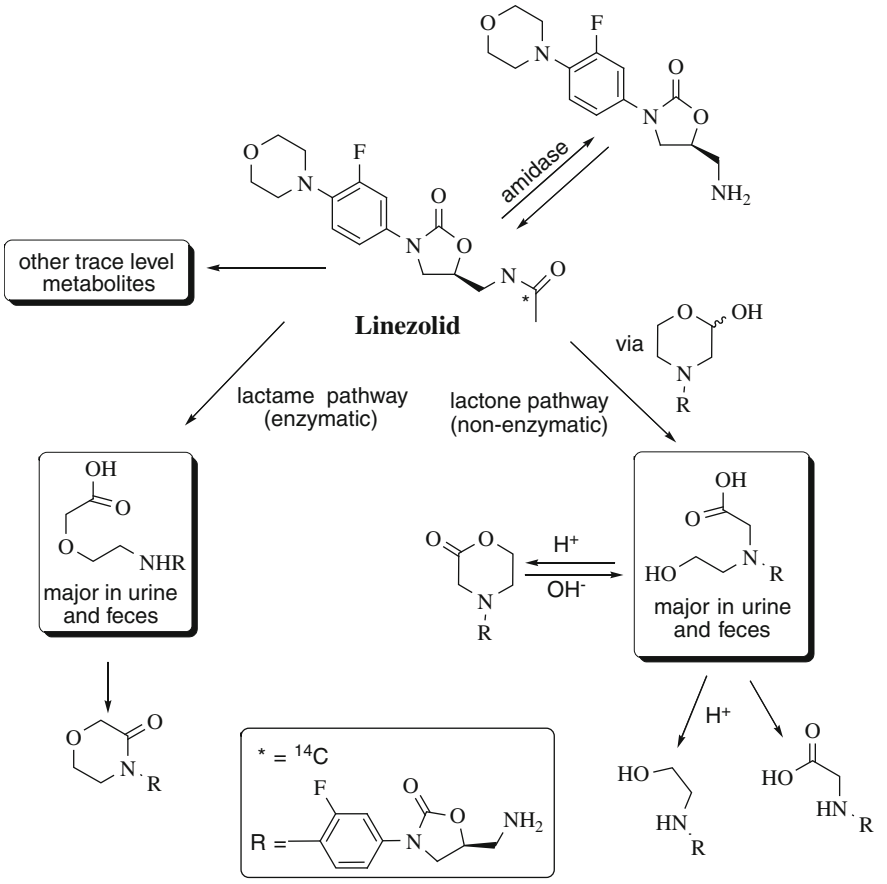


Fig. 13.5 Metabolic transformations of linezolid

The linezolid metabolism is not affected by cytochrome P450 and the compound does not inhibit the P450 pathway by any significant amount.

The co-administration of antacids had no effect on the pharmacokinetics of linezolid, with no significant difference in any pharmacokinetic parameters (Grunder et al. 2006).

13.2.3 Mechanism of Action and Resistance of Oxazolidin-2-Ones

The oxazolidin-2-ones are considered bacteriostatic for enterococci and staphylococci, but bactericidal for penicillin-susceptible *S. pneumoniae* and *Bacteroides fragilis* (Zurenko et al. 1996, 1997). Continuous protein synthesis in a bacterial

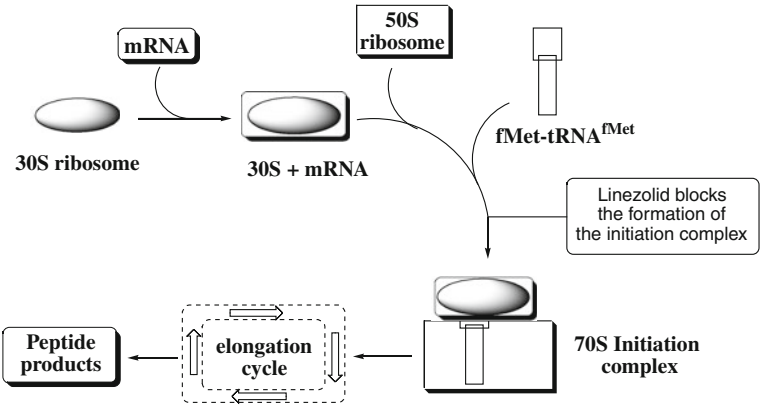


Fig. 13.6 Mode of action of linezolid inhibiting bacterial protein synthesis

cell is crucial for its survival and antibiotics have been targeted at different steps involving this synthesis. Protein synthesis begins with the formation of the 70S initiation complex when the ribosome (made up of 30S and 50S subunits) comes together with an array of initiation factors, themselves proteins, and *m*-RNA and *t*-RNA (Fig. 13.6). Once the complex is formed, protein chain elongation occurs by the sequential addition of single amino acids as programmed by the *m*-RNA, being released from the 70S complex after completion of the synthesis. According to the current literature, linezolid binds to 50S subunit and prevents protein biosynthesis by inhibiting the formation of the 70S ribosomal initiation complex. Oxazolidinones cross-link the conserved nucleotide A2602 in the 23S rRNA with the ribosomal protein L27 and LepA, a ribosome-associated protein, homologous to translation factors. Recent studies (Leach et al. 2007), together the publication of the crystal structures of linezolid bound to the 50S subunit of *Haloarcula marismortui* and *Deinococcus radiodurans* (Ippolito et al. 2008; Wilson et al. 2008), showed that oxazolidin-2-ones bind at the A site of the peptidyl transferase center (PTC), further supporting the previous findings of inhibition by competition with the incoming A site substrates.

Naturally occurring resistance to linezolid is unlikely, and in vitro induced resistance is difficult. The spontaneous frequency of resistance to oxazolidinones is relatively low as compared to other antimicrobial agents, and the frequency of linezolid resistance in staphylococci and enterococci, is reported to be $<10^{-9}$ (Prystowsky et al. 2001).

Nevertheless, the first linezolid resistance was reported clinically in *E. faecium* infections (Zurenko et al. 1999) followed by reports on resistant staphylococci and enterococci strains (Gonzales et al. 2001; Tsiodras et al. 2001).

Resistance to linezolid occurs mainly by mutation in chromosomal genes encoding 23S rRNA (Swaney et al. 1998; Long et al. 2010). A number of mutations in the PTC, more specifically mutations in the genes encoding 23S rRNA: (*E. coli* numbering) G2447T, T2500A, A2503G, T2504C, G2505A, and G2576T,

were reported (Long 2012) to confer resistance to linezolid in staphylococci and enterococci. Other mutations were observed in linezolid-resistant strains only in combination with G2576T or mutations in the gene encoding ribosomal protein L4. Additional mutations found in linezolid-resistant strains were mapped in different areas than PTC (Wong et al. 2010; Livermore et al. 2009).

A nonmutational new mechanism of linezolid resistance was recently reported after the identification in 2005 of chloramphenicol-florfenicol resistance (*cfr*) methyltransferase gene in a linezolid-resistant clinical strain of MRSA from a Colombian patient (Toh et al. 2007). The *cfr* gene was initially described in a bovine *Staphylococcus sciuri* isolate (Schwarz et al. 2000) and it is capable of horizontal transfer among staphylococci (Kehrenberg et al. 2007). The product of the *cfr* gene is a methyltransferase that catalyzes methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit, conferring resistance to chloramphenicol, florfenicol, and clindamycin (Kehrenberg et al. 2005). More recent reports (Morales et al. 2010; Nian et al. 2012) highlighted on the importance of the *cfr*-mediated resistance to linezolid in staphylococcal strains where no G2576T mutation were detected.

A recent paper (Locke et al. 2010) from Trius Therapeutics focused on the structure-activity relationships (SAR) of new and old oxazolidinones (Fig. 13.7), using a panel of clinical and laboratory derived *S. aureus* strains possessing ribosomal resistance mutations or the *cfr* methyltransferase gene. The experimental and molecular modeling studies showed that potency against all strains is correlated with optimization of C- and D-rings, which interact with highly conserved regions of the peptidyl transferase center binding site.

Thus, activity against *cfr* strains was retained in compounds bearing a D-ring substituent and with either hydroxyl-methyl or 1,2,3-triazole C-5 groups, but was reduced by two to eightfold in compounds with acetamide substituents. According to these observations, linezolid which possesses a C-5 acetamide group and lacks a D-ring substituent, showed the lowest potency against all strains tested, particularly against *cfr* strains. As a consequence, it is clear that a careful design of the substituents to C- and D-ring can give rise to new oxazolidinones that may circumvent the known resistance mechanisms.

13.2.4 Oxazolidin-2-Ones Under Active Clinical Investigation

Significant efforts have been made in the last 25 years, from academia and the industry, to design new oxazolidinone-based antibacterial agents (Zappia et al. 2007a; Poce et al. 2008; Shaw and Barbachyn 2011; Pandit et al. 2012). The greatest challenge in the oxazolidinone chemistry is developing a compound with adequate coverage against linezolid-resistant bacterial strains and overcoming the myelosuppression signal intrinsic to this class under an acceptable antibacterial profile.

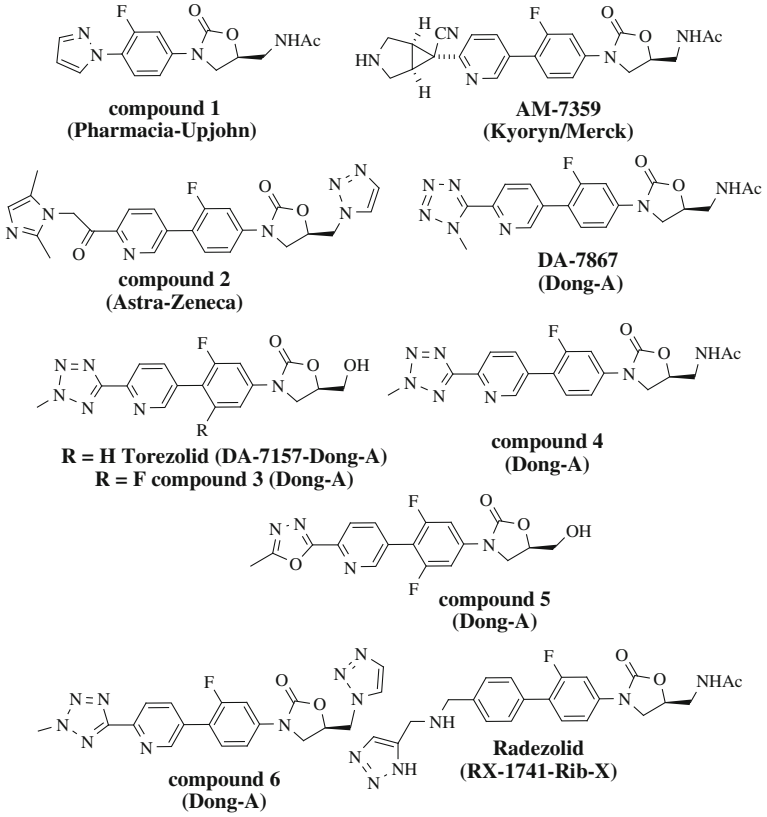


Fig. 13.7 Structures of new and old oxazolidinones studied by Trius Therapeutics (Locke et al. 2010)

More than 30 companies have been leading the preclinical development of almost 20 compounds. Some of them were terminated largely due to (a) inadequate PK properties, (b) low solubility, precluding thereby IV formulations, and (c) a poor safety profile. In general all these candidates did not demonstrate significant improvement over the progenitor linezolid. A short number of oxazolidinones are currently undergoing clinical evaluation (Fig. 13.8) and their main features will be discussed below.

Radezolid (Rx-01 667,RX-1741), an N-biphenyl substituted oxazolidinone patented by Rib-X Pharmaceuticals (Rib-X), demonstrated a significant better activity than linezolid with a MIC_{90} of 0.25 μg/mL against both *S. pneumonia* and *S. pyogenes*. Radezolid MIC_{90} values for staphylococci range from 1–4 μg/mL and 0.5–1 μg/mL for enterococci (Lawrence et al. 2008). In addition, radezolid shows strong activity versus *Haemophilus influenzae* and *Moraxella catarrhalis*, with an MIC_{90} of 1 μg/mL and 0.5 μg/mL, respectively.

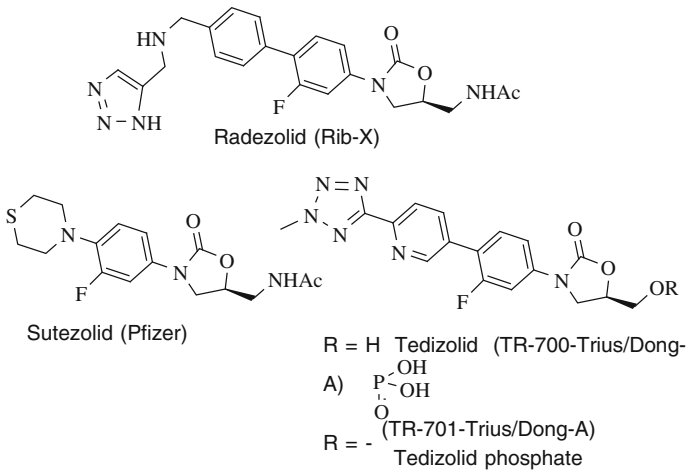


Fig. 13.8 Structures of oxazolidinones currently undergoing clinical evaluation

Radezolid outperformed linezolid in an *E. faecium* VanA peritonitis model when dosed IV daily (Luo et al. 2005), as well as in *S. pneumoniae* pneumonia and peritonitis infection models when dosed *per os* (PO). According to these data, radezolid is twofold more active in vitro than linezolid against the staphylococci and 4- to 16-fold more potent against the streptococci and enterococci (Lawrence et al. 2008). Notably, radezolid offers coverage of the fastidious Gram-negative organisms. Radezolid recently completed successfully two-Phase 2 clinical trials: one for community acquired pneumonia (CAP) and the second for uncomplicated skin and skin structure infections (USSSI). The results from a 3-month rat toxicology study on radezolid were recently reported (Moore et al. 2012). The study was conducted to evaluate the long-term safety of radezolid, at 10, 50, and 200 mg/kg/day, compared to linezolid at 40 and 100 mg/kg/day. Radezolid had no effect on clinical observations, mortality or organ weights any dose level studied, resulting in a No Observed Adverse Effect Level (NOAEL) at 200 mg/kg/day. On the other hand, rats in the high-dose groups (100 mg/kg/day) exhibited a decrease in body weight and food consumption, with associated clinical and hematological effects, when treated with linezolid.

The thiomorpholinyl oxazolidinone sutezolid (PNU-100480) was identified by Pfizer as a potent antimycobacterial agent with in vitro potency and in vivo efficacy superior to that of linezolid (Barbachyn et al. 1996; Cynamon et al. 1999) (Fig. 13.8). The low water solubility (0.2 mg/mL in pH 7 phosphate buffer), precluding it from IV administration, as well as a complex oxidative metabolism profile in rodents stopped further investigations. Recently Williams et al. (2009a, b) confirmed the strong bactericidal activity of sutezolid against established infections with *Mycobacterium tuberculosis* in a murine model; in addition they documented that the addition of sutezolid (100 mg/kg of body weight/day) to the standard daily regimen of rifampicin, isoniazid, and pyrazinamide resulted in an additional

2.0-$\log_{10}$-unit reduction in lung colony forming units (CFU) counts during the first 2 months of treatment. The authors reported that the principal circulating antibacterial agent was the sulfoxide metabolite, in presence of small amount of the sulfone. A study (Wallis et al. 2010) conducted to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of multiple ascending doses of sutezolid in healthy volunteers, using biomarkers for safety and efficacy, showed positive results.

The activity of PNU-100480 was explored against clinical isolates from multidrug and extensively drug-resistant tuberculosis (MDR/XDR-TB) patients with a known level of susceptibility to linezolid (Alffenaar et al. 2011). The reported mean MIC for PNU-100480 was 3.2 times lower than that for linezolid and, according to the authors, it is a promising candidate to be developed further as an adjunct in the treatment of multidrug and extensively drug-resistant tuberculosis (MDR/XDR-TB).

In addition to the above antibacterial agents, particular attention was dedicated to tedizolid phosphate, a potent prodrug of tedizolid (TR-700), with a solubility of $\geq$130 mg/mL in aqueous solution at pH $\geq$ 5.0, and 91.7 % oral bioavailability (Bien et al. 2010).

Tedizolid, formerly known as torezolid phosphate (TR-701/DA-7218), was developed by scientists at Dong-A as a consequence of the revision of the early DuPont SAR studies (Fig. 13.8). In fact, the N-aryl-5-hydroxymethyloxazolidinones showed, in general, lower antimicrobial activity than acetamide analogs (Barbachyn and Ford 2003), but an intensive exploration of the N-substituents revealed that compounds with a good antimicrobial activity could be obtained through incorporation of favorable C-D ring structures. These studies led to identify the C5 hydroxymethyl oxazolidinone DA-7157 (tedizolid) with potent antimicrobial activity (Im et al. 2011). Data from phase 1 studies showed that tedizolid phosphate is rapidly absorbed and converted to TR-700, with a mean half-life ranging from 8 to 11 h, approximately 2-fold longer than that of linezolid and consistent with once-a-day dosing. Tedizolid demonstrated 8- to 16-fold greater potency than linezolid against all strains tested, including MRSA and vancomycin-resistant enterococci (VRE) (Schaadt et al. 2009; Shaw et al. 2008). TR-700 was less active against *M. catarrhalis* and *H. influenzae* but was twofold more active than linezolid. Moreover, torezolid retains activity against linezolid-resistant strains of *S. aureus* including *cfr*-harboring strains. A value of 2 mg/mL for the MIC_{90} of tedizolid against linezolid-resistant *S. aureus* was indicated. Based on molecular modeling studies of binding of tedizolid to 23S rRNA, Shaw et al. (2008) proposed a model in which the increased potency of TR-700 is due to additional target site interactions, mainly for the substituent in the D-ring; TR-700 binding is less reliant on target residues associated with the resistance to linezolid. In this context, the presence of the smaller-CH_2OH in A-ring of tedizolid respect to the -CH_2NHAc in the linezolid should be less affected by the increased steric bulk of the methylated A2503 residue generated by posttranscriptional modification in strains expressing a *cfr* methyltransferase (Toh et al. 2007).

Novel results were disclosed by Trius Therapeutics Inc. (Prokocimer et al. 2011) for a randomized, double-blind phase 2 trial with 200, 300, or 400 mg of oral tedizolid phosphate once daily for 5–7 days in patients with complicated skin and skin structure infections (CSSSI). According to these authors, clinical cure rates were very high for each infection type and at all dose levels. Efficacy, safety, and pharmacokinetic/pharmacodynamic results supported the selection of 200 mg daily of tedizolid phosphate for the oral treatment of patients with CSSSI.

An in vitro evaluation on activity of tedizolid and linezolid against Gram-positive pathogens isolated from patents in the study was described (Prokocimer et al. 2012).

The MIC_{50} and MIC_{90} of tedizolid against both MSSA and MRSA were 0.25 μg/mL, compared with MIC_{50} of 1 μg/mL and MIC_{90} of 2 μg/mL with linezolid. For coagulase-negative staphylococci (n = 7), viridans group streptococci (n = 15), and β-hemolytic streptococci (n = 3), MICs ranged from 0.03 to 0.25 μg/mL for tedizolid and 0.12–1 μg/mL for linezolid. Microbiological eradication rates at the test-of-cure visit (7–14 days posttreatment) showed overall eradication rates of 97.7 % for all pathogens, 97.9 % for MRSA, and 95.7 % for MSSA.

These studies confirm the important activity of tedizolid against pathogenic Gram-positive cocci, including MRSA, and 4-fold greater potency compared with linezolid.

Positive results of a randomized, double-blind, multicenter Phase 3 study of oral tedizolid phosphate, 200 mg daily for 6 days versus oral linezolid 600 mg every 12 h for 10 days for the treatment of acute bacterial skin and skin structure infection (ABSSSI) were recently described (De Anda et al. 2012). According to the authors, tedizolid phosphate for 6 days is noninferior to linezolid for 10 days.

13.3 Synthetic Approaches to the Construction of Oxazolidin-2-One Ring

Reviews on the chemistry of oxazolidin-2-ones chemistry were first published by Dyen and Swern (1967) and by Ager et al. (1996) on the use of oxazolidin-2-ones as chiral auxiliaries. Different synthetic routes were extensively studied and followed by academic and industrial (i.e., DuPont and Upjohn) laboratories for preparing 1,3-oxazolidin-2-one nucleus and linezolid derivatives. A detailed analysis of the vast literature regarding the different synthetic strategies to prepare these molecules is out of the scope of this chapter, but readers can refer to reviews on the synthetic approaches to the construction of the cyclic carbamate nucleus and on the use of oxazolidin-2-ones as chiral auxiliaries as building blocks in the design and preparation of foldamers or as organocatalysts (Zappia et al. 2007b, c; Tomasini et al. 2011). By far, 1,2-aminoalcohols and *N*-alkoxycarbonyl aminoalcohols are the most used substrate for the preparation of oxazolidin-2-ones.

1,2-aminoalcohols are readily available by numerous routes (Bergmeier 2000) and can be converted into cyclic carbamates using different reagents and catalysts (Barco et al. 1999; Bratulescu 2007; Davies et al. 1997; Delle Monache et al. 2000; Knolker et al. 1995; Knolker and Braxmeier 1996; Lindsay and Pyne 2002; Lohray et al. 1999; Mellon et al. 1992; Petrini et al. 2002). Base- or acid-promoted cyclization is another general method to prepare the oxazolidin-2-one nucleus (Casado-Bellever et al. 2002; Knapp 1999; Langlois 1999; Larsen et al. 1990; Nugent 1998; Tai and Imperiali 1998). Such approach was developed at DuPont labs for the first asymmetric synthesis of oxazolidin-2-ones employed as the key step for preparing compound DUP 721 (Wang et al. 1989). Another interesting method to obtain oxazolidin-2-ones is the halocyclofunctionalization of allyl-carbamates, a reaction resulting very often in high regio and stereo control. This approach was utilized by the Upjohn researchers to prepare racemic oxazolidinone derivatives in their initial SAR studies (Grega et al. 1995). A significant improvement in this approach was reported by Zappia et al. (Delle Monache et al. 1997; Di Giovanni et al. 1997; Guindon et al. 1992; Misiti and Zappia 1990) on the use as substrates of chiral allylic carbamates derived from the corresponding α-aminoacids. Other examples of efficient intramolecular cyclizations were described by Nicolaou et al. (2000a, b). Functionalization reactions such as acylation, alkylation and arylation reactions were mainly studied in connection with the use of chiral oxazolidin-2-ones as chiral auxiliaries (Evans and Ellmann 1989; Shamszat and Crimmins 2012), but the N-arylation of oxazolidin-2-ones was investigated as an alternative way to prepare new linezolid analogs (Cacchi et al. 2001; Ghosh et al. 2003; Madar et al. 2001; Mallesham et al. 2003; Moràn-Ramallal et al. 2008; Yin and Buchwald 2002).

13.4 Naturally Occurring Biological Active Oxazolidin-2-Ones

Natural products bearing an oxazolidin-2-one moiety are quite rare and antibacterial activities have not been associated to these compounds. (-)-Cytoxazone was isolated in 1998 from a soil sample of *Streptomyces* sp. and assigned the structure (4*R*, 5*R*)-5-(hydroxymethyl)-4-(4-methoxy-phenyl)-2-oxazolidinone (Fig. 13.9). The compound showed a cytokine-modulating activity (Kakeya et al. 1998, 1999). Interestingly, the synthesis and evaluation of the biological activity of the four stereoisomers confirmed the dose-dependent inhibition of Interleukin (IL)-10 production by (-)-Cytoxazone relative to IL-2 in pokeweed mitogen-stimulated murine splenocytes, in addition to an irrelevant influence of the stereochemistry on the biological activity (Carter et al. 2003).

(+)-Streptazolin, a lipophilic neutral tricyclic compound, was first isolated in 1981 from a culture of *Streptomyces viridochromogenes* (Drautz et al. 1981) and later rediscovered by chemical screening of *Streptomyces luteogriseus* (Grabley et al. 1993) (Fig. 13.9). Notably, in spite of the low antimicrobial activity

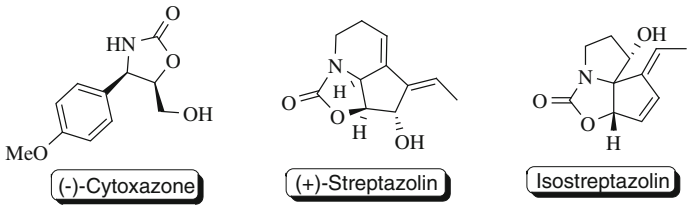


Fig. 13.9 Structures of natural products bearing an oxazolidin-2-one moiety

exhibited by streptazolin, some Diels–Alder adducts of the compound with naphthoquinones have been reported to possess antitumor activity comparable to that of adriamycin on leukemia L1210 cells (Grabley et al. 1987).

Biosynthetic studies on streptazolin were conducted by Mayer and Thiericke (1993) by feeding *Streptomyces* sp. (strain FH-S 2184) with sodium [^{13}C]acetate, sodium [^{13}C]formate, [^{13}C]urea, L-[methyl-^{13}C]methionine, [$^{15}N_2$]ammonium sulfate, and L-[^{15}N]glutamic acid. The unique tricyclic skeleton was shown to be produced via a mixed the polyketide pathway. Recently, a structurally analog of streptazolin, isostreptazolin (Fig. 13.9), was isolated by Zheng et al. (2012), from the culture broth of *Streptomyces sannanensis* and the cytotoxic effects were tested against human large-cell lung carcinoma cell line (H460) and human cervix carcinoma cell lina (HeLa). Isostreptazolin was inactive (100 mM, inhibition rate <50 %) against both cell lines.

13.5 Conclusions

Oxazolidinones represent a landmark in antimicrobial research being the first new class of antibiotics to enter clinical usage in 2000, 30 years after a new class of antibiotics had reached the marked. Linezolid is still a very active antibiotic and its value to address serious emerging resistance among Gram-positive cocci has been well documented. The size of the market success of Linezolid can be assessed by considering that the worldwide sales only for 2010 totaled $222,555,000.

However, the recent acquisition of a linezolid resistance mechanism based on chromosomal genes mutations and mediated by the *cfr* gene localized on transferable elements, indicates a potential to be disseminated among Gram-positive pathogenic strains.

Despite a great deal of efforts that have been made both from academia and industry to find new oxazolidin-2-one based compounds with improved potency, significant activity against linezolid-resistant strains, and improved safety profile, especially with regard to the elimination of the reversible myelosuppressive effects, as well as the MAO inhibition profile, only a few oxazolidinones are under active investigation. According to the current literature, the most successful potential next-generation oxazolidinone candidates in the clinic are tedizolid

(phase 3) and radezolid (phase 2). Within a global strategy to create a stable research infrastructure for antimicrobial development, the oxazolidin-2-one based antibacterial agents occupy a central position in order to bridge the gap in antibacterial development.

References

Ager DJ, Prakash I, Schaad DR (1996) 1,2-Amino alcohols and their heterocyclic derivatives as chiral auxiliaries in asymmetric synthesis. Chem Rev 96:835–875

Alffenaar JWC, van der Laan T, Simons S et al (2011) Susceptibility of clinical *Mycobacterium tuberculosis* isolates to a potentially less toxic derivate of linezolid, PNU-100480. Antimicrob Agents Chemother 55:1287–1289

Barbachyn MR, Brickner SJ, Ford CW et al (1996) Identification of a novel oxazolidinone (U-100480) with potent antimyco-bacterial activity. J Med Chem 39:680–685

Barbachyn MR, Ford CW (2003) Oxazolidinone structure-activity relationships leading to linezolid. Angew Chem Int Ed Engl 42:2010–2023

Barco A, Benetti S, Bergamini P et al (1999) Diastereoselective synthesis of β-amino-α-hydroxy phosphonates via oxazaborolidine catalyzed reduction of β-phthalimido-α-keto phosphonates. Tetrahedron Lett 40:7705–7708

Bergmeier SC (2000) The synthesis of amino alcohols. Tetrahedron 56:2561–2576

Bien P, Prokocimer P et al (2010) Absolute bioavailability of TR-701 FA and pharmaco-kinetics after single and multiple dose intravenous administration in healthy adult subjects, abstr. A1–013. In: 50th Inter-science Conference on Antimicrobial Agents and Chemotheraphy, Sept 12–15. Boston, MA

Bratulescu G (2007) An excellent procedure for the synthesis of oxazolidin-2-ones. Synthesis 20:3111–3112

Brickner SJ, Hutchinson DK, Barbachyn MR et al (1996) Synthesis and antibacterial activity of U-100592 and U-100766, two oxazolidi-none antibacterial agents for the potential treatment of multi-drug resistant Gram-positive bacterial infections. J Med Chem 39:673–679

Brickner S, Barbachyn MR, Hutchinson DK, Manninen PR (2008) Linezolid (ZYVOX), the first member of a completely new class of antibacterial agents for treatment of serious Gram-positive infections. J Med Chem 51:1982–1990

Cacchi S, Fabrizi G, Goggiamani A, Zappia G (2001) 3-Aryl-2-oxazolidinones through the palladium-catalyzed N-arylation of 2-oxazolidinones. Org Lett 3:2539–2541

Carter PH, LaPorte JR, Scherle PA, Decicco CP (2003) A new synthesis of cytoxazone and its diastereomers provides key initial SAR information. Bioorg Med Chem Lett 13:1237–1239

Casado-Bellever FJ, Gonzalez-Rosende ME et al (2002) Synthesis of chiral oxazolidin-2-ones from N-alkoxycarbonyl amino epoxides: a computational study. J Chem Soc 14:1650–1654

Cynamon MH et al (1999) Activities of several novel oxazolidinones against *Mycobacterium tuberculosis* in a murine model. Antimicrob Agents Chemother 43:1189–1191

Davies SG, Fenwick DR, Ichihara O (1997) The use of lithium (α-methylbenzyl)allylamide for the asymmetric synthesis of unsaturated β-amino acid derivatives. Tetrahedron: Asymmetry 8:3387–3391

De Anda C, Fang E et al (2012) Comparison of investigator assessed and programmatic clinical outcomes of tedizolid phosphate *vs* linezolid in a phase 3 study in patients with ABSSSI L1-1665. In: 52nd Inter-science Conference on Antimicrobial Agents and Chemotheraphy, Sept 9–12. San Francisco

Di Giovanni MC, Misiti D, Zappia G, Delle Monache G (1997) Chiral 4,5-disubstituted oxazolidin-2-ones: stereoselective synthesis of beta-hydroxy alpha-amino acids. Gazz Chim Ital 127:475–487

Delle Monache G, Di Giovanni MC, Misiti D, Zappia G (1997) An enantioselective, stereodivergent synthesis of threonine analogs. Tetrahedron: Asymmetry 8:231–243
Delle Monache G, Misiti D, Salvatore P, Zappia G (2000) Synthesis of both enantiomers of 4-vinyl oxazolidin-2-one from a single precursor: D-isoascorbic acid. Chirality 12:143–148
Drautz H, Zahner H, Kupfer E, Keller-Schierlein W (1981) Stoffwechselprodukte von mikroorganismen 205. Mitteilung isolierung und struktur von streptazolin. Helv Chim Acta 64:1752–1765
Dryden MS (2011) Linezolid pharmacokinetics and pharmacodynamics in clinical treatment. J Antimicrob Chemother 66 (S4):7–15
Dyen MA, Swern D (1967) 2-Oxazolidinones. Chem Rev 67:197–246
Evans DA, Takacs JM, McGee LR, Ennis MD, Mathre DJ, Bartroli J (1981) Chiral enolate design. PureAppl Chem 53:1109–1127
Evans DA, Ellman JA (1989) The total syntheses of the isodityrosine-derived cyclic tripeptides OF4949-I11 and K-13. Determination of the absolute configuration of K-13. J Am Chem Soc 111:1063–1072
Falagas ME, Bliziotis IA (2007) Pandrug resistant Gram-negative bacteria: the dawn of the post-antibiotic era? Int J Antimicrob Agents 29:630–636
French G (2003) Safety and tolerability of linezolid. J Antimicrob Chemother 51 (S2):45–53
Ghosh A, Sieser JE, Riou M, Cai W, Rivera-Ruiz L (2003) Palladium-catalyzed synthesis of N-aryloxazolidinones from aryl chlorides. Org Lett 5:2207–2210
Gonzales RD et al (2001) Infections due to vancomycin resistant *Enterococcus faecium* resistant to linezolid. Lancet 357:1179
Gorchynski J, Rose JK (2008) Complications of MRSA treatment: linezolid-induced myelosuppression presenting with pancytopenia. West J Emerg Med 9:177–178
Grabley S, Hammann P, Kluge H, Wink J, Kricke P (1993) Secondary metabolites by chemical screening, 21. Clonostachydiol, a novel anthelmintic macrodiolide from the fungus *Clonostachys cylindrospora* (strain FH-A 6607). J Antibiot (Tokyo) 58:343–345
Grabley S, Kluge H, Hoppe H-U (1987) New lead structures by Diels-Alder reactions of streptazolin with naphthoquinones. Angew Chem Int Ed Engl 26:664–665
Grega KC, Basbachyn MR, Brickner SJ, Mizsak SA (1995) Regioselective metalation of fluoroanilines. An application to the aynthesis of fluorinated oxazolidinone antibacterial agents. J Org Chem 60:5255–5261
Gregory WA (1984) *p*-oxooxazolidinylbenzene compounds as antibacterial agents. U.S. Patent 4461773
Grunder G, Zysset-Aschmann Y, Vollenweider F et al (2006) Lack of pharmacokinetic interaction between linezolid and antacid in healthy volunteers. Antimicrob Agents Chemother 50:68–72
Guindon Y, Slassi A, Ghiro E, Bantle G, Yung G (1992) Stereoselective silver triflate-mediated iodocyclization of carbamates. Tetrahedron Lett 33:4257–4260
Humphrey SJ et al (2001) Cardiovascular sympathomimetic amine interactions in rats treated with monoamine oxidase inhibitors and the novel oxazolidinone antibiotic linezolid. J Cardiovasc Pharmacol 37:548–563
Im WB et al (2011) Discovery of torezolid as a novel 5-hydroxymethyl-oxazolidinone antibacterial agent. Eur J Med Chem 46:1027–1039
Ippolito JA et al (2008) Crystal structure of the oxazolidinone antibiotic linezolid bound to the 50S ribosomal subunit. J Med Chem 51:3353–3356
Kakeya H, Morishita M, Koshino H, Morita T, Kobayashi K, Osada H (1999) Cytoxazone: a novel cytokine modulator containing a 2-oxazolidinone ring produced by *Streptomyces* sp. J Org Chem 64:1052–1053
Kakeya H, Morishita M, Kobinata K, OsonoM Ishizuka M, Osada H (1998) Isolation and biological activity of a novel cytokine modulator, cytoxazone. J Antibiot 51:1126
Kehrenberg C, Aarestrup FM, Schwarz S (2007) IS21-558 insertion sequences are involved in the mobility of the multiresistance gene *cfr*. Antimicrob Agents Chemother 51:483–487

Kehrenberg C, Schwarz S et al (2005) A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Mol Microbiol 57:1064–1073

Knapp S (1999) The tethered nitrogen in natural products synthesis. Chem Soc Rev 28:61–72

Knolker H-J, Braxmeier T (1996) Isocyanates - Part 3. Synthesis of carbamates by DMAP-catalyzed reaction of amines with di-*tert*butyldicarbonate and alcohols. Tetrahedron Lett 37:5861–5864

Knolker H-J, Braxmeier T, Schlechtingen G (1995) A novel method for the synthesis of isocyanates under mild conditions. Angew Chem Int Ed Engl 34:2497–2500

Langlois N, Moro A (1999) Regio- and stereoselective opening of oxiranes through neighbouring group participation: stereocontrolled synthesis of enantiopure hydroxylated oxazolidin-2-ones. Eur J Org Chem 3483–3488

Larsen RD, Davis EG, Corley PJ, Reider TR, Grabowski EJ (1990) Synthesis of (5R,10S,11R)-(+)-10,11-dihydro-5-methyl-5H-dibenzo[a, d]cyclohepten-5,10-imin-11-ol: a hydroxylated metabolite of MK-0801. J Org Chem 55:299–304

Lawrence L, Danese P, DeVito J et al (2008) In vitro activities of the Rx-01 oxazolidinones against hospital and community pathogens. Antimicrob Agents Chemother 52:1653–1662

Leach KL et al (2007) The site of action of oxazolidinone antibiotics in living bacteria and in human mitochondria. Mol Cell 26:393–402

Lindsay KB, Pyne SG (2002) Asymmetric synthesis of (−)-swainsonine, (+)-1,2-di-epi-swainsonine, and (+)-1,2,8-tri-epi-swainsonine. J Org Chem 67:7774–7780

Livermore DM et al (2009) Activity of oxazolidinone TR-700 against linezolid-susceptible and -resistant staphylococci and enterococci. J Antimicrob Chemother 63:713–715

Locke JB et al (2010) Structure-activity relationships of diverse oxazolidinones for linezolid-resistant *Staphylococcus aureus* strains possessing the *cfr* methyltransfe-rase gene or ribosomal mutations. Antimicrob Agents Chemother 54:5337–5343

Lohray B, Baskaran S, Rao BS, Reddy Y, Rao N (1999) A short synthesis of oxazolidinone derivatives linezolid and eperezolid: a new class of antibacterials. Tetrahedron Lett 40:4855–4856

Long KS et al (2010) Mutations in 23S rRNA at the peptidyl transferase center and their relationship to linezolid binding and cross-resistance. Antimicrob Agents Chemother 54:4705–4713

Long KS, Vester B (2012) Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob Agents Chemother 56:603–612

Luo X et al (2005) In vivo efficacy of designer oxazolidinones in murine infectious models, abstr. F-1260. In: Abstr 45th Interscience Conference on Antimicrobial Agents and Chemotheraphy, Washington, DC, 16–19 Dec

Madar DJ, Kopecka H et al (2001) Synthesis of *N*-arylated oxazolidinones via a palladium catalyzed cross coupling reaction. Application to the synthesis of the antibacterial agent Dup-721. Tetrahedron Lett 42:3681

Mallesham B, Rajesh BM et al (2003) Highly efficient cui-catalyzed coupling of aryl bromides with oxazoli-dinones using Buchwald's protocol: a short route to linezolid and toloxatone. Org Lett 5:963–965

MacGowan A (2003) Pharmacokinetic and pharmacodynamic profile of linezolid in healthy volunteers and patients with Gram-positive infections. J Antimicrob Chemother 51 (S2):17–25

Mayer M, Thiericke R (1993) Biosynthesis of streptazolin. J Org Chem 58:3486–3489

Mellon D, Gravier-Pellettier C, Le Merrer Y, Depezay JC (1992) Enantiomerically pure 3-amino-2-hydroxy and 5-amino-4-hydroxy acids from D-isoascorbic acid. Bull Soc Chim Fr 129:585–593

Mellon D, Gravier- Matsunaga S, Kumagai N, Harada S, Shibasaki M (2003) Anti-selective direct catalytic asymmetric Mannich-type reaction of hydroxyketone providing beta-amino alcohols. J Am Chem Soc 125:4712–4713

Misiti D, Zappia G (1990) A stereoselective synthesis of (3S,4S) statine and related compounds. Tetrahedron Lett 31:7359–7362

Moore C, Dedhia H, Duffy E (2012) Radezolid demonstrates favorable safety compared to linezolid in a three-month rat toxicology study. In: Abstract 52nd Interscience Conference on Antimicrobial Agents and Chemotheraphy, San Francisco, 9–12 Sept

Morales G, Picazo E, Candel FJ (2010) Resistance to linezolid is mediated by the *cfr* gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. Clin Infec Dis 50:821–825

Moràn-Ramallal R, Liz R, Gotor V (2008) Regioselective and stereospecific synthesis of enantiopure 1,3-oxazolidin-2-ones by intramolecular ring opening of 2-(Boc-aminomethyl)-aziri-dines. Preparation of the antibiotic linezolid. Org Lett 10:1935–1938

Nian H, Chu Y, Tian S, Sun G, Ding L, Guo L, Shang H (2012) First report of clinical linezolid-resistant *Staphylococcus cohnii* mediated by the *cfr* gene. African J Microb Res 6:5043

Nicolaou KC, Baran PS, Zhong YL (2000a) Novel IBX-mediated processes for the synthesis of amino sugars and libraries. Thereof Angew Chem Int Ed 39:2525–2529

Nicolaou KC, Zhong YL, Baran PS (2000b) New synthetic technology for the rapid construction of novel heterocycles-part 2. the reaction of IBX with anilides and related compounds. Angew Chem Int Ed 39:625–628

Nugent WA (1998) Desymmetrization of meso epoxides with halides: a new catalytic reaction based on mechanistic insight. J Am Chem Soc 120:7139–7140

Pandit N, Singla RK, Shrivastava B (2012) Current updates on oxazolidinone and its significance. Int J Med Chem 1–24

Petrini M, Profeta R, Righi P (2002) Reaction of allylzinc reagents and zinc enolates of ketones with α-amidoalkylphenyl sulfones. J Org Chem 67:4530–4535

Pfizer(2010) Zyvox (R)(linezolid) package insert. http://www.pfizer.com/files/products/uspi_zyvox.pdf

Poce G, Zappia G, Porretta GC, Botta B, Biava M (2008) New oxazolidinone derivatives as antibacterial agents with improved activity. Expert Opin Ther Patents 18:97–121

Prokocimer P, Bien P, Surber J et al (2011) 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 infection. Antimicrob Agents Chemother 55:583–592

Prokocimer PP, Bien P, De Anda C, Pillar CM, Bartizal K (2012) In vitro activity and microbiological efficacy of tedizolid (TR-700) against Gram-positive clinical isolates from a phase 2 study of oral tedizolid phosphate (TR-701) in patients with complicated skin and skin structure infections. Antimicrob Agents Chemother 56:4608–4613

Prystowsky J, Siddiqui F, Chosay J, Shinabarger DL, Millichap J, Peterson LR, Noskin GA (2001) Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. Antimicrob Agents Chemother 45:2154–2156

Rogers GS, Belloff GB, Paul MF, Yurchenco JA, Gever G (1956) Furazolidone: a new antimicrobial nitrofuran-A review of laboratory and clinical data. Antibiot Chemother 6:231–241

Schaadt R, Sweeney D, Shinabarger D, Zurenko G (2009) In vitro activity of TR-700, the active ingredient of the antibacterial prodrug TR-701, a novel oxazolidinone antibacterial agent. Antimicrob Agents Chemother 53:3236–3239

Schwarz S, Werckenthin C, Kehrenberg C (2000) Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. Antimicrob Agents Chemother 44:2530–2533

Shamszat M, Crimmins MT (2012) Amino acid derived heterocyclic chiral auxiliaries: the use of oxazolidinones, oxazolidinethiones, thiazolidinethiones, and imidazolidinones. In: Carreira EM, Yamamoto H (eds) Comprehensive Chirality, vol 3: Synthetic Methods II–Chiral Auxiliaries, Elsevier Science, Philadelphia

Shaw KJ, Poppe S, Schaadt D et al (2008) In vitro activity of TR-700, the antibacterial moiety of the prodrug TR-701, against linezolid-resistant strains. Antimicrob Agents Chemother 52:4442–4447

Shaw KJ, Barbachyn MR (2011) The oxazolidinones: past, present, and future. Ann N Y Acad Sci 1241:48–70

Slatter JG, Stalker DJ, Feenstra KL et al (2001) Pharmacokinetics, metabolism, and excretion of linezolid following an oral dose of [^{14}C]linezolid to healthy human subjects. Drug Metab Dispos 29:1136–1145

Slee AM, Wuonola MA, McRipley RJ, Zajac I, Zawada MJ, Bartholomew PT, Gregory WA, Forbes M (1987) Oxazolidinones, a new class of synthetic antibacterial agents: in vitro and in vivo activities of DuP 105 and DuP 721. Antimicrob Agents Chemother 31:1791–1797

Sisson TL, Jungbluth GL, Hopkins NK (1999) A pharmacokinetic evaluation of concomitant administration of linezolid and aztreonam. J Clin Pharmacol 39:1277–1282

Stalker DJ, Jungbluth GL (2003) Clinical pharmacokinetics of linezolid, a novel oxazolidinone antibacterial. Clin Pharmacokinet 42:1129–1140

Swaney SM et al (1998) Oxazolidinone resistance is associated with a mutation in the peptidyl transferase region of 23S rRNA. In: Abstract 38th Interscience Conference on Antimicrobial Agents and Chemotheraphy, San Diego, 24–27 Sept

Tai VW-F, Imperiali B (1998) Model study for the incorporation of the (syn, anti)-2-amino-1,3-diol functionality in carbocycles. Tetrahedron Lett 39:7215–7218

Toh SM, Xiong L, Arias CA et al (2007) Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol 64:1506–1514

Tomasini C, Angelici G, Castellucci N (2011) Foldamers based on oxazolidin-2-ones. Eur J Org Chem 3648–3669

Tsiodras S et al (2001) Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. Lancet 38:207–208

Wallis RS, Jakubiec W, Kumar V, Bedarida G, Silvia A, Paige D, Zhu T, Mitton-Fry M, Ladutko L, Campbell S, Miller PF (2010) Biomarker-assisted dose selection for safety and efficacy in early deve-lopment of PNU-100480 for tuberculosis. Antimicrob Agents Chemother 55:567–574

Wang CJ, Gregory WA, Wuonola MA (1989) Chiral synthesis of dup 721, a new antibacterial agent. Tetrahedron 45:1323–1326

Welshman IR, Stalker DJ, Wajsczuk CP (1998) Assessment of absolute bioavailability and evaluation of the effect of food on oral bioavailability of linezolid. Anti-Infect Drugs Chemother 16:54

Wilson DN, Schluenzen F, Harms JM, Starosta AL, Connell SR, Fucini P (2008) The oxazolidinone antibiotics perturb the ribosomal peptidyl-transferase center and effect tRNA positioning. Proc Natl Acad Sci U S A 105:13339–13344

Williams KN, Brickner SJ, Stover CK, Zhu T, Ogden A, Tasneen R, Tyagi S, Grosset JH, Nuermberger EL (2009a) Addition of PNU-100480 to first-line drugs shortens the time needed to cure murine tuberculosis. Am J Respir Crit Care Med 180:371–376

Williams KN, Stover CK, Zhu T, Tasneen R, Tyagi S, Grosset JH, Nuermberger E (2009b) Promising antituberculosis activity of the oxazolidinone PNU-100480 relative to that of linezolid in a murine model. Antimicrob Agents Chemother 53:1314–1319

Wong A, Reddy SP, Smyth DS, Aguero-Rosenfeld ME, Sakoulas G, Robinson DA (2010) Polyphyletic emergence of linezolid resistant staphylococci in the United States. Antimicrob Agents Chemother 54:742–748

Yin J, Buchwald SL (2002) The pd-catalyzed intermolecular amidation of aryl halides: the discovery that xantphos can be *trans*-chelating in a palladium complex. J Am Chem Soc 124:6043–6048

Zappia G, Menendez P, Delle Monache G, Misiti D, Nevola L, Botta B (2007a) The contribution of oxazolidinone frame to the biological activity of pharmaceutical drugs and natural products. Med Chem 7:389–409

Zappia G, Cancelliere G, Gacs-Baitz E, Delle Monache G, Misiti D, Nevola L, Botta B (2007b) Oxazolidin-2-one ring, a popular framework in synthetic organic chemistry. Applications and modifications. Curr Org Synth 4:238–307

Zappia G, Gacs-Baitz E, Delle Monache G, Misiti D, Nevola L, Botta B (2007c) Oxazolidin-2-one ring, a popular framework in synthetic organic chemistry. The construction of the oxazolidin-2-one ring. Curr Org Synth 4:81–135

Zheng D, Han L, Li Y, Li J, Rong H, Leng Q, Zhao L, Huang X (2012) Isostreptazolin and Sannaphenol, two new metabolites from *Streptomyces sannanensis*. Molecules 17:836–842

Zurenko GE, Yagi BH, Schaadt RD, Allison JV et al (1996) In vitro activities of U-100592 and U-100766, novel oxazolidinone antibacterial agents. Antimicrob Agents Chemother 40:839–845

Zurenko GE, Ford CW, Hutchinson DK, Brickner SJ, Barbachyn MR (1997) Oxazolidinone antibacterial agents: development of the clinical candidates eperezolid and linezolid. Expert Opin Investig Drugs 6:151–158

Zurenko GE et al (1999) Development of linezolid resistant *Enterococcus faecium* in two compassionate use program patients treated with linezolid. In: Abstract 39th Interscience Conference on Antimicrobial Agents and Chemotheraphy, San Francisco, CA, 17–20 Sept

Chapter 14
Protein Synthesis Inhibitors from Smaller Antibiotic Classes

Herbert A. Kirst

Abstract Inhibition of microbial protein synthesis is one of the most commonly used and important mechanisms of action for antibiotics. In addition to the four larger antibiotic classes (aminoglycosides, tetracyclines, oxazolidinones and macrolides) that operate by this mechanism, several smaller antibiotic classes also employ it. This review covers key members and important developments in the lincosaminide, streptogramin, phenicol, pleuromutilin, fusidane, pseudomonic acid, and thiopeptide classes of fermentation-derived antibiotics. Retapamulin is the most recently approved antibiotic from this group while some other members of these seven classes are currently in the antibiotic development pipeline. In addition, many other smaller classes of previously discovered antibiotics remain under-explored and under-utilized, which, if further examined, could represent additional opportunities and starting materials for creating new scaffolds for novel antibiotics.

14.1 Introduction

The steadily rising levels of microbial resistance to antibiotics have been thoroughly publicized for over two decades and accompanied by many calls for action (Boucher et al. 2009; Bush et al. 2011; Fong and Drlica 2008; Freire-Moran et al. 2011; IDSA 2010; Pulcini et al. 2012). In one response to such calls, the current antibiotic pipeline of new agents at some stage of clinical development is slowly being filled with representatives from many different antibiotic classes (Bush and Pucci 2011; Butler and Cooper 2011; Coates et al. 2011; Donadio et al. 2010; Kirst 2010a, 2012). Inhibition of protein biosynthesis in bacteria is one of the most

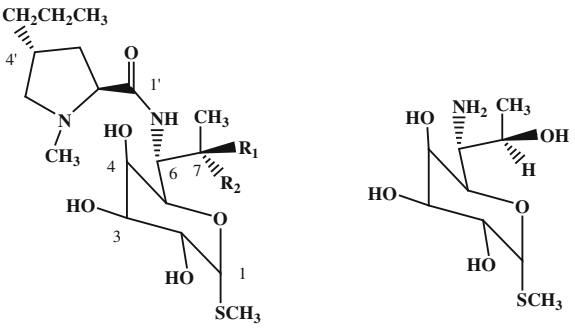
H. A. Kirst (✉)
7840 West 88th Street, Indianapolis, IN 46278, USA
e-mail: herbertkirst1@comcast.net

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_14,

utilized mechanisms of action (MOA) for eliciting antibacterial activity (Lange et al. 2007). The ribosome is the target site of action for microbial protein biosynthesis which different antibiotics disrupt in many diverse ways (Blanchard et al. 2010; Hermann 2005; McCoy et al. 2011; Yonath 2005). The importance of this MOA is confirmed by four other chapters of this book (see Kirst and Marinelli, Kirst, Genilloud and Vincente, Zappia et al. this volume) that are each devoted to one of the large antibiotic classes of protein synthesis inhibitors, namely, aminoglycosides, tetracyclines, macrolides, and oxazolidinones. Complementing those four reviews, this chapter summarizes several smaller classes of other antibiotics that also exert their antimicrobial activity by inhibition of protein synthesis.

14.2 Lincosaminides

Lincomycin is an older antibiotic produced from fermentation of *Streptomyces lincolnensis* (Mason et al. 1963). It is a relatively small molecule whose structure is composed of an uncommon amino acid attached via an amide bond to a unique amino-octose named lincosamine (Fig. 14.1) (Mitscher 2010). Based on the latter name, this antibiotic class is known as lincosaminides (or lincosamides). Clindamycin is a more potent semi-synthetic derivative of lincomycin in which chlorine has replaced the 7-hydroxyl group (Fig. 14.1) (Lewis 1974). Clindamycin has generally superseded lincomycin in clinical use due to features such as higher potency and better oral absorption. Clindamycin is used to treat infections caused by Gram-positive bacteria, many anaerobes, and certain other pathogens (Guay 2007; Sivapalasingam and Steigbigel 2010). It became particularly useful upon clinical introduction due to its greater activity against the Gram-negative anaerobe, *Bacteroides fragilis*. However, it was further noted that the opportunistic anaerobe *Clostridium difficile* could proliferate and cause a dangerous condition known as pseudomembranous colitis if the beneficial gastrointestinal bacteria became too



Lincomycin: R_1 = OH, R_2 = H
Clindamycin: R_1 = H, R_2 = Cl

Lincosamine

Fig. 14.1 Structures of lincomycin, clindamycin, and lincosamine

severely depleted or overrun due to antibiotic activity against them in the gastrointestinal tract (Bartlett 2008). Another important clinical use of clindamycin is to treat infections caused by Gram-positive bacteria including community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) (Dryden 2010; Guay 2007). Current levels of clindamycin resistance appear to be relatively low, but as for all antibiotics, resistance is becoming more prevalent (Frei et al. 2010; Kluka 2011; Marcinak and Frank 2006). Hospital-acquired MRSA strains are more resistant (Dryden 2010).

Clindamycin binding occurs at the peptide exit tunnel and extends to the peptidyl transferase center (PTC) of the ribosome (McCoy et al. 2011; Tenson et al. 2003). As discussed in the preceding chapter for macrolides (see Kirst, this volume), the binding site for lincosaminides in the exit tunnel region overlaps the binding sites for macrolide and streptogramin B antibiotics. As a consequence, either *erm* gene-governed N-methylation of an adenine residue or nucleotide point mutations at this site can reduce antibiotic binding for members of all three of these structurally-unrelated classes. This mutual reduction in binding results in cross-resistance between the macrolide, lincosaminide, and streptogramin B (MLS_B resistance) antibiotics (Leclercq and Courvalin 1991; Roberts 2011; Weisblum 1995). At the PTC, clindamycin inhibits transpeptidation where its binding site overlaps and competes with chloramphenicol and tiamulin. X-ray studies have been published illustrating structures for co-crystals of clindamycin bound in the large ribosomal subunits from *Deinococcus radiodurans*, *Haloarcula marismortu*, and *Escherichia coli* (Dunkle et al. 2010; Schlunzen et al. 2001; Tu et al. 2005). Analysis of these complexes identified differences in binding between the three of them, although the latter two were more consistent with each other at several positions. These differences emphasized the need for caution in assuming that ribosomes from different species are so conserved that they always bind antibiotics in the same arrangements (Kannan and Mankin 2011; Wilson 2011).

Other mechanisms of resistance to lincosaminides are efflux systems and enzymatic inactivation, but they are less common than target site modifications. Efflux systems transport unaltered antibiotic out of the cell to counteract its uptake, maintain low intracellular concentrations, and keep it from the ribosome. Lincosaminide efflux is controlled by *lmr*, *car*, and *lsa* genes (Roberts 1999, 2008, 2011). Enzymatic inactivation occurs by O-adenylylation that is coded by *lnu* (formerly *lin*) genes (Morar et al. 2009). Adenylylation occurs at the 4-hydroxyl group in *S. aureus* and the 3-hydroxyl group in *Enterococcus faecium* for clindamycin, but at the 3-hydroxyl group of lincomycin in both bacteria (Mitscher 2010).

Pirlimycin is an old member of this class that is used for veterinary applications such as treatment of bovine mastitis (Saini et al. 2011). Mirincamycin is an old pair of *cis/trans* isomers that has more recently been further tested for anti-malarial activity (Khemawoot et al. 2011). However, only a few efforts to synthesize new derivatives in this class have been recently reported (Kirst 2010b).

14.3 Streptogramins

The streptogramins are an old antibiotic class originally obtained from fermentation of *Streptomyces graminofaciens* (Charney et al. 1953). They have been re-isolated on several later occasions and given different names at those times, such as pristinamycins from *Streptomyces pristinaespiralis*, virginiamycins from *Streptomyces virginiae*, and others (Johnston et al. 2002). The class is composed of two separate and structurally unrelated series (Fig. 14.2). Compounds in the streptogramin A series have a 23-membered mixed polyketide and amino acid macrocyclic core whereas compounds in the streptogramin B series are macrocyclic depsipeptides (Kirst 2010b; Mitscher 2010). Each series by itself possesses insufficient potency and is only bacteriostatic, but a combination of the A and B series has a dual target and exhibits synergistic bactericidal activity (Canu and Leclercq 2001).

The first product, Pyostacine®, was a fixed combination containing a 7:3 ratio of streptogramin A:B (Bacque et al. 2005). Extensive SAR studies were conducted over many years especially to improve water solubility and efficacy upon injection. These efforts resulted in the creation and development of a 7:3 combination by weight of dalfopristin:quinopristin named Synercid® (Fig. 14.2). It is used for

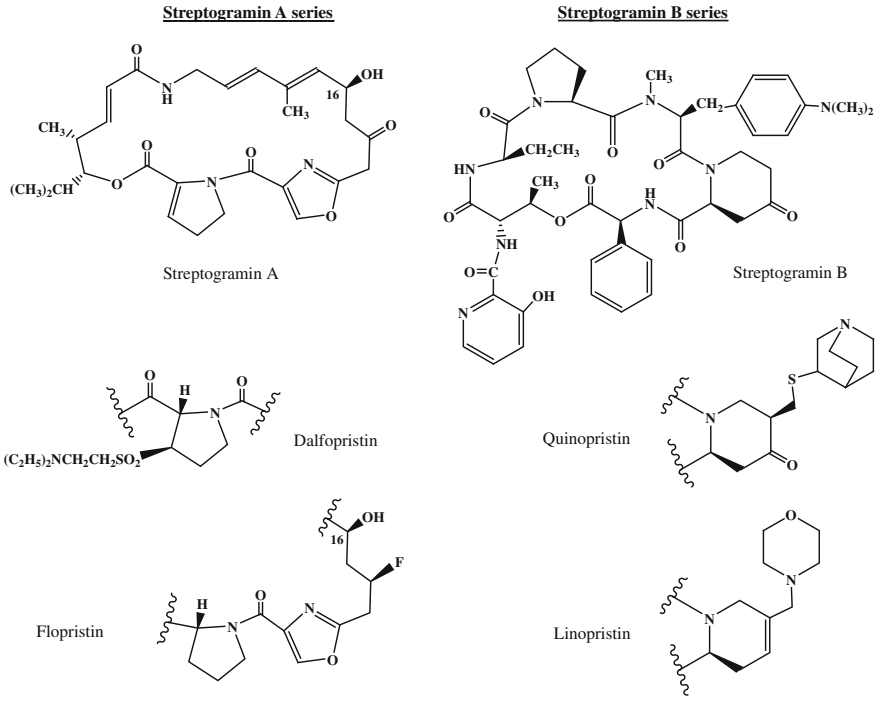


Fig. 14.2 Structures of streptogramins and derivatives

parenteral treatment of multidrug-resistant Gram-positive bacterial infections, including vancomycin-resistant *E. faecium* (Manfredi 2005; Nailor and Sobel 2009). Upon approval in the U.S. in 1999, it became the first approved new antibiotic to have activity against vancomycin-resistant Gram-positive bacteria, but newer antibiotics have now generally replaced it (Anstead and Owens 2004; Dang et al. 2007). Further SAR studies that were focused on oral administration have subsequently yielded another combination containing 70 % flopristin and 30 % linopristin (Fig. 14.2) (Bacque et al. 2005). This latter orally bioavailable combination coded NXL-103 (formerly XRP2868) has been undergoing Phase II clinical trials (Devasahayam et al. 2010; Politano and Sawyer 2010).

As previously mentioned, streptogramin B is the third component in the MLS_B resistance phenotype that occurs due to overlapping binding sites of MLS_B antibiotics around the peptide exit tunnel of the ribosome (Canu and Leclercq 2001; Leclercq and Courvalin 1991; Tenson et al. 2003; Weisblum 1995). The A series binds to a site at the PTC that changes ribosome conformation in a manner which strengthens binding of the B series. As a result, binding of both streptogramin components produces a synergistic irreversible bactericidal effect due to greater stabilization of binding relative to each component alone (Hermann 2005; Yonath 2005). X-ray crystallographic analyses have been reported for complexes between ribosomes from *H. marismortui* and either streptogramin B (Tu et al. 2005) or streptogramin A (Hansen et al. 2003) and for ribosomes from *D. radiodurans* complexed with both Synercid components (Harms et al. 2004).

In addition to ribosomal modifications that reduce antibiotic binding, clinically important resistance to streptogramins arises from enzymatic inactivation of the antibiotics (Mlynarczyk et al. 2010). Inactivation of the B series by *vgb* gene-coded lyases produces a linear peptide in which the macrocycle is cleaved at the ester linkage via a beta-elimination reaction involving dehydration of the threonine subunit to a 2-amino-2-butenoic acid residue (Korczynska et al. 2007). The A series is inactivated by *vat* genes coding for acetylation of the 16-hydroxyl group. Efflux utilizes ATP-binding transporters coded by *msr* genes for the B series and *vga* genes for the A series (Roberts 1999).

14.4 Phenicols

Chloramphenicol is another old antibiotic that was initially obtained as a fermentation product from *Streptomyces venezuelae* (Ehrlich et al. 1947). However, its relatively small and uncomplicated structure lends it to efficient manufacturing by total synthesis, which long ago superseded fermentation as the product source (Yang et al. 2009). The only active antimicrobial isomer of the four possibilities is the one that possesses *2R, 3R* stereochemistry (Fig. 14.3) (Loncaric and Wulff 2001). Clinical use is limited by toxicity, especially a relatively low incidence of aplastic anemia but which is unpredictable, untreatable, and generally fatal (Maviglia et al. 2009). However, chloramphenicol is valuable for treating typhoid

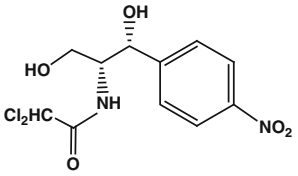


Fig. 14.3 Structure of chloramphenicol

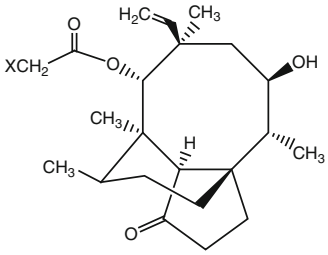
fever or other serious infections for which less toxic alternatives are not available. Due to the absence of many new Gram-negative antibiotics in development, chloramphenicol is one of several older antibiotics being reconsidered for possible uses (Falagas et al. 2008; Maviglia et al. 2009). Florphenicol and thiamphenicol are analogs that are used to treat respiratory infections in veterinary medicine (Schwarz and Kehrenberg 2006).

The MOA for chloramphenicol is inhibition of ribosomal protein synthesis at the PTC by interfering with binding of tRNA at the A site (McCoy et al. 2011). X-ray studies of chloramphenicol bound to large ribosomal subunits from three different species identified a different binding site in *H. marismortui* compared to the binding site in *D. radiodurans* (Dunkle et al. 2010; Hansen et al. 2003; Schlunzen et al. 2001). The results observed from *E. coli* were more consistent with those from *H. marismortui*. However, both binding sites may be relevant targets for chloramphenicol since each site overlaps sites used by other antibiotics to which cross-resistance has been observed (McCoy et al. 2011; Yonath 2005).

The most significant mechanism of clinical resistance is inactivation by chloramphenicol acetyltransferase (CAT) that acetylates the primary hydroxyl group (Schwarz et al. 2004). CAT does not confer resistance to thiamphenicol or florphenicol. Efflux of chloramphenicol only is coded by the *cml* gene, while efflux via the *flo, cfr,* and *fex* genes produce resistance to all members of this class (Doublet et al. 2005; White et al. 2000).

14.5 Pleuromutilins

The parent compound of this class is pleuromutilin, a tricyclic diterpenoid antibiotic (Fig. 14.4) that was isolated from culture broths of *Pleurotis* species (now reclassified as the genus *Clitopilus*) (Hartley et al. 2009; Kavanagh et al. 1951). Although clinical applications of pleuromutilin and a semisynthetic derivative, azamulin, were explored, neither compound became a successful product. However, due to the unique structure and MOA, SAR studies of this class were continued. Those efforts produced two useful semi-synthetic derivatives, first with tiamulin and later with valnemulin (Fig. 14.4). Both derivatives became important commercial products in the veterinary field for treatment and control of infectious diseases in swine and poultry (Hu and Zou 2009; Novak and Shlaes 2010; Tang et al. 2012).



Pleuromutilin: X = OH
Tiamulin: X = $SCH_2CH_2N(CH_2CH_3)_2$
Valnemulin: X = $S\text{-}C(CH_3)_2\text{-}CH_2\text{-}NH\text{-}CO\text{-}CH(NH_2)\text{-}CH(CH_3)_2$

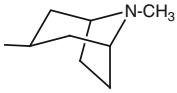
Retapamulin: X = S 

Fig. 14.4 Structures of pleuromutilin and derivatives

Early efforts to develop commercial products for human medicine in the pleuromutilin class were generally unsuccessful due to problems such as unsatisfactory pharmacokinetics, short half-life, and too rapid metabolism. However, a later semi-synthetic derivative, retapamulin (Fig. 14.4), has recently received U.S. regulatory approval for the topical treatment of certain human skin infections (Jacobs 2010; Weinberg and Tyring 2010). This approval is especially noteworthy for being the second natural product-originated antibiotic to be recently approved for human use (after daptomycin, see Baltz, this volume) and the first representative coming from a class that has been previously unrepresented in human medicine (pleuromutilins). Additional semi-synthetic derivatives are being evaluated in clinical trials, which may result in additional compounds from this class becoming useful antibiotics in human medicine (Butler and Cooper 2011; Donadio et al. 2010; Kirst 2012).

In spite of six decades between the discovery of pleuromutilin and the approval of retapamulin, interest in this class has remained high due to the unique structure and good spectrum of activity against Gram-positive bacteria. Very little cross-resistance has been reported thus far between the mutilins and other antibiotic classes, and in vitro studies have shown a low potential for development of resistance (Hu and Zou 2009; Tang et al. 2012). Its MOA has been well-established as an inhibitor of protein synthesis (Poulsen et al. 2001). Pleuromutilin binds at the PTC where it inhibits the formation of peptide bonds. Its binding site overlaps those of clindamycin, chloramphenicol, and the disaccharide substituent of carbomycin (McCoy et al. 2011). X-ray crystallographic studies of tiamulin complexed with the large ribosomal subunits from both *H. marismortui* and *D. radiodurans* have identified the antibiotic binding site (Gurel et al. 2009; Schlunzen et al. 2004). In one report, resistance to tiamulin in *E. coli* was caused by a point mutation in ribosomal protein L3 that hindered antibiotic binding (Bøsling et al. 2003).

14.6 Fusidanes

Fusidic acid is an old steroidal antibiotic that was obtained from fermentation of *Fusidium coccineum* (Godtfredsen et al. 1962). Its structure consists of a tetracyclic triterpenoid scaffold whose perimeter is arrayed with a variety of substituents, including a doubly unsaturated carboxylic acid moiety attached to C-17 in the D-ring (Fig. 14.5) (Godtfredsen et al. 1965; Cooper 1966). Fusidic acid is the only developed member of this small group of antibiotics known as fusidanes, which includes helvolic acid, helvolinic acid, and cephalosporin P1. Fusidic acid is structurally unrelated to other antibiotics that are used in human or veterinary medicine. It is currently used principally as a topical antibiotic against Gram-positive bacteria, especially staphylococci.

Given the dearth of new antibiotics, fusidic acid is one of several older antibiotics that have been targeted for potentially increased use and importance (Maviglia et al. 2009; Pulcini et al. 2012). It has been noted for its good Gram-positive activity including MRSA, oral bioavailability, minimal toxicity, and low development of cross-resistance with other antibiotics (Schofer and Simonsen 2010). But despite these several favorable features, it has never been previously registered for the U.S. market. However, a novel loading dose regimen has been developed to improve clinical effects and to hinder resistance development (Cempra 2011). Fusidic acid has completed a Phase II clinical trial for ABSSSI and a Phase I trial for prosthetic joint infections to support registration in the U.S. (Cempra 2012; Corey et al. 2011; Sutcliffe 2011).

Fusidic acid possesses a unique antimicrobial MOA in which protein synthesis is inhibited through binding to a protein called elongation factor G (EF-G). EF-G is one component of the complex that performs the successive steps in the ribosomal elongation cycle in which the amide bonds of a growing protein are sequentially formed with the incoming amino acids (Agrawal et al. 2000). The system is driven by hydrolysis of GTP using the GTP-ase which is EF-G. Binding of fusidic acid to EF-G stabilizes the complex following GTP hydrolysis and hinders dissociation of EF-G from the ribosome, which interrupts the continuity of

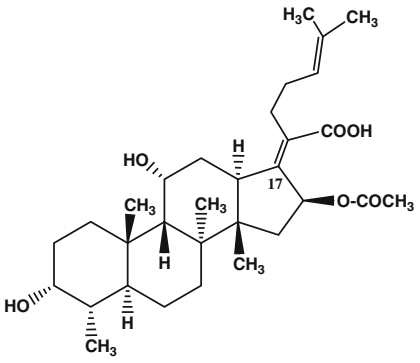


Fig. 14.5 Structure of fusidic acid

the elongation cycle and stalls further protein synthesis. A crystal structure of the complex has been published (Gao et al. 2009). Bacterial resistance to fusidic acid has been recently summarized (Farrell et al. 2011). The most common mechanism involves ways to reduce binding affinity and to protect EF-G from binding with fusidic acid, either by alterations of EF-G (*fusA*) or protein L6 (*fusE*) or by formation of protective proteins (*fusB, fusC, fusD*). Less common mechanisms are enzymatic inactivation and reduced uptake. Combinations with other antibiotics and limited duration of use have been used to help reduce development of resistance.

14.7 Pseudomonic Acids

The pseudomonic acids constitute a small antibiotic class produced from fermentation of the bacterium, *Pseudomonas fluorescens* (Fuller et al. 1971). Mupirocin is a mixture of pseudomonic acids in which pseudomonic acid A is the predominant component. Thus, mupirocin is often used synonymously with pseudomonic acid A (Hothersall et al. 2007). Its structure is a highly oxygenated polyketide-derived carboxylic acid (Fig. 14.6) (Chain and Mellows 1977). It is used as a topical antibiotic to treat a variety of skin and related infections caused by several Gram-positive bacteria and for decolonization of *S. aureus* from nasal passages (Coates et al. 2009). It exhibits bactericidal activity against Gram-positive bacteria that include MRSA and beta-lactamase producing strains.

Mupirocin is an inhibitor of protein synthesis that exerts its activity by a unique MOA (Thomas et al. 2010). It binds to bacterial isoleucyl-tRNA synthetase, which prevents binding between ATP and isoleucine and thus prevents the synthesis of isoleucyl-tRNA. Consequently, isoleucyl-tRNA is unavailable when needed for incorporation into growing proteins in the ribosome (Vondenhoff and Van Aerschot 2011). As anticipated for a unique MOA, cross-resistance to other antibiotics has not been demonstrated (Lee 2007). However, as eventually happens for all antibiotics, development of resistance to mupirocin has been slowly occurring (Thomas et al. 2010). Such resistance to mupirocin has been observed due to either alterations in the native isoleucyl-tRNA synthetase gene (*ileS*) that reduce binding of mupirocin or incorporation of another synthetase gene (*mupA*) (Patel et al. 2009).

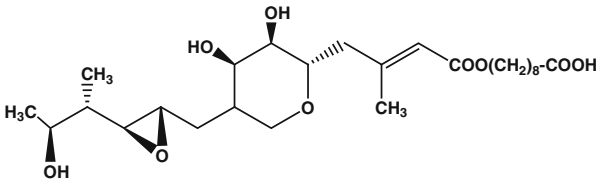


Fig. 14.6 Structure of pseudomonic acid A

Inhibition of aminoacyl-tRNA synthetases has been proposed as a novel MOA to use in the search for new antibiotics, but thus far the impact of this approach has been limited (Pohlmann and Brotz-Oesterhelt 2004).

14.8 Thiopeptides

The thiopeptide class is so-named due to its high sulfur content from the numerous sulfur-containing heterocycles within their highly modified peptidyl-based structures (Bagley et al. 2005; Hughes and Moody 2007). This class has been steadily expanding as new members continue to be isolated and identified. Many thiopeptides are high molecular weight compounds possessing very complex structures, although some smaller members are also known. One of the oldest and best-known members of this class is thiostrepton, which was initially isolated from the fermentation of *Streptomyces azureus* (Pagano et al. 1956). Its complex structure was not fully elucidated until 1983 (Fig. 14.7a) (Anderson et al. 1970; Hensens and Albers-Schonberg 1983). Total synthesis of thiostrepton was not achieved until 2004 (Nicolaou et al. 2004).

Although many thiopeptides exhibit good antimicrobial activity, especially against Gram-positive bacteria including MRSA, they have not yet been successfully developed for treating serious infections in human medicine. Low water solubility of these large and often lipophilic molecules creates problems for parenteral administration and their size hinders oral bioavailability. One advantage is their relatively low toxicity to mammalian systems since there is minimal overlap or similarity between their mammalian and bacterial targets. Some efforts have been made to overcome the problems by creation of semi-synthetic derivatives (Kirst 2012). However, because of the size and complexity of most thiopeptide structures, chemical derivatization of such compounds has thus far been generally slow and limited to modifying those functional groups located around the periphery of the molecule that can be readily accessed and selectively manipulated. Two semi-synthetic derivatives of GE2270A are a recent example of efforts to find important clinical candidates within this class (Fig. 14.7b) (LaMarche et al. 2011; Leeds et al. 2011). Some veterinary applications for another thiopeptide, thiopeptin, have been investigated, such as treatment of liver abscesses and lactic acidosis in ruminents (Lechtenberg et al. 1998; Muir et al. 1981).

Many thiopeptides interfere with some component(s) of bacterial protein synthesis, although they often differ on the distinct steps being affected (Lentzen et al. 2003; Mikolajka et al. 2011). Thiostrepton inhibits protein synthesis by binding to the complex of 23S rRNA and the L11 protein (Harms et al. 2008). This location is associated with the binding sites of translation factors (EF-Tu, EF-G) and GTPase-promoted hydrolysis of GTP. Both thiostrepton and micrococcin inhibit the same target, EF-G dependent translation, but their small structural differences lead to opposite effects; thiostrepton decreases turnover of EF-G while micrococcin increases turnover (Lentzen et al. 2003; Mikolajka et al. 2011). This site(s) does

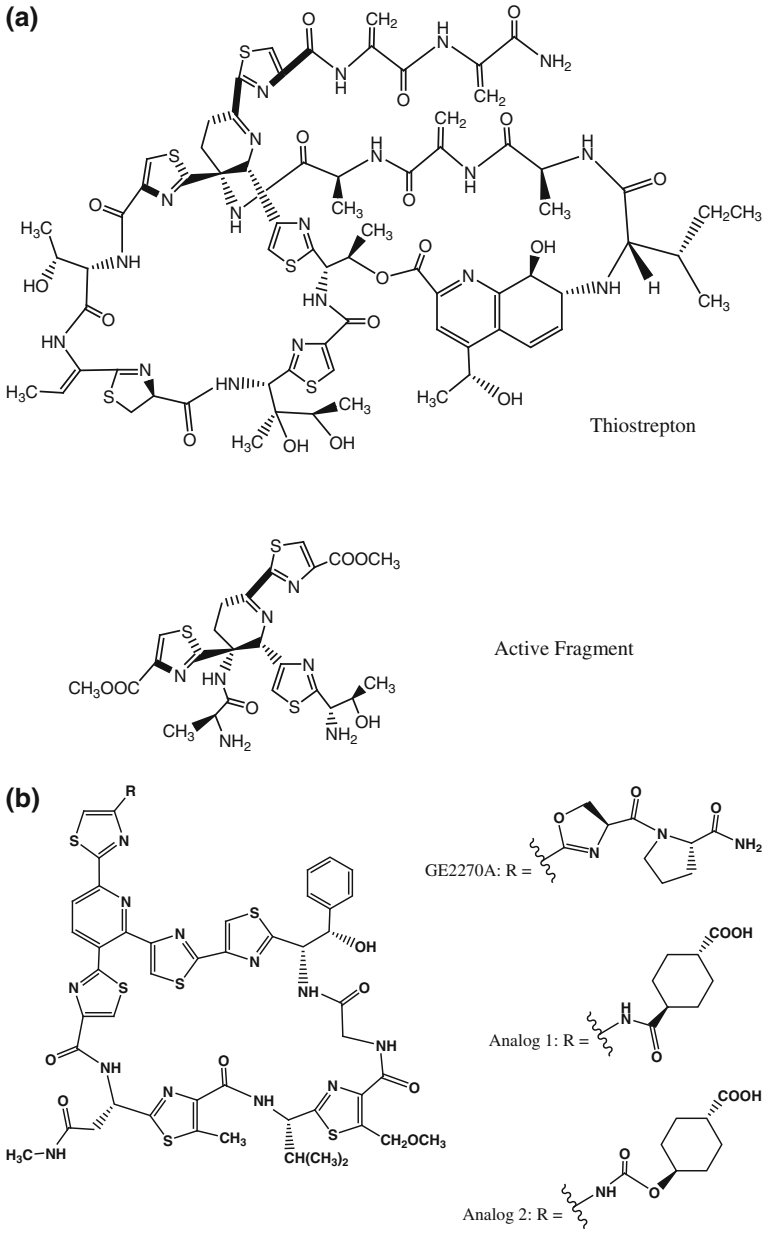


Fig. 14.7 **a** Structures of thiostrepton and an active fragment. **b** Structures of GE2270A and derivatives

not overlap the binding site of other antibiotics, so cross-resistance is not a problem. Other thiopeptides show both similarities and differences in their MOA compared to thiostrepton. For example, GE2270A inhibits bacterial protein synthesis by a mechanism involving binding with elongation factor Tu (EF-Tu) (Leeds et al. 2011).

Some recent effort has been successful in reducing the size of these compounds while still retaining some antimicrobial activity. During a total synthesis program, a fragment from the structure of thiostrepton (Fig. 14.7a) was discovered to possess moderate anti-MRSA activity (Nicolaou et al. 2005). This result demonstrates that a substantial portion of the large structure is unnecessary for moderate antimicrobial activity and could be replaced. Another study constructed a synthetic library of compounds based upon fragments that were identified from analyzing a model of thiostrepton-ribosome binding (Bower et al. 2003). The thiostrepton-ribosome binding site was also examined by proximity-induced covalent capture (Baumann et al. 2008). It will prove interesting to see if these or other strategies can identify smaller pharmacophores that SAR studies can use as starting points to create improved analogs having stronger antibiotic features.

Synthetic challenges with this class offer opportunities for biosynthetic engineering to provide a complementary strategy for producing new analogs. The intriguing origins of the many uncommon components assembled in thiopeptide structures have been attributed to extensive post-translational modifications of peptides initially formed by biosynthesis on the ribosome (Arndt et al. 2009; Li et al. 2011). Further studies are being conducted by several research groups to learn further details about the biosynthetic pathways and then to manipulate and modify them to produce new entities and to eventually achieve combinatorial biosynthesis in this class (Li and Kelly 2010, Li et al. 2011).

14.9 Miscellaneous

In addition to the seven smaller classes reviewed in this chapter, many other antibiotics are known that employ some aspect of inhibition of microbial protein synthesis as their MOA. Such a list includes some agents that have never experienced much if any significant clinical development, such as sparsomycin, pactamycin, anisomycin, viomycin, puromycin, kirromycin, negamycin, TAN-1057, and edeine (Hermann 2005; Lange et al. 2007; McCoy et al. 2011; Yonath 2005). Some of these agents use a unique MOA not shared by other antibiotics and represent new uncharted territory.

Another example is capreomycin, an old but still useful second-line anti-tuberculosis drug used in cocktails of multiple agents in order to hinder development of resistance and to combat multi-drug resistant (MDR) strains of *M. tuberculosis* (Ma et al. 2007). One member of the orthosomycin class, everninomicin D (Ziracin®), experienced some clinical development before it was eventually dropped due to toxicity (Lee 2007). While more details on MOA are

still needed, orthosomycins inhibit protein synthesis through a unique mechanism of binding at a site relatively far removed from the PTC, resulting in no cross-resistance with other antibiotics (Mikolajka et al. 2011). However, there appears to be no follow-up after everninomicin D was dropped from development.

Completely synthetic compounds that target critical steps in microbial protein biosynthesis are also being investigated. GSK2251052 (AN3365) is a novel boron-containing antibiotic that was discovered at Anacor Pharmaceuticals and licensed to GSK (Fig. 14.8). It functions as an inhibitor of leucyl-tRNA synthetase and is one of the few new antibiotics that exhibit activity against Gram-negative bacteria and anaerobes (Citron and Goldstein 2011). It is currently in Phase II clinical trials for treatment of hospital-acquired Gram-negative infections (Bush and Pucci 2011; Butler and Cooper 2011; Coates et al. 2011; Sutcliffe 2011).

Another inhibitor of protein synthesis is GSK1322322, a synthetic analog of the original natural product lead structure of actinonin (Chen et al. 2000). GSK1322322 has a hydrazino-pyrimidine structure (Fig. 14.8) and functions as an inhibitor of peptide deformylase (PDF), a ubiquitous and critical bacterial enzyme long considered as a potential new target for antibiotics (Sharma et al. 2009, see East, this volume). PDF cleaves the N-formyl group from the N-terminus of the

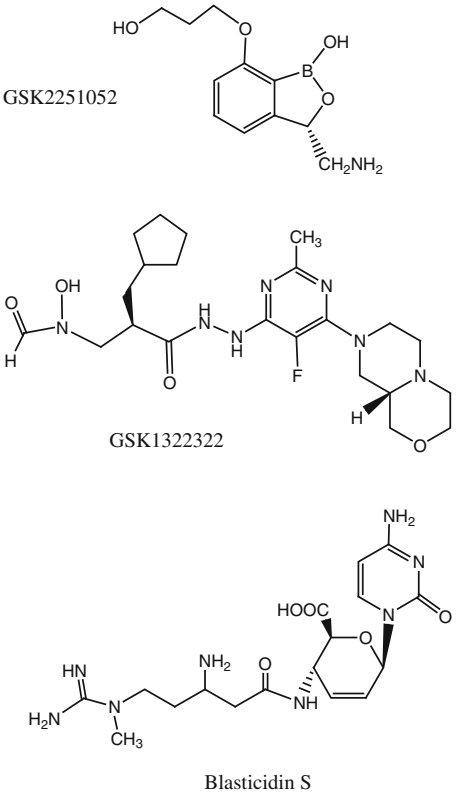


Fig. 14.8 Structures of GSK2251052, GSK1322322, and blasticidin S

protein synthesis initiator f-met on the peptide chain in preparation for the final steps to release and activate the desired peptide product. Failure to properly cleave the N-formyl moiety at the correct time during protein biosynthesis disrupts the ribosomal process. Although several previous PDF inhibitors have been studied and dropped from further development, GSK1322322 is in Phase II clinical trial (Bush and Pucci 2011; Butler and Cooper 2011; Coates et al. 2011; Kirst 2012; Sutcliffe 2011; see East, this volume).

Rib-X Pharmaceuticals presented their RX-04 program in fourteen posters at the 2011 ICAAC (Rib-X 2012). A starting point was provided by blasticidin S, an old nucleoside antibiotic (Fig. 14.8) which is obtained from fermentation of *Streptomyces griseochromogenes* and is a known inhibitor of protein synthesis (Kumasaka et al. 2007; Takeuchi et al. 1958). An initial structure for a PTC—blasticidin S complex has been described (Hansen et al. 2003). In the current effort, X-ray crystallography and computational analyses of ribosome-blasticidin S co-crystals were used to identify potential new scaffold fragments for SAR elaborations and to suggest new directions for synthesizing novel entities that would exhibit stronger ribosomal binding and greater antimicrobial activity (Kanyo et al. 2011). From these studies, three separate new platforms were presented as leads for future synthetic efforts and SAR studies in order to create new broad spectrum antibiotic structures (Marra et al. 2011).

14.10 Conclusions

Recent events described in this review clearly illustrate that creative ideas for new antibiotics are still being generated even within such a highly investigated field as protein synthesis inhibitors. In addition to new antibiotics coming from the four large classes of protein synthesis inhibitors, several smaller classes are also starting to contribute new agents into the antibiotic development pipeline. One member of the pleuromutilin class, retapamulin, recently received regulatory approval. Other members from these (and other) smaller classes should follow in due course.

In addition to the seven classes in this chapter, a vast wealth of other previously isolated fermentation-derived antibiotics remains under-explored and under-developed. This large collection of known natural products is a rich lode that is nowhere yet fully mined for either chemistry or microbiology and it warrants more serious attention. It is reasonable to expect that many of these compounds inhibit protein synthesis, but they have never been (fully) tested to learn their detailed MOA. New targets may be discovered that are currently unused or unknown within that large and complex MOA of protein synthesis inhibitors. Thus, new opportunities await researchers to again use antibiotics to discover new microbiology. The fermentative origin of compounds along with advances in microbial genetics opens greater opportunities for controlled manipulations of biosynthetic

pathways and combinatorial biosynthesis to complement chemical synthesis in creating new antibiotics.

From the chemistry side, the compounds are certainly a valuable resource as leading structures for medicinal chemistry programs. Natural products tend to occupy unfilled chemical spaces that prompt new synthetic strategies and diversity-oriented chemical libraries for screening (Cordier et al. 2008). Applications of fragment-based design and creation of new natural product-derived scaffolds for elaboration by analog synthesis and SAR studies are other encouraging developments. Thus, multiple opportunities are available to discover new agents that target bacterial protein synthesis (as well as other MOAs). All of these potential developments indicate that inhibition of protein synthesis should continue to flourish as an important MOA for new antimicrobial agents.

References

Agrawal RK, Heagle AB, Frank J (2000) Studies of elongation factor G-dependent tRNA translocation by three-dimensional cryo-electron microscopy. In: Garrett RA, Douthwaite SR, Liljas A, Matheson AT, Moore PB, Noller HF (eds) The ribosome: structure, function, antibiotics, and cellular interactions. ASM Press, Washington DC

Anderson B, Hodgkin DC, Viswamitra MA (1970) The structure of thiostrepton. Nature 225:233–235

Anstead GM, Owens AD (2004) Recent advances in the treatment of infections due to resistant *Staphylococcus aureus*. Curr Opin Infect Dis 17:549–555

Arndt H-D, Schoof S, Lu J-Y (2009) Thiopeptide antibiotic biosynthesis. Angew Chem Int Ed 48:6770–6773

Bacque E, Barriere J-C, Berthaud N (2005) Recent progress in the field of antibacterial pristinamycins. Curr Med Chem Anti-Infect Agents 4:185–217

Bagley MC, Dale JW, Merritt EA, Xiong X (2005) Thiopeptide antibiotics. Chem Rev 105:685–714

Bartlett JG (2008) Historical perspectives on studies of *Clostridium difficile* and *C. difficile* infection. Clin Infect Dis 46(Suppl 1): S4–S11

Baumann S, School S, Harkal SD, Arndt H-D (2008) Mapping the binding site of thiopeptide antibiotics by proximity-induced covalent capture. J Am Chem Soc 130:5664–5666

Blanchard SC, Cooperman BS, Wilson DN (2010) Probing translation with small-molecule inhibitors. Chem Biol 17:633–645

Bøsling J, Poulsen SM, Vester B, Long KS (2003) Resistance to the peptidyl transferase inhibitor tiamulin caused by mutation of ribosomal protein L3. Antimicrob Agents Chemother 47:2892–2896

Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48:1–12

Bower J, Drysdale M, Hebdon R, Jordan A, Lentzen G, Matassova N, Murchie A, Powles J, Roughley S (2003) Structure-based design of agents targeting the bacterial ribosome. Bioorg Med Chem Lett 13:2455–2458

Bush K, Pucci MJ (2011) New antimicrobial agents on the horizon. Biochem Pharmacol 82:1528–1539

Bush K et al (2011) Tackling antibiotic resistance. Nat Rev Microbiol 9:894–896

Butler MS, Cooper MA (2011) Antibiotics in the clinical pipeline in 2011. J Antibiot 64:413–425

Canu A, Leclercq R (2001) Overcoming bacterial resistance by dual target inhibition: the case of streptogramins. Curr Drug Targets Infect Disord 1:215–225
Cempra Pharmaceuticals (2011) Fusidic acid dosing regimens for treatment of bacterial infections. WO/2011/008193
Cempra Pharmaceuticals (2012) http://www.cempra.com
Chain EB, Mellows G (1977) Pseudomonic acid. Part 1. The structure of pseudomonic acid A, a novel metabolite produced by *Pseudomonas fluorescens*. J Chem Soc Perkin Trans I 1977:294–309
Charney J, Fisher WP, Curran C, Machlowitz RA, Tytell TA (1953) Streptogramin, a new antibiotic. Antibiot Chemother 3:1283–1286
Chen DZ, Patel DV, Hackbarth CJ, Wang W, Drever G, Young DC, Margolis PS, Wu C, Ni CJ, Trias J, White RJ, Yuan Z (2000) Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochem 39:1256–1262
Citron DM, Goldstein JC (2011) Comparative in vitro activity of GSK2251052, a novel boron leucyl-tRNA synthetase inhibitor, against 916 anaerobic organisms. In: 51st interscience conference on antimicrobial agents and chemotherapy, Chicago, IL, E-111
Coates T, Bax R, Coates A (2009) Nasal decolonization of *Staphylococcus aureus* with mupirocin: strengths, weaknesses and future prospects. J Antimicrob Chemother 64:9–15
Coates ARM, Halls G, Hu Y (2011) Novel classes of antibiotics or more of the same? Br J Pharmacol 163:184–194
Cooper A (1966) The crystal structure of fusidic acid methyl ester 3-*p*-bromobenzoate. Tetrahedron 22:1379–1381
Cordier C, Morton D, Murrison S, Nelson A, O'Leary-Steele C (2008) Natural products as an inspiration in the diversity-oriented synthesis of bioactive compound libraries. Nat Prod Rep 25:719–737
Corey GR, Moellering R, Grayson ML (eds) (2011) Fusidic acid enters the United States. Clin Infect Dis 52(Suppl 7):S467–S546
Dang V, Nanda N, Cooper TW, Greenfield RA, Bronze MS (2007) Macrolides, azalides, ketolides, lincoamides, and streptogramins. J Okla State Med Assoc 100:75–81
Devasahayam G, Scheld WM, Hoffman PS (2010) Newer antibacterial drugs for a new century. Expert Opin Investig Drugs 19:215–234
Donadio S, Maffioli S, Monciardini P, Sosio M, Jabes D (2010) Antibiotic discovery in the twenty-first century: current trends and future perspectives. J Antibiot 63:423–430
Doublet B, Schwarz S, Kehrenberg C, Cloeckaert A (2005) Florfenicol resistance gene *floR* is part of a novel transposon. Antimicrob Agents Chemother 49:2106–2108
Dryden MS (2010) Complicated skin and soft tissue infection. J Antimicrob Chemother 65(Suppl 3):35–44
Dunkle JA, Xiong L, Mankin AS, Cate JHD (2010) Structures of the *Escherichia coli* ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. Proc Nat Acad Sci USA 107:17152–17157
Ehrlich J, Bartz QR, Smith RM, Joslyn DA, Burkholder PR (1947) Chloromycetin, a new antibiotic from a soil actinomycete. Science 106:417
Falagas ME, Grammatikos AP, Michalopoulos A (2008) Potential of old-generation antibiotics to address current need for new antibiotics. Expert Rev Anti Infect Ther 6:593–600
Farrell DJ, Castanheira M, Chopra I (2011) Characterization of global patterns and the genetics of fusidic acid resistance. Clin Infect Dis 52(Suppl 7):S487–S492
Fong IW, Drlica K (eds) (2008) Antimicrobial resistance and implications for the twenty-first century. Springer, New York
Frei CR, Miller ML, Lewis JS, Lawson KA, Peddaiahgari R, Talbert RL (2010) Retrospective cohort study of hospitalized adults treated with vancomycin or clindamycin for methicillin-resistant *Staphylococcus aureus* skin infections. Clin Ther 32:2024–2029
Freire-Moran L, Aronsson B, Manz C, Gyssens IC, So AD, Monnet DL, Cars O (2011) Critical shortage of new antibiotics in development against multi-resistant bacteria. Drug Resist Update 14:118–124

Fuller AT, Mellows G, Woolford M, Banks GT, Barrow KD, Chain EB (1971) Pseudomonic acid: an antibiotic produced by *Pseudomonas fluorescens*. Nature 234:416–417

Gao Y-G, Selmer M, Dunham CM, Weixlbaumer A, Kelley AC, Ramakrishnan V (2009) The structure of the ribosome with elongation factor G trapped in the posttranslational state. Science 326:694–699

Godtfredsen WO, Jahnsen S, Lorck H, Roholt K, Tybring L (1962) Fusidic acid: a new antibiotic. Nature 193:987

Godtfredsen WO, von Daehne W, Vangedal S, Marquet A, Arigoni D, Melera A (1965) The stereochemistry of fusidic acid. Tetrahedron 21:3505–3530

Guay D (2007) Update on clindamycin in the management of bacterial, fungal and protozoal infections. Expert Opin Pharmacother 8:2401–2444

Gurel G, Blaha G, Moore PB, Steitz TA (2009) U2504 determines the species specificity of the A-site cleft antibiotics. J Mol Biol 389:146–156

Hansen JL, Moore PB, Steitz TA (2003) Structures of five antibiotics bound at the peptidyl transferase center of the large ribosomal subunit. J Mol Biol 330:1061–1075

Harms JM, Schlunzen F, Fucini P, Bartels H, Yonath A (2004) Alterations at the peptidyl transferase centre of the ribosome induced by the synergistic action of the streptogramin dalfopristin and quinupristin. BMC Biol 2:4

Harms JM, Wilson DN, Schluenzen F, Connell SR, Stachelhaus T, Zaborowska Z, Spahn CM, Fucini P (2008) Translational regulation via L11: molecular switches on the ribosome turned on and off by thiostrepton and micrococcin. Mol Cell 30:26–38

Hartley AJ, de Mattos-Shipley K, Collins CM, Kilaru S, Foster GD, Bailey AM (2009) Investigating pleuromutilin-producing *Clitopilus* species and related basidiomycetes. FEMS Microbiol Lett 297:24–30

Hensens OD, Albers-Schonberg G (1983) 13C NMR study of thiostrepton and thiopeptin components. J Antibiot 36:832–845

Hermann T (2005) Drugs targeting the ribosome. Curr Opin Struct Biol 15:355–366

Hothersall J, Wu J, Rahman AS, Shields JA, Haddock J, Johnson N, Cooper SM, Stephens ER, Cox RJ, Crosby J, Willis CL, Simpson TJ, Thomas CM (2007) Mutational analysis reveals that all tailoring region genes are required for production of polyketide antibiotic mupirocin by *Pseudomonas fluorescens*. J Biol Chem 282:15451–15461

Hu C, Zou Y (2009) Mutilins derivatives: from veterinary to human-used antibiotics. Mini-Rev Med Chem 9:1397–1406

Hughes RA, Moody CJ (2007) From amino acids to heteroaromatics—thiopeptide antibiotics, nature's heterocyclic peptides. Angew Chem Int Ed 46:7930–7954

IDSA (2010) The 10 × 20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin Infect Dis 50:1081–1083

Jacobs MR (2010) Retapamulin: focus on its use in the treatment of uncomplicated superficial skin infections and impetigo. Expert Rev Dermatol 5:505–513

Johnston NJ, Mukhtar TA, Wright GD (2002) Streptogramin antibiotics: mode of action and resistance. Curr Drug Targets 3:335–344

Kannan K, Mankin AS (2011) Macrolide antibiotics in the ribosome exit tunnel: species-specific binding and action. Ann N Y Acad Sci 1241:33–47

Kanyo Z, Devivo M, Bhattacharjee A, Ippolito J, Wimberly B, Duffy E (2011) Structural basis for the binding of RX-04, a novel broad spectrum antibacterial class, to bacterial ribosomes. In 51st interscience conference on antimicrobial agents chemotherapy, Chicago, IL, F1-1842

Kavanagh F, Hervey A, Robbins WJ (1951) Antibiotic substances from Basidiomycetes. Proc Natl Acad Sci U S A 37:570–574

Khemawoot P, Saunders D, Rasameesoraj M, Melendez V, Imerbsin R, Ohrt C, Fracisco S, Tejaisavadharm P (2011) Absolute bioavailability of cis-mirincamycin and trans-mirincamycin in healthy rhesus monkeys and ex vivo antimalarial activity against *Plasmodium falciparum*. Antimicrob Agents Chemother 55:5881–5886

Kirst HA (ed) (2010a) Antibiotics versus resistant bacteria: a continual challenge. Expert Opin Ther Patents 20: 1271–1420

Kirst HA (2010b) New macrolide, lincosaminide and streptogramin B antibiotics. Expert Opin Ther Patents 20:1343–1357
Kirst HA (2012) Recent derivatives from smaller classes of fermentation-derived antibacterials. Expert Opin Ther Patents 22:15–35
Kluka EA (2011) Emerging dilemmas with methicillin-resistant *Staphylococcus aureus* infections in children. Curr Opin Otolaryngol Head Neck Surg 19:462–466
Korczynska M, Mukhtar TA, Wright GD, Berghuis AM (2007) Structural basis for streptogramin B resistance in *Staphylococcus aureus* by virginiamycin B lyase. Proc Natl Acad Sci U S A 104:10388–10393
Kumasaka T, Yamamoto M, Furuichi M, Nakasako M, Teh A-H, Kimura M, Yamaguchi I, Ueki T (2007) Crystal structures of blasticidin S deaminase (BSD). J Biol Chem 282:37103–37111
LaMarche MJ, Leeds JA, Dzink-Fox J, Gunderson K, Krastel P, Memmert K, Patane MA, Rann EM, Schmitt E, Tiamfook S, Wang B (2011) 4-Aminothiazolyl analogues of GE2270 A: antibacterial lead finding. J Med Chem 54:2517–2521
Lange RP, Locher HH, Wyss PC, Then RL (2007) The targets of currently used antibacterial agents: lessons for drug discovery. Curr Pharm Des 13:3140–3154
Lechtenberg KF, Nagaraja TG, Chengappa MM (1998) Antimicrobial susceptibility of *Fusobacterium necrophorum* isolated from bovine hepatic abscesses. Am J Vet Res 59:44–47
Leclercq R, Courvalin P (1991) Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob Agents Chemother 35:1267–1272
Lee VJ (2007) Anti-Gram-positive agents of natural product origins. In: Triggle DJ, Taylor JB (eds) Comprehensive medicinal chemistry II, vol 7. Elsevier, Oxford
Leeds JA, LaMarche MJ, Brewer JT, Bushell SM, Deng G, Dewhurst JM et al (2011) In vitro and in vivo activities of novel, semisynthetic thiopeptide inhibitors of bacterial elongation factor Tu. Antimicrob Agents Chemother 55:5277–5283
Lentzen G, Klinck R, Matassova N, Aboul-ela F, Murchie AIH (2003) Structural basis for contrasting activities of ribosome binding thiazole antibiotics. Chem Biol 10:769–778
Lewis C (1974) Clinically useful antibiotics obtained by directed chemical modification—lincomycins. Fed Proc 33:2303–2306
Li C, Kelly WL (2010) Recent advances in thiopeptide antibiotic biosynthesis. Nat Prod Rep 27:153–164
Li C, Zhang F, Kelly WL (2011) Heterologous production of thiostrepton A and biosynthetic engineering of thiostrepton analogs. Mol BioSyst 7:82–90
Loncaric C, Wulff WD (2001) An efficient synthesis of (-)-chloramphenicol via asymmetric catalytic aziridination. Org Lett 3:3675–3678
Ma Z, Ginsberg AM, Spigelman M (2007) Antimycobacterium agents. In: Triggle DJ, Taylor JB (eds) Comprehensive medicinal chemistry II, vol 7. Elsevier, Oxford
Manfredi R (2005) A re-emerging class of antimicrobial agents: streptogramins (quinupristin/dalfopristin) in the management of multiresistant Gram-positive nosocomial cocci in hospital setting. Mini-Rev Med Chem 5:1075–1081
Marcinak JF, Frank AL (2006) Epidemiology and treatment of community-associated methicillin-resistant *Staphylococcus aureus* in children. Expert Opin Anti-Infect Ther 4:91–100
Marra A, Bortolon E, Molstad D, Wu Y, Jing H, Duffy E (2011) Novel ribosome inhibitors are efficacious in a murine peritonitis model caused by different bacterial pathogens. In 51st interscience conference on antimicrobial agents and chemotherapy, Chicago, IL, F1-1854
Mason DJ, Dietz A, DeBoer C (1963) Lincomycin, a new antibiotic. In: Antimicrobial agents and chemotherapy 1962. Williams & Wilkins, Baltimore, pp 554–559
Maviglia R, Nestorini R, Pennisi MA (2009) Role of old antibiotics in multidrug resistant bacterial infections. Curr Drug Targets 10:895–905
McCoy LS, Xie Y, Tor Y (2011) Antibiotics that target protein synthesis. WIREs RNA 2:209–232
Mikolajka A, Liu H, Chen Y, Starosta AL, Marquez V, Ivanova M, Cooperman BS, Wilson DN (2011) Differential effects of thiopeptide and orthosomycin antibiotics on translational GTPases. Chem Biol 18:589–600

Mitscher LA (2010) Tetracycline, aminoglycoside, macrolide, and miscellaneous antibiotics. In: Abraham DJ, Rotella DP (eds) Burger's medicinal chemistry, drug discovery, and development, 7th edn. Wiley, New York

Mlynarczyk B, Mlynarczyk A, Kmera-Muszynska M, Majewski S, Mlynarczyk G (2010) Mechanisms of resistance to antimicrobial drugs in pathogenic Gram-positive cocci. Mini-Rev Med Chem 10:928–937

Morar M, Bhullar K, Hughes DW, Junop M, Wright GD (2009) Structure and mechanism of the lincosamide antibiotic adenylyltransferase LinB. Structure 17:1649–1659

Muir LA, Rickes EL, Duquette PF, Smith GE (1981) Prevention of induced lactic acidosis in cattle by thiopeptin. J Anim Sci 52:635–643

Nailor MD, Sobel JD (2009) Antibiotics for Gram-positive infections. Infect Dis Clin N Am 23:965–982

Nicolaou KC, Zak M, Safina BS, Lee SH, Estrada AA (2004) Total synthesis of thiostrepton, Part 2. Construction of the quinaldic acid macrocycle and final stages of the synthesis. Angew Chem Int Ed 43:5092–5097

Nicolaou KC, Zak M, Rahimipour S, Estrada AA, Lee SH, O'Brate A, Giannakakou P, Ghadiri MR (2005) Discovery of a biologically active thiostrepton fragment. J Am Chem Soc 127:15042–15044

Novak R, Shlaes DM (2010) The pleuromutilin antibiotics: a new class for human use. Curr Opin Investig Drugs 11:182–191

Pagano JF, Weinstein MJ, Stout HA, Donovick R (1956) Thiostrepton, a new antibiotic. 1. In vitro studies. Antibiot Annu 1955–1956(3):554–559

Patel JB, Gorwitz RJ, Jernigan JA (2009) Mupirocin resistance. Clin Infect Dis 49:935–941

Pohlmann J, Brotz-Oesterhelt H (2004) New aminoacyl-tRNA synthetase inhibitors as antibacterial agents. Curr Drug Targets Infect Disorders 4:261–272

Politano AD, Sawyer RG (2010) 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 11:225–236

Poulsen SM, Karlsson M, Johansson LB, Vester B (2001) The pleuromutilin drugs tiamulin and valnemulin bind to the RNA at the peptidyl transferase centre on the ribosome. Mol Microbiol 41:1091–1099

Pulcini C et al (2012) Forgotten antibiotics: an inventory in Europe, the United States, Canada, and Australia. Clin Infect Dis 54:268–274

Rib-X Pharmaceuticals (2012) www.rib-x.com/research-discovery/publications-presentations.php#RX04

Roberts MC (1999) Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob Agents Chemother 43:2823–2830

Roberts MC (2008) Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. FEMS Microbiol Lett 282:147–159

Roberts MC (2011) Environmental macrolide-lincosamide-streptogramin and tetracycline resistant bacteria. Frontiers Microbiol 2:40–47

Saini V, Olde Riekerink RGM, McClure JT, Barkema HW (2011) Diagnostic accuracy assessment of sensititre and agar disk diffusion for determining antimicrobial resistance profiles of bovine clinical mastitis pathogens. J Clin Microbiol 49:1568–1577

Schlunzen F, Zarivach R, Harms J, Bashan A, Tocilj A, Albrecht R, Yonath A, Franceschi F (2001) Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. Nature 413:814–821

Schlunzen F, Pyetan E, Fucini P, Yonath A, Harms JM (2004) Inhibition of peptide bond formation by pleuromutilins: the structure of the 50S ribosomal subunit from Deinococcus radiodurans in complex with tiamulin. Mol Microbiol 54:1287–1294

Schofer H, Simonsen L (2010) Fusidic acid in dermatology: an updated review. Eur J Dermatol 20:6–15

Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A (2004) Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev 28:519–542

Schwarz S, Kehrenberg C (2006) Old dogs that learn new tricks: modified antimicrobial agents that escape pre-existing resistance mechanisms. Int J Med Microbiol 296(Suppl 2):45–49

Sharma A, Khuller GK, Sharma S (2009) Peptide deformylase—a promising therapeutic target for tuberculosis and antibacterial drug discovery. Expert Opin Ther Targets 13:753–765

Sivapalasingam S, Steigbigel NH (2010) Macrolides, clindamycin, and ketolides. In: Mandell GL, Bennett JE, Dolin R (eds) Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 7th edn. Churchill Livingstone, Philadelphia

Sutcliffe JA (2011) Antibiotics in development targeting protein synthesis. Ann N Y Acad Sci 1241:122–152

Takeuchi S, Hirayama K, Ueda K, Yonehara H (1958) Blasticidin S, a new antibiotic. J Antibiot 11:1–5

Tang YZ, Liu YH, Chen JX (2012) Pleuromutilin and its derivatives—the lead compounds for novel antibiotics. Mini-Rev Med Chem 12:53–61

Tenson T, Lovmar M, Ehrenberg M (2003) The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. J Mol Biol 330:1005–1014

Thomas CM, Hothersall J, Willis CL, Simpson TJ (2010) Resistance to and synthesis of the antibiotic mupirocin. Nature Rev Microbiol 8:281–289

Tu D, Blaha G, Moore PB, Steitz TA (2005) Structures of MLS_BK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell 121:257–270

Vondenhoff GHM, van Aerschot A (2011) Aminoacyl-tRNA synthetase inhibitors as potential antibiotics. Eur J Med Chem 46:5227–5236

Weinberg JM, Tyring SK (2010) Retapamulin: an antibacterial with a novel mode of action in an age of emerging resistance to *Staphylococcus aureus*. J Drugs Dermatol 9:1198–1204

Weisblum B (1995) Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother 39:577–585

White DG, Hudson C, Maurer JJ, Ayers S, Zhao S, Lee MD, Bolton L, Foley T, Sherwood J (2000) Characterization of chloramphenicol and florphenicol resistance in *Escherichia coli* associated with bovine diarrhea. J Clin Microbiol 38:4593–4598

Wilson DN (2011) On the specificity of antibiotics targeting the large ribosomal subunit. Ann N Y Acad Sci 1241:1–16

Yang K, Fang H, Gong J, Su L, Xu W (2009) An overview of highly optically pure chloramphenicol bases: applications and modifications. Mini-Rev Med Chem 9:1329–1341

Yonath A (2005) Antibiotics targeting ribosomes. Annu Rev Biochem 74:649–679

Chapter 15
Actinonin and Analogs: Inhibitors of Bacterial Peptide Deformylase

Stephen P. East

Abstract The naturally occurring hydroxamic acid actinonin was isolated from species of actinomycetes and characterised as an antibiotic in the 1960s. More than 30 years later the antibacterial activity of actinonin was attributed to inhibition of the metallohydrolase peptide deformylase (PDF), an enzyme that performs an essential step in bacterial protein synthesis. To date, three PDF inhibitors have progressed into clinical trials and one compound, GSK1322322, is currently in Phase II for the treatment of respiratory tract infections and skin and soft tissue infections. This chapter provides an introduction to PDF as an antibacterial target and discusses PDF inhibitors described in the literature that are structurally related to actinonin. Structure–activity relationships, in vivo data, potential resistance mechanisms and recent developments in the area of PDF inhibition are presented.

15.1 Introduction

The role of hydroxamic acids (HAs) as efficient metal chelators and the examination of this class of compounds as pharmacologically important molecules has been well documented (Muri et al. 2002). Indeed the launch of the histone deacetylase (HDAC) inhibitor drug Vorinostat for use in an oncology setting is an excellent illustration of the clinical relevance of HAs. In addition to synthetic HAs, there are also many compounds isolated from natural sources that contain this functional group (Neilands 1967; Maehr 1971) and nature has contributed to the discovery of new pharmaceutical agents containing HAs. For example, trichostatin A (Fig. 15.1), first described as an antifungal antibiotic isolated from *Streptomyces hygroscopicus* (Tsuji et al. 1976), more recently gained attention as a prototypical

S. P. East (✉)
Evotec (UK) Ltd, 114 Innovation Drive, Milton Park, Abingdon, Oxfordshire, OX14 4RZ, UK
e-mail: stephen.east@evotec.com

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_15,

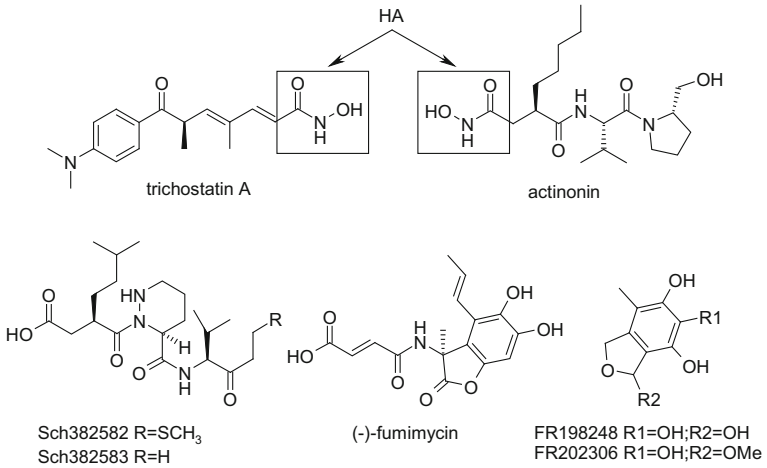


Fig. 15.1 Naturally occurring HAs and PDF inhibitors

HDAC inhibitor. Another example of a natural product containing an HA group is actinonin, which was first discovered in 1962 (Gordon et al. 1962, 1975) and found to be a potent antibacterial agent on Gram-positive and Gram-negative species. Nearly 40 years later, Chen et al. (2000) elucidated the mechanism of action of actinonin to be via inhibition of the metallohydrolase enzyme peptide deformylase (PDF). This discovery helped to prompt the search for other small molecules that inhibit bacterial PDF. In this chapter, an overview of PDF inhibition as a novel target for the treatment of antibacterial infections together with recent developments in this area of research will be discussed.

15.2 Peptide Deformylase

The first step of protein synthesis in bacteria is N-formylation of methionyl-tRNA (Marcker and Sanger 1964). Formation of the polypeptide chain is then initiated, and, typically, the cleavage of both the N-formyl group by PDF (Adams 1968) and the terminal methionine amino acid residue by methionine aminopeptidase occur as part of the processing of new proteins. In bacteria, the formylation–deformylation steps are essential for the release of mature proteins and hence maintaining bacterial growth. Blocking the hydrolytic cleavage step performed by PDF through small molecule inhibition has therefore been considered as a novel mechanism of action in the search for a new class of antibacterial drugs. It has also been shown that a secondary, and potentially beneficial, effect of PDF inhibition might occur as a result of the increase in bacterial N-formyl peptides at the site of infection. These peptides activate human formyl peptide receptors and trigger an innate immune response that might help to clear the infection (Fu et al. 2003; Mader et al. 2010).

The major obstacle that thwarted the progression of research into PDF and contributed to a 30+ year gap between the identification of the enzyme and its full characterisation was its observed instability. In its native active form PDF is an iron (II)-containing metalloenzyme; however, exposure to atmospheric oxygen readily converts the ferrous iron in functional PDF to its ferric state and this irreversible oxidative process renders PDF catalytically inactive (Rajagopalan and Pei 1998). A critical finding for PDF research was that the ferrous iron could be replaced with other metals, most commonly nickel for bacterial PDFs, and this metal switch increases PDF's stability to oxidation. Importantly, the modified nickel enzyme retains full catalytic activity (Groche et al. 1998). This discovery led to the development of robust in vitro biochemical assays, garnered the interest of the drug discovery community and paved the way for high-throughput screens and X-ray structure-guided drug design of PDF inhibitors (Genilloud and Vicente, this volume).

Four types of PDF have been characterised. Two of these, termed PDF1b and PDF2, have been found in bacterial species with PDF2 only being observed in Gram-positive strains. PDF3 has been identified in parasitic species of archaeal and trypanosomatids (Bouzaidi-Tiali et al. 2007). The PDF homologue PDF1a has been found in plant and mammalian systems including human mitochondria. As cytoplasmic protein synthesis in mammals does not require a formylation step for initiation (and consequently a deformylation step for protein maturation) the functional relevance of a mammalian mitochondrial PDF is still largely unknown. Human PDF (hsPDF) has been proposed as a novel cancer target (Lee et al. 2004) as actinonin and structurally related compounds were shown to inhibit hsPDF and consequently cell proliferation in a variety of cancer cell lines. More recently Escobar-Alvarez et al. (2010) elucidated the role of hsPDF in the respiratory function of mitochondria and such emerging evidence on the function of hsPDF adds a complexity to the identification of bacteria-specific inhibitors of PDF.

15.3 PDF Inhibitors

15.3.1 Structure and Kinetics of PDF Inhibition

Protein NMR, and more predominantly, X-ray crystallography have been used to provide information on the 3D structures of both apo and ligand complexes of PDF. Actinonin has been crystallised with several forms of bacterial PDF and an illustration of the key protein–ligand interactions as shown in Fig. 15.2 for the *E. coli* zinc actinonin complex (Guilloteau et al. 2002). This binding mode is reasonably representative of how actinonin interacts with other forms of PDF. The hydroxamic acid chelates in a bidentate mode to the metal ion and it is in close proximity to Gly45, Gln50, Leu91, and Glu133 in a secure hydrogen-bonding network. The backbone NH of Ile44 interacts with the C = O adjacent to the

n-pentyl P1′ side chain, which occupies the S1′ pocket (metalloproteinase nomenclature). The residue Gly89 seems to form H-bonds with both the NH and the C = O either side of the isopropyl P2′ moiety in the inhibitor, which is located in the S2′ region of the enzyme. Finally, the primary alcohol of actinonin is near to Glu87, although this residue is located at the edge of the binding site in a solvent exposed region and so this might only constitute a weak interaction.

Escobar-Alvarez et al. (2009) elucidated the first crystal structures of hsPDF (apo and actinonin bound) and compared them with non-mammalian type 1a as well as bacterial type 1b and type 2 PDF structures. Their conclusions were that, in comparison with the bacterial PDFs, the distinctive features of hsPDF were that the S1′ pocket was narrower and the S2′ and S3′ pockets were less well defined. Additionally the active site entrance for hsPDF is different to the bacterial PDFs. Despite the observation that the binding of actinonin is broadly conserved, when comparing hsPDF with bacterial PDFs the subtle differences in protein structure could offer opportunities for selective inhibitors of the different PDFs. Indeed some reports of selective bacterial PDF (Boularot et al. 2007) and selective hsPDF (Antczak et al. 2011) inhibitors have emerged. It should also be noted that selectivity on bacterial cells over mammalian cells can be achieved (see for example Waller et al. 2002). This finding might suggest that there is a disconnection between the selectivity observed in biochemical assays and observed efficacy in bacteria versus mammalian cellular systems, i.e. the selectivity window in cells is greater than the selectivity window in bacterial versus hsPDF enzymatic assays. Such differences might reduce the potential for mechanism of action-based toxicity with PDF inhibitors in the clinic.

Further to the wealth of structural information on PDF, Van Aller et al. (2005) and Tortoris et al. (2011) have performed detailed kinetic analysis on the binding of actinonin and other inhibitors to PDF. Their studies revealed that PDF inhibitors can show time-dependent inhibition despite the observation that there is little conformational change between the apo and ligand-bound protein structures. They rationalised that the interaction between the inhibitor and the metal plays a crucial

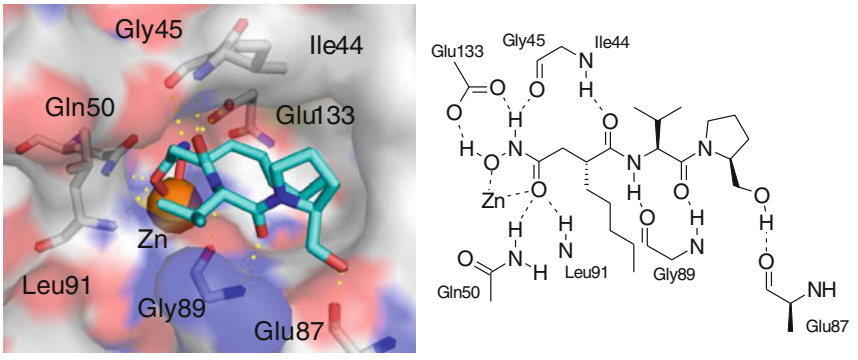


Fig. 15.2 Actinonin bound to *E. coli* PDF (PDB 1LRU)

role for the slow off-rate kinetics; however, neither the nature of the metal or the strain of bacterial PDF are influential in the time dependence. In additional experiments driven by judicious design of ligand structure rather than protein-mutation studies, Tortoris et al. (2011) showed that some hydrogen-bonding interactions can also contribute to the time dependence. They concluded that a long duration of target engagement might be advantageous for cellular efficacy or indeed responsible for the post-antibiotic effect observed for some PDF inhibitors in vivo (Waller et al. 2002). It was also hypothesised by Tortoris et al. (2011) that tuning specific hydrogen-bonding interactions in the chemical scaffold could have an impact on the rate of development of resistant mutations.

15.3.2 Natural Product Inhibitors of PDF

Several research groups identified actinonin as a potent inhibitor of PDF independently (Chen et al. 2000; Apfel et al. 2000; Clements et al. 2001). In the first of these disclosures, actinonin was reported to inhibit various forms of PDF in the low nanomolar range with IC_{50} values of 0.8, 3 and 11 nM recorded against *E. coli* (Fe), *E. coli* (Ni) and *Staphylococcus aureus* (Ni), respectively. Additionally the antibacterial activity across broad spectrum of bacterial strains was assessed and the data indicated that actinonin was effective against Gram-positive and Gram-negative strains (*S. aureus* MIC 8–16 μg/ml; *Streptococcus pneumonia*e MIC 8 μg/ml, *Haemophilus influenzae* MIC 1–2 μg/ml; *Moraxella catarrhalis* MIC 0.5 μg/ml). To verify that the antibacterial activity observed for actinonin was a consequence of PDF inhibition, Chen et al. (2000) used an arabinose promoter exchange technique to regulate the expression of the *def* gene that encodes PDF. In this study, they illustrated that the MIC of actinonin is influenced by PDF expression levels. Actinonin was also shown to act via a bacteriostatic mode of action in *S. aureus* in time-kill experiments. Despite encouraging in vitro data on actinonin, poor pharmacokinetics precluded the progression of this natural product into in vivo infection models (Clements et al. 2001).

Other naturally occurring molecules that are effective inhibitors of PDF have been described (Fig. 15.1). Chu et al. (2001) reported the isolation of two pseudopeptidic compounds, Sch 382582 and Sch 382583, from a *Streptomyces* sp. culture. These compounds containing a carboxylic acid, which is presumably the metal-binding motif, were found to have K_i* values of ~60 nM against PDF making them two of the most potent non-hydroxamate PDF inhibitors described to date. Both compounds showed some antibacterial activity against a sensitive *E. coli* strain (MIC 32 μg/ml). The absolute stereochemical configuration of these natural products was later elucidated via total synthesis (Coats et al. 2004). No further studies have been reported on these compounds.

(-)-Fumimycin, a non-peptidic natural product derived from *Aspergillus fumisynnematus*, has been described as a PDF inhibitor by Kwon et al. (2007). The reported IC_{50} against *S. aureus* PDF is 4.1 μM and it shows weak antibacterial

activity against strains of *S. aureus* (MRSA MIC 100 μg/ml). Through total synthesis, the natural configuration of (-)-fumimycin was determined as the *S*-isomer (Gross et al. 2010). This compound contains a carboxylic acid group and also a catechol-like moiety, either of which could be postulated as the metal-binding motif. Structurally related compounds, FR198248 and FR202306 (Kwon et al. 2010) from *Aspergillus flavipes* that also contain a dihydroxylated phenol moiety, but lack a carboxylic acid group, have been reported as PDF inhibitors. These compounds retain micromolar activity against *S. aureus* PDF and show modest antibacterial activity (*S. aureus* MICs ~25 μg/ml). Interestingly when the 6-position hydroxyl group is replaced with a methoxy group (i.e. R1 = OMe) no activity up to 100 μM on *S. aureus* PDF was detected, suggesting that the dihydroxy phenol might be crucial for binding. The same research group (Yoo et al. 2006) have presented the macrocyclic natural product macrolactin N (structure not shown) isolated from *Bacillus subtilis* as a PDF inhibitor with a *S. aureus* IC_{50} of 7.5 μM but only weak antibacterial activity (*S. aureus* MIC_{50} 100 μg/ml).

15.3.3 Structure–Activity Relationships (SAR) of Actinonin-Related PDF Inhibitors

Many of the PDF inhibitors described in the literature are structurally related to actinonin in that they contain a bidentate metal-chelating moiety (that interacts with the metal ion in PDF) attached to a pseudopeptidic backbone that occupies the S1′–S3′ region of PDF. An overview of the key enzymatic SAR features of the actinonin analogs is illustrated in Fig. 15.3. Smith et al. (2002) conducted a thorough study on the nature of the metal-binding group and concluded that either the HA found in actinonin or the related N-formyl hydroxylamine (reverse hydroxamate) is preferred. A methylene spacer between the metal-binding group and the P1′ substituent seems to be optimal and in the case of HAs Jain et al. (2003) demonstrated that α-substituted fluoro or hydroxyl groups might have advantages in terms of in vitro potency or in vivo properties. Side chains in the P1′ position of the inhibitor that mimic the methionine residue (found in the natural substrates) and occupy the hydrophobic S1′ pocket are favoured. Either an *n*-butyl or a cyclopentylmethyl group in this position seems to provide the best shape and electrostatic match (Davies et al. 2003). It has been demonstrated that there is much more flexibility with the substituents that can be introduced in the P2′ and P3′ regions of the inhibitor (Aubart and Zalacain 2006) and fine tuning the region of the inhibitor, especially to balance a desirable antibacterial profile and appropriate pharmacokinetic properties, has been the focus of many research groups.

There have been other noteworthy SAR discoveries reported in the literature. Hu et al. (2004) noted from X-ray structures that the P1′ and P3′ side chains are in close proximity and they prepared several examples of potent macrocyclic PDF inhibitors. Removal of asymmetric centres has been investigated; for example,

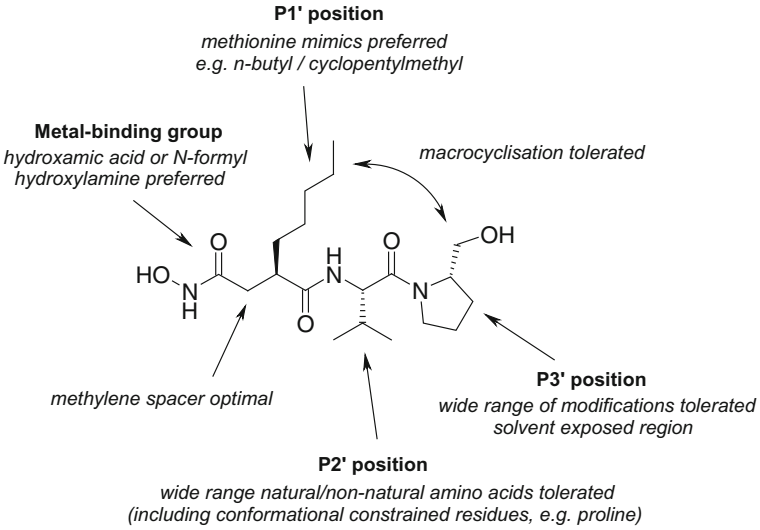


Fig. 15.3 Summary of bacterial PDF IC_{50} SAR of actinonin

Hackbarth et al. (2002) synthesised a series of N-alkyl urea HAs in which the P1′ side chain is attached to a nitrogen atom rather than to a carbon. A representative compound in this subseries of PDF inhibitors is VRC4307 (Fig. 15.4). Aubart et al. (2010) made stepwise modifications in P2′ and P3′ to a series of reverse hydroxamates before combining these changes to provide a series of hydrazino-pyrimidines with GSK246 identified as a key representative of this series. In addition, many groups have published non-peptidic PDF inhibitors, although this falls outside the scope of this review. Aubart and Zalacain (2006) have provided a comprehensive analysis of the literature up to 2006 on both pseudopeptidic and non-peptidic PDF inhibitors.

The antibacterial activity of pseudopeptidic PDF inhibitors has been examined in detail. The opportunity for broad-spectrum antibacterials as a consequence of similar potencies being noted on Gram-positive and Gram-negative PDF enzymes in vitro was not realised because of the limited activity observed on fastidious Gram-negative strains such as *Pseudomonas aeruginosa*. This lack of activity is largely the result of recognition by effective efflux systems in these strains (see Sect. 15.6). What has emerged from the plethora of literature available is that this class of antibacterials is perhaps best suited for the treatment of respiratory tract infections (RTIs) because these compounds demonstrate good antibacterial activity against strains of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Many studies on drug-sensitive and drug-resistant strains of *S. aureus* have also shown that PDF inhibitors work effectively on these pathogens and so their application in skin and soft tissue infections (SSTIs) is also being investigated and there is emerging in vivo data to support this application.

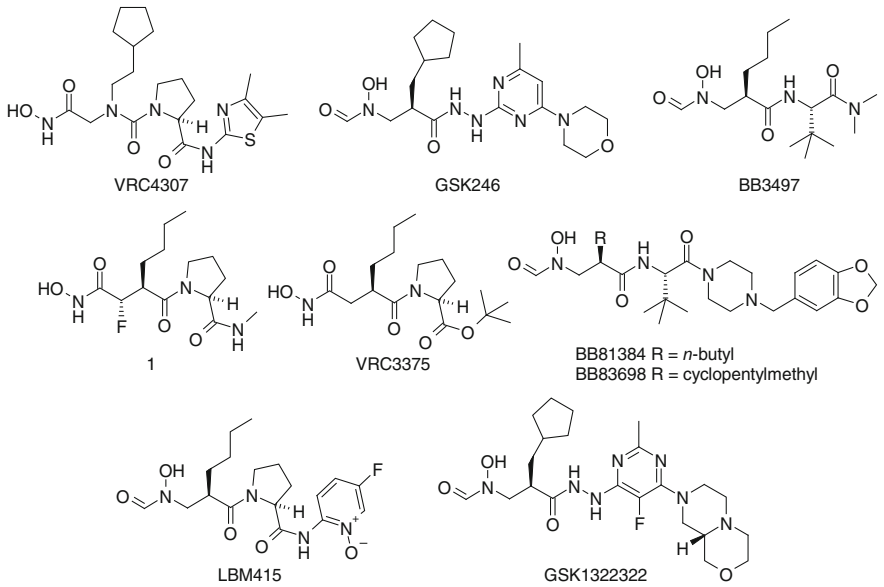


Fig. 15.4 Preclinical and clinical PDF inhibitors

15.4 In vivo Profiling of PDF Inhibitors

15.4.1 Preclinical Compounds

Several in vivo proof-of-concept studies with PDF inhibitors have been presented in the literature and the in vitro profiles of these compounds are shown in Table 15.1. A prototypical synthetic PDF inhibitor BB3497 was reported to have ED_{50} values of 7–14 mg/kg in *S. aureus* systemic infection models (including an MRSA strain) when dosed intravenously or orally (Clements et al. 2001). Hackbarth et al. (2002) identified compounds in the N-alkyl urea series that demonstrated modest in vivo activity in a mouse *S. aureus* septicaemia model of infection, via subcutaneous administration. VRC4307 showed an ED_{50} of 18 mg/kg in this model. No protection was given to mice when they were dosed orally at 30 mg/kg in this model.

Jain et al. (2003) reported ED_{50} values for several compounds from the α-substituted HAs, including compound 1, which when dosed orally in the mouse *S. aureus* septicaemia model showed an ED_{50} of 10 mg/kg, despite its modest oral bioavailability (F = 11 %). The prototype compound in this series, VRC3375 (Jain et al. 2003; Chen et al. 2004), also showed efficacy in the mouse *S. aureus* model with an ED_{50} of 26 mg/kg via oral administration. In depth characterisation of the compound BB81384, structurally related to BB3497, was reported by Gross et al. (2004). This study was the first positive data reported on a PDF inhibitor in pneumococcal infection models. BB81384 shows an ED_{50} of 30 mg/kg in a lethal

Table 15.1 In vitro profile of in vivo active preclinical PDF inhibitors

Assay		BB-3497	VRC4307	1	VRC3375	BB-81384	GSK246
E. coli PDF (Ni) IC_{50} [nM]		7	2	1	4	60[a]	4[b]
MIC [μg/ml]	*S. pneumoniae*	8	1	2–4	8–32	0.06–0.5	2[c]
	H. influenzae	0.25	2–4	0.5–1	2–4	2–4	2[c]
	M. catarrhalis	nd	0.06	nd	0.25	0.125	nd
	S. aureus	4–16	0.5–1	2–4	1–4	4	8[c]
In vivo model		*S. aureus*	*S. aureus*	*S. aureus*	*S. aureus*	*S. pneumoniae*	*S. aureus* *S. pneumoniae* *H. influenzae*

nd = no data

[a] *S. pneumoniae* PDF (Ni) IC_{50} 9 nM

[b] IC_{50} is against *S. pneumoniae* PDF (Ni)

[c] MIC_{90} values

S. pneumoniae peritonitis model and it also demonstrates efficacy in lung and neutropenic mouse thigh murine infection models when dosed orally. Aubart et al. (2010) presented data on GSK246 in mice infection models. Oral efficacy was achieved using strains of *S. pneumoniae* and *S. aureus*, but also the first positive data in a *H. influenzae* model were demonstrated when the compound was dosed at 300 mg/kg.

15.4.2 Clinical Candidates

By 2011, three PDF inhibitors had been progressed into clinical trials (Fig. 15.4). The frontrunner compounds, both reverse hydroxamates, were the intravenous BB83698 (British Biotech/Genesoft/Oscient) and the orally available LBM415 (Novartis/Vicuron); however, these compounds either dropped out of the clinic or at least development activities have stalled. More recently GSK1322322 (Glaxo-SmithKline), also a reverse hydroxamate, advanced into the clinic and as of early 2012 was in Phase II (see Kirst, this volume). Head-to-head comparisons of selected in vitro pharmacology data for all three of the clinical candidates are reported in Table 15.2 (Bouchillon et al. 2010; Lofland et al. 2004; Watters et al. 2006). The antibacterial activity profiles are broadly similar although LBM415 and GSK1322322 seem to have a better spectrum of activity against *H. influenza*e species and BB83698 a better spectrum of activity against *S. pneumoniae*. These differences in the activities are perhaps indicative of the delicate balance between activity on Gram-positive and Gram-negative organisms.

Azoulay-Dupuis et al. (2004) investigated BB83698 in multiple challenging models of mouse pneumococcal pneumonia. The studies involved penicillin-susceptible, penicillin-susceptible/macrolide-resistant and two quinoline-resistant strains of *S. pneumoniae*. In all four studies, BB83698 was dosed subcutaneously (80 mg/kg 12 h post infection or 160 mg/kg 24 h post infection) and the survival rates were assessed after 10 days. BB83698 provided excellent protection to the mice (70–100 % survival) compared with the control animals that typically died at 2–4 days post infection. In additional studies, Azoulay-Dupuis et al. (2004) showed that BB83698 exerts a bactericidal effect in vivo following experiments on *S. pneumoniae* (penicillin-susceptible strain) infected mice. In a neutropenic mouse *S. pneumoniae* infection model, BB83698 was also shown to demonstrate efficacy with the potential for once a day dosing on the basis of the AUC/MIC ratio-driving efficacy (Waller et al. 2002). A bactericidal effect was also confirmed in this study.

A detailed summary of the preclinical animal PK data on BB83698 together with the first human PK data from the Phase I trial was reported by Ramanathan-Girish et al. (2004). Dose-dependent increases in C_{max} and AUC were observed in mice (10 mg and 50 mg single iv doses) and rats (10 mg, 22 mg, 50 mg iv q.d for 28 days) with a slightly more than dose-proportional effect observed in both animals at the highest dose. There were no adverse effects recorded in these

Table 15.2 Selected in vitro data for clinically relevant PDF inhibitors

Organism	BB-83698[a] MIC (μg/ml)			LBM-415[b] MIC (μg/ml)			GSK1322322[c] MIC (μg/ml)		
	50 %	90 %	Range	50 %	90 %	Range	50 %	90 %	Range
S. pneumoniae	0.25	0.25–0.5	0.015–1 (n = 213)	0.5	2	≤0.016– > 4 (n = 2159)	1	2	≤0.03–4 (n = 961)
H. influenzae	8–16	32–64	0.06–128 (n = 110)	2	4	≤0.016–32 (n = 2845)	2	4	≤0.03–32 (n = 2553)
M. catarrhalis	0.06–0.12	0.12	0.004–0.25 (n = 50)	0.25	0.5	0.03–2 (n = 135)	1	1	0.06–2 (n = 115)
S. aureus	4	8	1–256 (n = 154)	0.5	1	≤0.016– ≥ 32 (n = 8886)	2	4	0.12–8 (n = 809)
S. pyogenes	0.06	0.12	0.015–0.25 (n = 21)	nd	nd	nd	0.5	0.5	0.06–1 (n = 398)

n = number of isolates; nd = no data

[a] Lofland et al. (2004)

[b] Watters et al. (2006)

[c] Bouchillon et al. (2010)

animals. In dogs (10 mg, 22 mg, 50 mg q.d for 28 days) the PK showed good linearity during dose infusion range finding studies, but in addition to emesis which was common in all animals, CNS side effects were observed following doses of ≥75 mg when dosed over 1 h. These adverse events were attributed to high C_{max} levels because when an infusion, even at the highest dose (175 mg), was administered over 6 h the side effects were managed despite an increase in the AUC.

In the clinical study, BB83698 was administered to healthy volunteers in incremental doses ranging from 10 mg to 475 mg. The PK show good linearity with only minor deviations observed at the lowest and highest doses. Exposure levels were in good agreement with levels predicted on the basis of allometric scaling and at the highest dose sufficient blood levels (and hence AUC/MIC ratio) were obtained to suggest that this dose would be therapeutically relevant (theoretical on the basis of an MIC 0.25 μg/ml for BB83698 in *S. pneumoniae* strain). Overall the first clinical study on BB83698 indicated the potential for once-daily dosing in humans. The reason for the cessation of BB83698 development has not been disclosed.

Efficacy data for LBM415 in several Gram-positive mouse infection models have been reported by Osbourne et al. (2009). In two *S. aureus* systemic model (MSSA strains), ED_{50} values for LBM415 following oral doses at 1 h and 5 h post infection were 2.3 mg/kg and 13.2 mg/kg. The compound was also active following oral administration in an MRSA systemic infection model with an ED_{50} of 5.9 mg/kg (doses at 0 h, 4 h and 23 h post infection). Further support for the potential of PDF inhibitors in the treatment of MRSA was provided by examining LBM415 in a *S. aureus* thigh infection experiment. Bacterial counts dropped from 7.5 $\log_{10}$ in the control animals to 3.6 $\log_{10}$ at the highest oral dose of LBM415 (50 mg/kg t.i.d for 2 days). In *S. pneumoniae* systemic models, LBM415 was efficacious following oral administration in a PSSP infection model (ED_{50} 6.4 mg/kg t.i.d for 4 days) and multi-drug-resistant infection study (ED_{50} 36.6 mg/kg b.i.d for 3 days). LBM415 also showed efficacy in a *S. pneumoniae* lung infection model with an ED_{50} of 23.3 mg/kg when dosed orally (b.i.d for 3 days at 16 h post infection).

Rolan et al. (2011) reported the human pharmacokinetic and safety profile of LBM415 from the Phase I clinical trial. In single-dose experiments (100–3,000 mg) LBM415 showed approximately dose-dependent pharmacokinetics and it was well tolerated by the study groups. In the multiple-dose experiments, there were no significant differences in peak concentration and exposure levels during the 11 days of dosing. At the projected therapeutic dose in humans (1,000 mg b.i.d) LBM415 was well tolerated with no significant adverse events recorded but when the drug was dosed at 1,000 mg t.i.d, cyanosis that could be reversed was observed. It was proposed that this adverse effect of LBM415 on multiple dosing at the highest concentration was the result of methemoglobinemia caused by a metabolite of the drug that subsequently was found to form in vivo only in monkeys but not in rats and dogs, the species in which the original preclinical toxicity studies were performed. This publication findings suggest why LBM415 failed to progress in clinical trials.

GSK1322322 has been evaluated in three mouse *S. aureus* abscess infection models (Lewandowski et al. 2010a). Efficacy was demonstrated at the lowest dose (37.5 mg or 75 mg po b.i.d) in all three studies. The *S. aureus* infections in these experiments were generated from quinoline/macrolide-resistant, multi-drug-resistant and Panton–Valentine positive strains. Efficacy has also been demonstrated in a rat abscess model (Singley et al. 2010) and in mice *S. pneumoniae* and *H. influenzae* infections (Lewandowski et al. 2010b). Preliminary human pharmacokinetics studies from Phase I trials revealed that GSK1322322 was well tolerated in healthy volunteers up to a dose of 1,500 mg q.d via oral administration (Naderer et al. 2010). No adverse events other than headache were reported in the study.

15.5 Recent Developments in PDF Inhibition

Recent literature on PDF inhibitors has been largely focused on compounds that contain features either inspired by or closely related to the structures of the three clinical candidates (Fig. 15.5). Shi et al. (2010, 2011) explored analogs of LBM415 where the P2′ proline was replaced with either a 2,5-dihydropyrrole or a 3-methylenepyrrolidine. Analogues 2 and 3 were two of the most promising compounds in these chemical series and they exhibit good antibacterial activities on drug-sensitive and drug-resistant Gram-positive organisms (MICs 0.0625–1 μg/ml) but consistent with previous findings they are less active against strains of *H. influenzae* (Compound 2 MIC 2–8 μg/ml). Yu et al. (2011) replaced the P2′ proline with an oxazolidine and they reported antibacterial activities for compound 4 were comparable with LBM415 in a head-to-head study. East et al. (2011) substituted the P2′ proline motif with an azaproline or azapipecolinic acid to give compounds with a single stereocentre such as 5. In several cases the trend was towards PDF inhibitors

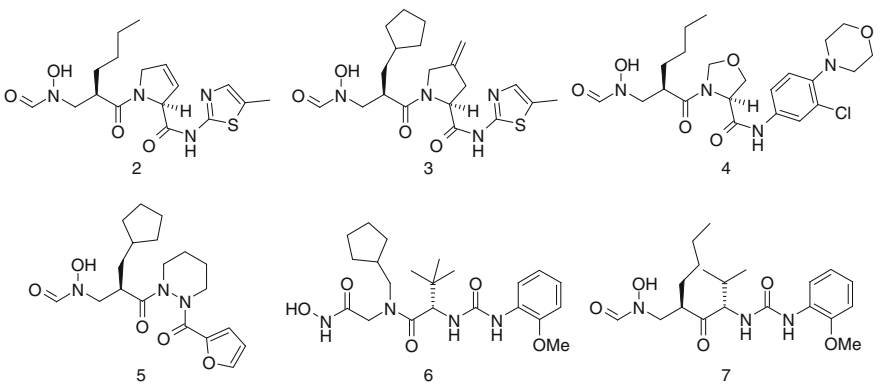


Fig. 15.5 Recent PDF inhibitors (2010–2011)

with improved activity against *H. influenzae* (Compound 5: PDF Ni.*E. coli* IC_{50} 5 nM; *S. pneumoniae* MIC 0.5–2 μg/ml; *H. influenzae* MIC < 0.125–0.25 μg/ml).

Lee et al. (2010, 2011) described PDF inhibitors containing a urea functionality. The retro-amide hydroxamic scaffold as exemplified by compound 6 contains only one stereocentre with the P1′ side chain appended to the nitrogen atom on the backbone of the inhibitor. In a related report the N-formyl hydroxylamine 7 is described. Activities of both classes of compounds are sub 50 nM on *P. aeruginosa* PDF with MICs in the range of 0.1–0.2 μg/ml on RTI organisms. In all of the recent examples only in vitro properties are described and it remains to be seen whether modifications to the pseudopeptidic backbone can offer advantages in terms of an in vivo profile.

15.6 Resistance Mechanisms

Spontaneous resistance in Gram-positive and Gram-negative organisms following incubation with pseudopeptidic PDF inhibitors has been reported to arise at frequencies from 10^{-6} to 10^{-10}. Mechanisms of resistance can be divided into three categories: (i) circumventing the formylation–deformylation pathway; (ii) target-based modification/overexpression and (iii) efflux.

Mutations in the formyltransferase gene (*fmt*) have been observed in Gram-positive organisms such as *S. aureus* and also Gram-negatives such as *E. coli*, *H. influenzae* and *P. aeruginosa* as summarised by Giglione and Meinnel (2001). As a consequence of *fmt* mutations, transformylase activity to install the N-formyl group on methionine-tRNA is compromised and consequently deformylation is rendered unnecessary. This has been confirmed in follow-up experiments which showed that PDF inhibitors are ineffective when tested against the mutated strains. Without the formyltransferase activity, the bacteria have slower growth rates that could compromise the development of this mechanism of resistance in the clinic. Indeed, Margolis et al. (2000) and Clements et al. (2001) showed, albeit in pre-clinical models, that mutant strains resistant to PDF inhibitors require higher inocula to generate infections in mice. Two additional modes of bypassing the formylation–deformylation cycle have been described by Duroc et al. (2009). In *B. subtilis* mutations to the *folD* and *glyA* genes that are upstream in the pathway from formyltransferase were observed. These genes encode proteins that are responsible for the production of the N-formyl group.

Two types of target-based resistance to PDF inhibitors have been demonstrated in vitro. In *S. pneumoniae*, spontaneous resistance mutants were generated at a frequency $<10^{-8}$ using actinonin (Margolis et al. 2001). The mutations were found to occur in the *def* gene that encodes PDF and the mutated bacteria were less susceptible to actinonin compared with the wild type upon retreatment. Knockout studies in *S. pneumoniae* demonstrated that the *fmt* and *def* genes were both crucial for survival. These results, together with the observation that the mutations were found to occur in the ligand/metal-binding site, suggest that a PDF inhibitor might

select specific resistant deformylase mutants but these mutants might remain susceptible to structurally different inhibitors. Serial passage experiments in *S. pneumoniae* using the clinical compound LBM415 also showed that mutations in the deformylase ligand-binding site were responsible for reduced MICs upon retreatment of inhibitor (Kosowska-Shick et al. 2007). Dean et al. (2007) described overexpression of deformylase in *H. influenzae* as an alternative target-based resistance mechanism that reduces the effectiveness of PDF inhibitors such as LBM415.

Several groups (see for example Chen et al. 2000 and Clements et al. 2001) observed that *acr* efflux pump knockout strains of Gram-negative organisms *H. influenzae* and *E. coli* showed increased susceptibility to PDF inhibitors such as actinonin and BB3497, suggesting that pseudopeptidic compounds were good substrates for efflux pumps. Dean et al. (2005) demonstrated that this was also the case for the clinical compound LBM415, which showed reduced susceptibility to strains of *H. influenzae* with AcrAB-TolC pump activity. More recently, Mamelli et al. (2009) indicated that efflux pumps are also effective in other Gram-negative bacteria such as *Enterobacter aerogenes*. The same group reported that non-peptidic PDF inhibitors might be less susceptible to the efflux mechanisms but instead have compromised uptake rates.

Preclinically, Azoulay-Dupuis et al. (2004) searched for evidence of resistance in an in vivo setting by dosing suboptimal levels of BB83698 for 3 days to *S. pneumoniae* infected mice. There was no evidence for resistant strains emerging from this study and the authors concluded that resistance frequencies were low in this lung model. In the absence of any clinical data, the emergence and prevalence of resistant mechanisms to PDF inhibitors have yet to be determined.

15.7 Conclusions

Following pioneering work by the academic community in the 1990s, bacterial PDFs emerged as biological targets of significant importance in the search for new antibacterial agents with novel mechanisms of action. The flurry of interest in these targets, spurred on by structure-based drug design, culminated in the progression of two PDF inhibitors into the clinic in the early 2000s; however, both of these compounds subsequently stalled in development. With the identification of eukaryotic PDFs and concerns about their role, particularly in mammalian systems, the early enthusiasm for PDF as a druggable antibacterial target faded somewhat in parallel with many pharmaceutical companies moving away from antibacterial drug discovery. More recently, the outlook seems promising as GlaxoSmithKline moved the third PDF inhibitor, GSK1322322, into the clinic and by early 2012 this compound had progressed into Phase II trials for RTIs and SSTIs. It therefore remains to be seen whether bacterial PDF will be proven to be a safe, relevant and new molecular target for antibacterial chemotherapy in hospital and community settings.

References

Adams JM (1968) On the release of the formyl group from nascent protein. J Mol Biol 33:571–589

Antczak C, Shum D, Bassit B, Frattini MG, Li Y, de Stanchina E, Scheinberg DA, Djaballah H (2011) Identification of benzofuran-4,5-diones as novel and selective non-hydroxamic acid, non-peptidomimetic based inhibitors of human peptide deformylase. Bioorg Med Chem Lett 21:4528–4532

Apfel C, Banner DW, Bur D, Dietz M, Hirata T, Hubschwerlen C, Locher H, Page MGP, Pirson W, Rossé G, Specklin J-L (2000) Hydroxamic acid derivatives as potent peptide deformylase inhibitors and antibacterial agents. J Med Chem 43:2324–2331

Aubart K, Zalacain M (2006) Peptide deformylase inhibitors. Prog Med Chem 44:109–143

Aubart K, Benowitz A, Campobasso N, Dreabit J, Fang Y, Karpinski J, Kelly S, Liao X, Lee D, Mercer D, Lewandowski T, Van Aller G, Zonis R, Christensen S, Zalacain M (2010) Hydrazinopyrimidines as a new class of peptide deformylase inhibitors. F1-2110 paper presented at 50th annual interscience conference on antimicrobial agents and chemotherapy meeting, Boston, MA, USA, 12–15 Sept 2010

Azoulay-Dupuis E, Mohler J, Bédos JP (2004) Efficacy of BB-83698, a novel peptide deformylase inhibitor, in a mouse model of pneumococcal pneumonia. Antimicrob Agents Chemother 48:80–85

Bouchillon S, Hackel M, Hoban D, Zalacain M, Butler D (2010) In vitro activity of GSK1322322, a novel peptide deformylase inhibitor, against 4,836 pathogens from skin and soft tissue infections and respiratory tract infections. F1-2112 paper presented at 50th annual interscience conference on antimicrobial agents and chemotherapy meeting, Boston, MA, USA, 12–15 Sept 2010

Boularot A, Giglione C, Petit S, Duroc Y, Alves de Sousa R, Larue V, Cresteil T, Dardel F, Artaud I, Meinnel T (2007) Discovery and refinement of a new structural class of potent peptide deformylase inhibitors. J Med Chem 50:10–20

Bouzaidi-Tiali N, Giglione C, Bulliard Y, Pusnik M, Meinnel T, Schneider A (2007) Type 3 peptide deformylases are required for oxidative phosphorylation in *Trypanosoma brucei*. Mol Microbiol 65:1218–1228

Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC, Margolis PS, Wu C, Ni Z-J, Trias J, White RJ, Yuan Z (2000) Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry 39:1256–1262

Chen D, Hackbarth C, Ni ZJ, Wu C, Wang W, Jain R, He Y, Bracken K, Weidmann B, Patel DV, Trias J, White RJ, Yuan Z (2004) Peptide deformylase inhibitors as antibacterial agents: identification of VRC3375, a proline-3-alkylsuccinyl hydroxamate derivative, by using an integrated combinatorial and medicinal chemistry approach. Antimicrob Agents Chemother 48:250–261

Chu M, Mierzwa R, He L, Xu L, Gentile F, Terracciano J, Patel M, Miesel L, Bohanon S, Kravec C, Cramer C, Fischman TO, Hruza A, Ramanathan L, Shipkova P, Chan T-M (2001) Isolation and structure elucidation of two novel deformylase inhibitors produced by *Streptomyces* sp. Tetrahedron Lett 42:3549–3551

Clements JM, Beckett RP, Brown A, Catlin G, Lobell M, Palan S, Thomas W, Whittaker M, Wood S, Salama S, Baker PJ, Rodgers HF, Barynin V, Rice DW, Hunter MG (2001) Antibiotic activity and characterization of BB-3497, a novel peptide deformylase inhibitor. Antimicrob Agents Chemother 45:563–570

Coats RA, Lee S-L, David KA, Patel KM, Rhoads EK, Howard MH (2004) Stereochemical definition and chirospecific synthesis of the peptide deformylase inhibitor Sch 382583. J Org Chem 69:1734–1737

Davies SJ, Ayscough AP, Beckett RP, Bragg RA, Clements JM, Doel S, Grew C, Launchbury SB, Perkins GM, Pratt LM, Smith HK, Spavold ZM, Thomas SW, Todd RS, Whittaker M (2003) Structure-activity relationships of the peptide deformylase inhibitor BB-3497:

modification of the methylene spacer and the P1′ side chain. Bioorg Med Chem Lett 13:2709–2713

Dean CR, Narayan S, Daigle DM, Dzink-Fox JL, Puyang X, Bracken KR, Dean KE, Weidmann B, Yuan Z, Jain R, Ryder NS (2005) Role of the AcrAB-TolC efflux pump in determining susceptibility of *Haemophilus influenzae* to the novel peptide deformylase inhibitor LBM415. Antimicrob Agents Chemother 49:3129–3135

Dean CR, Narayan S, Richards J, Daigle DM, Esterow S, Leeds JA, Kamp H, Puyang X, Wiedmann B, Mueller D, Voshol H, van Oostrum J, Wall D, Koehn J, Dzink-Fox J, Ryder NS (2007) Reduced susceptibility of *Haemophilus influenzae* to the peptide deformylase inhibitor LBM415 can result from target protein overexpression due to amplified chromosomal def gene copy number. Antimicrob Agents Chemother 51:1004–1010

Duroc Y, Giglione C, Meinnel T (2009) Mutations in three distinct loci cause resistance to peptide deformylase inhibitors in *Bacillus subtilis*. Antimicrob Agents Chemother 53:1673–1678

East SP, Ayscough A, Toogood-Johnson I, Taylor S, Thomas W (2011) Peptidomimetic inhibitors of bacterial peptide deformylase. Bioorg Med Chem Lett 21:4032–4035

Escobar-Alvarez S, Goldgur Y, Yang G, Ouerfelli O, Li Y, Scheinberg DA (2009) Structure and activity of human mitochondrial peptide deformylase, a novel cancer target. J Mol Biol 387:1211–1228

Escobar-Alvarez S, Gardner J, Sheth A, Manfredi G, Yang G, Ouerfelli O, Heaney ML, Scheinberg DA (2010) Inhibition of human peptide deformylase disrupts mitochondrial function. Mol Cell Biol 30:5099–5109

Fu H, Dahlgren C, Bylund J (2003) Subinhibitory concentrations of the deformylase inhibitor actinonin increase bacterial release of neutrophil-activating peptides: a new approach to antimicrobial chemotherapy. Antimicrob Agents Chemother 47:2545–2550

Giglione C, Meinnel T (2001) Resistance to anti-peptide deformylase drugs. Expert Opin Ther Targets 5:415–418

Gordon JJ, Kelly BK, Miller GA (1962) Actinonin: an antibiotic substance produced by an actinomycete. Nature 195:701–702

Gordon JJ, Devlin JP, East AJ, Ollis WD, Sutherland IO, Wright DE, Ninet L (1975) Studies concerning the antibiotic actinonin. Part 1. The constitution of actinonin. A natural hydroxamic acid with antibiotic activity. J Chem Soc Perkin 1:819–825

Groche D, Becker A, Schlichting I, Kabsch W, Schultz S, Wagner AFV (1998) Isolation and crystallization of functionally competent *Escherichia coli* peptide deformylase forms containing either iron or nickel in the active site. Biochem Biophys Res Commun 246:342–346

Gross M, Clements J, Beckett RP, Thomas W, Taylor S, Lofland D, Ramanathan-Girish S, Garcia M, Difuntorum S, Hoch U, Chen H, Johnson KW (2004) Oral anti-pneumococcal activity and pharmacokinetic profiling of a novel peptide deformylase inhibitor. J Antimicrob Chemother 53:487–493

Gross PJ, Furche F, Nieger M, Bräse S (2010) Asymmetric total synthesis of (+)-fumimycin via 1,2-addition to ketimines. Chem Commun 46:9215–9217

Guilloteau J-P, Mathieu M, Giglione C, Blanc V, Dupuy A, Chevrier M, Gil P, Famechon A, Meinnel T, Mikol V (2002) The crystal structures of four peptide deformylases bound to the antibiotic actinonin reveal two distinct types: a platform for the structure-based design of antibacterial agents. J Mol Biol 320:951–962

Hackbarth CJ, Chen DZ, Lewis JG, Clark K, Mangold JB, Cramer JA, Margolis PS, Wang W, Koehn J, Wu C, Lopez S, Withers G III, Gu H, Dunn E, Kulathila R, Pan S-H, Porter WL, Jacobs J, Trias J, Patel DV, Weidmann B, White RJ, Yuan Z (2002) N-alkyl urea hydroxamic acids as a new class of peptide deformylase inhibitors with antibacterial activity. Antimicrob Agents Chemother 46:2752–2764

Hu X, Nguyen KT, Jiang VC, Lofland D, Moser HE, Pei D (2004) Macrocyclic inhibitors for peptide deformylase: a structure-activity relationship study of the ring size. J Med Chem 47:4941–4949

Jain R, Sundram A, Lopez S, Neckermann G, Wu C, Hackbarth C, Chen D, Wang W, Ryder NS, Weidmann B, Patel D, Trias J, White R, Yuan Z (2003) α-Substituted hydroxamic acids as novel bacterial deformylase inhibitor-based antibacterial agents. Bioorg Med Chem Lett 13:4223–4228 (Erratum in: Bioorg Med Chem Lett 2004 14:2373–2374)

Kosowska-Shick K, Credito KL, Pankuch GA, DeWasse B, McGhee P, Appelbaum PC (2007) Multistep resistance selection and postantibiotic-effect studies of the antipneumococcal activity of LBM415 compared to other agents. Antimicrob Agents Chemother 51:770–773

Kwon Y-J, Sohn M-J, Zheng C-J, Kim W-G (2007) Fumimycin: a peptide deformylase inhibitor with an unusual skeleton produced by *Aspergillus fumisynnematus*. Org Lett 9:2449–2451

Kwon Y-J, Zheng C-J, Kim W-G (2010) Isolation and identification of FR198248, a hydroxylated 1,3-dihydroisobenzofuran, from *Aspergillus flavipes* as an inhibitor of peptide deformylase. Biosci Biotechnol Biochem 74:390–393

Lee MD, She Y, Soskis MJ, Borella CP, Gardner JR, Hayes PA, Dy BM, Heaney ML, Philips MR, Bornmann WG, Sirotnak FM, Scheinberg DA (2004) Human mitochondrial peptide deformylase, a new anticancer target of actinonin-based antibiotics. J Clin Invest 114:1107–1116

Lee SK, Choi KH, Lee SJ, Suh SW, Kim BM, Lee BJ (2010) Peptide deformylase inhibitors with retro-amide scaffold: synthesis and structure-activity relationships. Bioorg Med Chem Lett 20:4317–4319

Lee SK, Choi KH, Lee SJ, Lee JS, Park JY, Kim BM, Lee BJ (2011) Peptide deformylase inhibitors with non-peptide scaffold: synthesis and structure-activity relationships. Bioorg Med Chem Lett 21:133–136

Lewandowski T, Demarsh P, Peters T, Kulkarni S (2010a) Potent activity of GSK1322322 a novel peptide deformylase inhibitor after oral dosing in a murine multi-drug resistant *Staphylococcus aureus* infection. F1-2113 paper presented at 50th annual interscience conference on antimicrobial agents and chemotherapy meeting, Boston, MA, USA, 12–15 Sept 2010

Lewandowski T, Peters T, Simon N, Kulkarni S (2010b) Potent activity of GSK1322322 a novel peptide deformylase inhibitor in a *Haemophilus influenzae* and *Streptococcus pneumoniae* respiratory tract infection model. F1-2115 paper presented at 50th annual interscience conference on antimicrobial agents and chemotherapy meeting, Boston, MA, USA, 12–15 Sept 2010

Lofland D, Difuntorum S, Waller A, Clements JM, Weaver MK, Karlowsky JA, Johnson K (2004) J Antimicrob Chemother 53:664–668

Mader D, Rabiet M-J, Boulay F, Peschel A (2010) Formyl peptide receptor-mediated proinflammatory consequences of peptide deformylase inhibition in *Staphylococcus aureus.* Microbes Infect 12:415–419

Maehr H (1971) Antibiotics and other naturally occurring hydroxamic acids and hydroxamates. Pure Appl Chem 28:603–636

Mamelli L, Petit S, Chevalier J, Giglione C, Lieutaud A, Meinnel T, Artaud I, Pagès J-M (2009) New antibiotic molecules: bypassing the membrane barrier of gram negative bacteria increases the activity of peptide deformylase inhibitors. PLoS ONE 4:e6443

Marcker K, Sanger F (1964) N-Formyl-methionyl-S-RNA. J Mol Biol 8:835–840

Margolis PS, Hackbarth CJ, Young DC, Wang W, Chen D, Yuan Z, White R, Trias J (2000) Peptide deformylase in *Staphylococcus aureus*: resistance to inhibition is mediated by mutations in the formyltransferase gene. Antimicrob Agents Chemother 44:1825–1831

Margolis P, Hackbarth C, Lopez S, Maniar M, Wang W, Yuan Z, White R, Trias J (2001) Resistance of *Streptococcus pneumoniae* to deformylase inhibitors is due to mutations in defB. Antimicrob Agents Chemother 45:2432–2435

Muri EMF, Nieto MJ, Sindelar RD, Williamson JS (2002) Hydroxamic acids as pharmacological agents. Curr Med Chem 9:1631–1653

Naderer OJ, Jones LS, Zhu JZ, Kurtinecz MT, Dumont EF (2010) A novel antibacterial peptide deformylase inhibitor (GSK1322322): first time in human safety and pharmacokinetics. F1-1966 paper presented at 50th annual interscience conference on antimicrobial agents and chemotherapy meeting, Boston, MA, USA, 12–15 Sept 2010

Neilands JB (1967) Hydroxamic acids in nature. Science 156:1443–1447
Osborne CS, Neckermann G, Fischer E, Pecanka R, Yu D, Manni K, Goldovitz J, Amaral K, Dzink-Fox J, Ryder NS (2009) In vivo characterization of the peptide deformylase inhibitor LBM415 in murine infection models. Antimicrob Agents Chemother 53:3777–3781
Rajagopalan PTR, Pei D (1998) Oxygen-mediated inactivation of peptide deformylase. J Biol Chem 273:22305–22310
Ramanathan-Girish S, McColm J, Clements JM, Taupin P, Barrowcliffe S, Hevizi J, Safrin S, Moore C, Patou G, Moser H, Gadd A, Hoch U, Jiang V, Lofland D, Johnson KW (2004) Pharmacokinetics in animals and humans of a first-in-class peptide deformylase inhibitor. Antimicrob Agents Chemother 48:4835–4842
Rolan P, Sun H, MacLeod C, Bracken K, Evans TG (2011) Pharmacokinetics and unexpected safety issues of LBM415, a novel oral peptide deformylase inhibitor. Clin Pharmacol Ther 90:256–262
Shi W, Duan Y, Qian Y, Li M, Yang L, Hu W (2010) Design, synthesis, and antibacterial activity of 2,5-dihydropyrrole formyl hydroxyamino derivatives as novel peptide deformylase inhibitors. Bioorg Med Chem Lett 20:3592–3595
Shi W, Ma H, Duan Y, Aubart K, Fang Y, Zonis R, Yang L, Hu W (2011) Design, synthesis and antibacterial activity of 3-methylenepyrrolidine formyl hydroxyamino derivatives as novel peptide deformylase inhibitors. Bioorg Med Chem Lett 21:1060–1063
Singley CM, Hoover J, Demarsh P, Elefante E, Zalacain M (2010) Efficacy of GSK1322322 against *Staphylococcus aureus* in a rat subcutaneous abscess model using a human exposure profile. F1-2114 paper presented at 50th annual interscience conference on antimicrobial agents and chemotherapy meeting, Boston, MA, USA, 12–15 Sept 2010
Smith HK, Beckett RP, Clements JM, Doel S, East SP, Launchbury SB, Pratt LM, Spavold ZM, Thomas W, Todd RS, Whittaker M (2002) Structure-activity relationships of the peptide deformylase inhibitor BB-3497: modification of the metal binding group. Bioorg Med Chem Lett 12:3595–3599
Totoritis R, Duraiswami C, Taylor AN, Kerrigan JJ, Campobasso N, Smith KJ, Ward P, King BW, Murrayz-Thompson M, Jones AD, Van Aller GS, Aubart KM, Zalacain M, Thrall SH, Meek TD, Schwartz B (2011) Understanding the origins of time-dependent inhibition by polypeptide deformylase inhibitors. Biochemistry 50:6642–6654
Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, Koizumi K (1976) A new antifungal antibiotic, trichostatin. J Antibiot 29:1–6
Van Aller GS, Nandigama R, Petit CM, DeWolf WE Jr, Quinn CJ, Aubart KM, Zalacain M, Christensen SB, Copeland RA, Lai Z (2005) Mechanism of time-dependent inhibition of polypeptide deformylase by actinonin. Biochemistry 44:253–260
Waller AS, Clements JM (2002) Novel approaches to antimicrobial therapy: peptide deformylase. Curr Opin Drug Discov Devel 5:785–792
Watters AA, Jones RN, Leeds JA, Denys G, Sader HS, Fritsche TR (2006) Antimicrobial activity of a novel peptide deformylase inhibitor, LBM415, tested against respiratory tract and cutaneous infection pathogens: a global surveillance report (2003–2004). J Antimicrob Chemother 57:914–923
Yoo J-S, Zheng C-J, Lee S, Kwak J-H, Kim W-G (2006) Macrolactin N, a new peptide deformylase inhibitor produced by *Bacillus subtilis*. Bioorg Med Chem Lett 16:4889–4892
Yu L, Zhou W, Wang Z (2011) Synthesis and in vitro antibacterial activity of oxazolidine LBM-415 analogs as peptide deformylase inhibitors. Bioorg Med Chem Lett 21:1541–1544

Chapter 16
Bacterial Topoisomerase Inhibitors: Quinolones and Beyond

Michael J. Pucci and Jason A. Wiles

Abstract Previous studies have established the essentiality of the bacterial DNA replication process and its requisite enzymes including DNA gyrase and topoisomerase IV. The quinolone class of antibiotics provided a clinical validation of these targets and has been widely used over the past 50 years. A number of more recent examples of these compounds are described herein. Two related, but structurally distinct quinolone-like compounds are also discussed. Non-quinolones, designated novel bacterial type II topoisomerase inhibitors (NBTIs), have been explored and several examples are provided in this work. Successful development of these latter compounds could enhance and extend the clinical utility of bacterial topoisomerase inhibitors beyond the clinically valuable and commercially successful quinolone antibiotics. Bacterial topoisomerase inhibitors should continue to play an important role in the battle against bacterial pathogens for many years to come.

16.1 Introduction

The quinolone class of antibiotics has proven to be an important option for the treatment of bacterial infections over the past several decades (Emmerson and Jones 2003). They possess a number of favorable characteristics that have contributed to both their economical and clinical successes. Quinolones target essential topoisomerase enzymes, DNA gyrase, and topoisomerase IV, involved in bacterial DNA replication and are bactericidal, typically, causing a rapid decrease in viable cells (Colin et al. 2011; Drlica et al. 2008; Wang et al. 2010; see Genilloud and Vicente, this volume). This dual target mechanism of action generally results in diminished rates of resistance development. These compounds can

M. J. Pucci (✉) · J. A. Wiles
Achillion Pharmaceuticals, 300 George Street, New Haven, CT 06511, USA
e-mail: mpucci@achillion.com

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_16,

have a relatively broad antimicrobial spectrum covering many important bacterial pathogens, both Gram-positive and Gram-negative, and can be orally and parenterally administered to patients. They also have relatively good pharmacokinetics and tissue distribution with efficacy in a number of different clinical indications including pneumonia, septicemia, and skin and soft tissue infections. Quinolones are also distinct from many other antibacterial drugs in that they are totally synthetic and not natural product derivatives, which can often translate into relatively easy chemical syntheses. Extensive structure-activity relationships (SAR) have been reported over the past several decades (Domagala and Hagen 2003; Mitscher 2005). Despite many advantages, there have been issues associated with members of this drug class including safety (Owens and Ambrose 2005; Rubenstein 2001) and the emergence of clinical resistance (Drlica et al. 2000). However, this has not halted efforts to discover and develop both new quinolones and other unrelated compounds that target bacterial DNA gyrase and/or topoisomerase IV with improved safety and/or coverage of antibiotic-resistant clinical isolates (Bradbury and Pucci 2008; Wiles et al. 2010).

16.2 Four Generations of Quinolones

The first quinolone for antibacterial use was nalidixic acid, based upon an impurity discovered in the chemical synthesis of the antimalarial drug chloroquine over 50 years ago (Lesher et al. 1962). Since then, numerous analogs have been synthesized, some with an improved antibacterial spectrum that has led to a classification into four generations of compounds (Owens and Ambrose 2000). Table 16.1 lists some of the more prominent agents that were developed into drugs. Peak annual sales for ciprofloxacin and levofloxacin have topped the $1 billion mark at some point in their lifecycles. This commercial success coupled with the desirable biological and chemical properties described above has driven continued research and development to the present day (Wiles et al. 2010). In this review, the goal is to highlight some of the more novel quinolone compounds (Table 16.2) that have been reported as well as some unique "quinolone-like" and non-quinolone bacterial topoisomerase inhibitors (Table 16.3) that have been reported over the past several years.

16.2.1 Besifloxacin

Besifloxacin (**1**, Fig. 16.1) is an 8-chloro-fluoroquinolone possessing a 7-membered nitrogen heterocycle at the 7 position. It is marketed by Bausch & Lomb and has potent, bactericidal activity against drug-resistant pathogens (Haas et al. 2009). This drug is sold as a topical ophthalmic agent that has demonstrated safety and efficacy in three bacterial conjunctivitis clinical trials (Tepedino et al. 2009).

Table 16.1 Examples of marketed quinolone antibiotics

Generation	Drug	Antimicrobial spectrum	Clinical indications
First	Nalidixic acid (NegGram)	Gram-negative (not *Pseudomonas*)	UTIs[a]
Second	Norfloxacin (Noroxin) Lomefloxacin (Maxaquin) Ofloxacin (Floxin) Ciprofloxacin (Cipro)	Gram-negatives (incl. *Pseudomonas*), some Gram-positives (not *S. pneumonia*) some atypicals	UTIs, cUTIs[b], STDs[c], SSSIs[d]
Third	Levofloxacin (Levaquin) Gatifloxacin (Tequin) Moxifloxacin (Avelox)	Same as 2nd generation plus expanded Gram-positive (incl. *S. pneumoniae*) and expanded atypicals	Acute exacerbations of chronic bronchitis, community-acquired pneumonia
Fourth	Trovafloxacin (Trovan)	Same as 3rd generation plus anaerobes	Same as preceeding generations (except cUTIs) plus IAIs[e], nosocomial pneumonia

[a] Uncomplicated urinary tract infections
[b] Complicated urinary tract infections
[c] Sexually transmitted diseases
[d] Skin and skin structure infections
[e] Intra-abdominal infections

Table 16.2 Recent quinolones in development

Compound	Company	Development status	Key reference
Besifloxacin	Bausch & Lomb	Market 2009; ophthalmic	Haas et al. (2009)
Delafloxacin	Rib-X	Completed Phase II	Almer et al. (2004)
Finafloxacin	MerLion	Phase II	Higgins et al. (2010)
Nemonoxacin	Taigen Biotechnology	Phase III	Adam et al. (2009)
Prulifloxacin	Optimer	Phase III	Rafailidis et al. (2011)
Zabofloxacin	Dong Wha/IASO	Phase II	Park et al. (2006)
DC159a	Daiichi Sankyo	Phase I; halted	Hoshino et al. (2008)
JNJ-Q2	Furiex	Phase II; awaiting partner	Morrow et al. (2010)

Table 16.3 Novel quinolone-like and other bacterial type II topoisomerase inhibitors

Chemical class	Representative compound	Company	Key reference
Isothiazoloquinolones	ACH-702	Achillion	Pucci et al. (2011)
Quinazolinediones	PD 0305970	Pfizer	Huband et al. (2007)
Alkylquinoline	NXL-101	Novexel/ AstraZeneca	Black et al. (2008)
Piperidinylalkylquinolines	GSK299423	GlaxoSmithKline	Bax et al. (2010)
Aminopiperidines	Compound 24l	AstraZeneca	Reck et al. (2011)
Pyrrolamides	Pyrrolamide 4	AstraZeneca	Eakin et al. (2012)
Aminobenzimidazoles	VRT-752586	Vertex	Grossman et al. (2007)
Triazolopyridines	Compound 27	Biota	East et al. (2009)
Pyrrolopyrimidines	Rx-701009	Trius	Tari et al. (2011a, b)

Pharmacokinetic studies demonstrated that after a single topical dose of 0.6 % besifloxacin suspension, mean levels in human tears ranged from 610 μg/mL at 10 min post administration to 1.6 μg/mL at 24 h post administration (Haas et al. 2009). Besifloxacin was especially potent against ciprofloxacin-resistant staphylococcal isolates with MIC_{90}s of 4 μg/mL compared with MIC_{90}s of >8 μg/mL for all other fluoroquinolones. A similar trend was noted for *S. epidermidis* and resistance to methicillin did not affect susceptibility. Potent antibacterial activity was also observed against streptococci including *Streptococcus pneumoniae* with MIC_{90}s of 0.06 and 0.5 μg/mL for levofloxacin-susceptible and non-susceptible isolates, respectively. Activity against Gram-negative organisms was more limited with the exception of the respiratory pathogens *H. influenzae* and *M. catarrhalis*. Mutant selection experiments indicated that DNA gyrase is the primary target and further biochemical and genetic studies showed that besifloxacin has potent, balanced activity against both essential gyrase and topoisomerase IV targets in *Staphylococcus aureus* and *S. pneumoniae* (Cambau et al. 2009).

16.2.2 Delafloxacin

Delafloxacin (ABT-492; WQ-3034; RX-3341) is an 8-chloro-fluoroquinolone (**2**, Fig. 16.1) that has completed Phase II clinical trials for community-acquired pneumonia (CAP) and acute bacterial skin and skin structure infections (ABSSSI). It was licensed to Rib-X Pharmaceuticals from Wakunaga Pharmaceutical Co. after Abbott Laboratories returned it back to Wakunaga after several years of development as ABT-492. Delafloxacin possesses a novel 6-amino-3,5-difluoro-pyridin-2-yl group at the N-1 position combined with a 3-hydroxyazetidin-1-yl group at the C-7 position of the quinolone core that confers improved antibacterial potency (Kuramato et al. 2003). This compound displays excellent antibacterial activity against Gram-positive bacterial pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA). An MIC_{90} of 1 μg/mL was reported for levofloxacin-resistant *S. aureus* as compared with 32 μg/mL and 8 μg/mL for levofloxacin and moxifloxacin, respectively (Almer et al. 2004). The improved antibacterial activity was attributed to equivalence of DNA gyrase and topoisomerase IV as targets for this compound (Nilius et al. 2003). In Phase I clinical studies, delafloxacin was found to be safe and well tolerated in normal healthy subjects at doses up to 900 mg. $AUC_{0\text{-}24}$ and C_{max} parameters increased proportionately with dose (Lawrence et al. 2011). These data support further development in Phase II and Phase III clinical trials.

16.2.3 Finafloxacin

Finafloxacin (**3**, Fig. 16.1) is an 8-cyano-fluoroquinolone under clinical development by MerLion Pharmaceuticals. It has the unique property of being activated under acidic conditions, pH 5.0–6.5, environments where other drugs often lose effectiveness. MICs compared with other fluoroquinolones at pH 5.8 were lower by a factor of 2–256-fold (Emrich et al. 2010). Finafloxacin has been shown to be

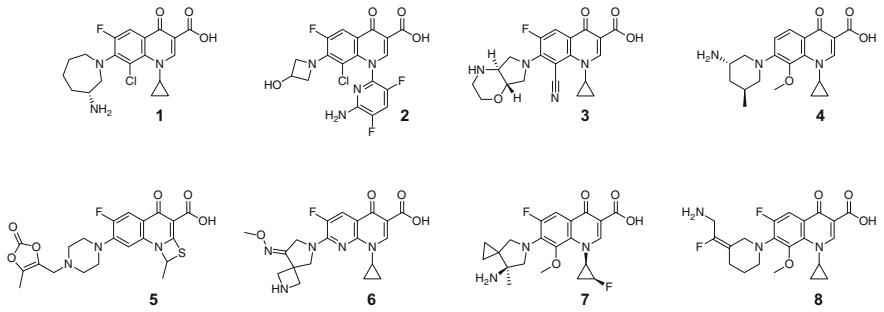


Fig. 16.1 Structures of quinolone compounds

superior to ciprofloxacin in the treatment of *Acinetobacter baumannii* infections under acidic conditions (Higgins et al. 2010). Both oral and intravenous formulations have shown excellent safety and good pharmacokinetics supportive of once daily dosing. Phase IIa trials with the oral formulation demonstrated efficacy in combatting urinary tract infections (UTIs) and in eradicating *Helicobacter pylori* infections (MerLion website http://www.merlionpharma.com, accessed 3/28/2012). Additional Phase II studies with the intravenous formulation for complicated urinary tract infections (cUTIs) and respiratory tract infections (RTIs) are planned for 2012. Other indications under investigation include cystic fibrosis, chronic obstructive pulmonary disease (COPD), and intraabdominal infections (IAIs). MerLion has also signed a licensing agreement with Alcon Pharmaceuticals to develop and commercialize the drug as an otic product for ear infections.

16.2.4 Nemonoxacin

Nemonoxacin (PGE-9602021; TG-873870) is an 8-methoxy-desfluoroquinolone (**4**, Fig. 16.1) that is being developed by Taigen Biotechnology Co. for use against antibiotic-resistant bacterial pathogens. Taigen in-licensed this compound from Procter & Gamble Healthcare and obtained worldwide rights in 2011. Nemonoxacin has a broad spectrum of activity against Gram-positive and Gram-negative bacteria and atypical pathogens. It displays first-in-class activity against MRSA (both hospital- and community-associated) and vancomycin-resistant isolates. Gram-positive MIC_{90} values have been reported for community-associated MRSA (0.5 μg/mL), methicillin-resistant *S. epidermidis* (MRSE) (2 μg/mL), *S. pneumoniae* (0.015 μg/mL), and *Enterococcus faecalis* (2 μg/mL) (Adam et al. 2009). Nemonoxacin was less potent against some Gram-negative pathogens with MIC_{90} values as follows: *E. coli* (32 μg/mL), *Klebsiella pneumoniae* (2 μg/mL), *Proteus mirabilis* (16 μg/mL), *Pseudomonas aeruginosa* (32 μg/mL), and *A. baumannii* (1 μg/mL) (Adam et al. 2009). In Phase I clinical trials, nemonoxacin was found to be generally safe and well-tolerated (Chung et al. 2009; Lin et al. 2010). The pharmacokinetics was favorable with free AUC/MIC_{90} ratios of more than 100, predictive of efficacy for most pathogens. Taigen has received positive clinical trial results including effective eradication of MRSA and quinolone-resistant MRSA in two Phase II trials for CAP and diabetic foot infection under a US IND (Taigen website http://www.taigenbiotech.com, accessed 3/28/2012). The company is now targeting to complete Phase III oral trials in China and Taiwan in 2012, and in developing an intravenous formulation for Phase II/III studies also scheduled for 2012.

16.2.5 Prulifloxacin

Prulifloxacin, originally NM 441, (**5**, Fig. 16.1) is an older fluoroquinolone that has recently been investigated for new indications. A patent was first filed in 1987 in Japan by Nippon Shinyaku Co. It has potent in vitro activity against commonly occurring Gram-positive and -negative pathogens and has been approved for use in the treatment of uncomplicated and complicated urinary tract infections, community-acquired respiratory tract infections in several European countries, and gastroenteritis including infectious diarrheas in Japan (Fritsche et al. 2009; Giannarini et al. 2009). Prulifloxacin appears to be a promising agent for the treatment of bacterial prostatitis and traveler's diarrhea (Rafailidis et al. 2011). This prodrug is converted to its active form, ulifloxacin (NM394), following oral administration and intestinal absorption (Araake et al. 2002; Keam and Perry 2004). This fluoroquinolone is administered orally as a 600-mg tablet once daily and has been shown to maintain high concentrations within the gastrointestinal tract (Matera 2006). Optimer Pharmaceuticals licensed prulifloxacin for development as therapy for acute gastroenteritis in adult travelers and has completed a Phase III clinical trial assessing the safety and efficacy for this indication. However, this compound is no longer listed on their website (http://www.optimerpharma.com accessed 3/28/2012) and current development status is unknown.

16.2.6 Zabofloxacin

Zabofloxacin (DW-224a) is a fluoronaphthyridone (**6**, Fig. 16.1) that is under clinical development by Dong Wha Pharmaceutical Co. This compound possesses a broad-spectrum antibacterial activity and it is especially active against Gram-positive organisms, particularly *S. pneumoniae*, including strains resistant to other fluoroquinolones. The MIC_{90}s of zabofloxacin against methicillin-sensitive *S. aureus* and MRSA were 0.03 and 4 μg/mL, respectively. It was also more active than ciprofloxacin and moxifloxacin against methicillin-sensitive coagulase-negative staphylococci (MSCNS) and methicillin-resistant CNS (MIC_{90}s, 0.125 and 2 μg/mL, respectively), and *Enterococcus faecium* (MIC_{90}, 16 μg/mL) (Park et al. 2006). Against *S. pneumoniae*, the activity of zabofloxacin (MIC_{90}, 0.03 μg/mL) was at least 16-fold better than those of moxifloxacin and ciprofloxacin. This strong pneumococcal activity initially suggested utility for the treatment of community-acquired respiratory tract infections. Against *Enterobacteriaceae* organisms, the activity of zabofloxacin was 2-fold lower than that of ciprofloxacin, but it was comparable to that of moxifloxacin (Park et al. 2006). MIC_{90} values for *E. coli, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae,* and *Enterobacter aerogenes* isolates were 1, 1, 0.25, 2, and 0.25 μg/mL, respectively. Against *A. baumannii* ($MIC_{90} = 4$ μg/mL) and *Stenotrophomonas maltophilia* ($MIC_{90} = 1$ μg/mL), zabofloxacin showed antibacterial activity comparable to

other quinolones. It was also highly active against *Haemophilus influenzae* (MIC_{90} = 0.008 μg/mL) and *Moraxella catarrhalis* (MIC_{90} = 0.03 μg/mL) and against *Neisseria gonorrhoeae* (MIC_{90} = 1 μg/mL) where it was 8-fold more active than ciprofloxacin. The activity of zabofloxacin against *P. aeruginosa* (MIC_{90} = 64 μg/mL) was inferior to ciprofloxacin, but comparable to that of moxifloxacin (Park et al. 2006). Therapeutic effects in mouse systemic infections with *S. aureus* and *S. pneumonia* as pathogens showed considerably better efficacy than that of ciprofloxacin, however, against Gram-positive organisms, similar or somewhat inferior activity was observed (Kwon et al. 2006). These results correlated with in vitro MIC data. In regard to safety pharmacology, zabofloxacin showed no adverse effects on the central nervous system, cardiovascular system, and respiratory system, with the exception of an effect on QT interval prolongation (Kim et al. 2004). Zabofloxacin, with its expanded anti-pneumococcal activity, is expected to be highly effective for the treatment of community-acquired respiratory tract infections, urinary tract infections, septicemia, systemic infections, skin and soft tissue infections, bacteremia, otitis media, and possibly endocarditis. Zabofloxacin (DW-224a) has recently completed a Phase I clinical study sponsored by Dong Wha and a Phase II study is in progress sponsored by IASO Pharma Inc. for CAP (http://clinicaltrials.gov/ct2/show/NCT01081964, accessed 3/28/2012).

16.2.7 DC-159a

DC-159a (**7**, Fig. 16.1), an 8-methoxy-fluoroquinolone discovered by Daiichi Sankyo Co., is intended to fill an unmet need for oral treatment of respiratory infections because of a desirable antibacterial spectrum and a low propensity to select for the emergence of resistance in key community-acquired respiratory pathogens. This compound was especially active against *S. pneumoniae* with an MIC_{90} of 0.12 μg/mL (Jones et al. 2008) and against quinolone-resistant *S. pneumoniae* strains with an MIC_{90} of 1 μg/mL against levofloxacin-resistant isolates (Hoshino et al. 2008). The MIC_{90}s against *H. influenzae*, *M. catarrhalis*, and *K. pneumoniae* were 0.015, 0.06, and 0.25 μg/mL, respectively. An attractive feature of DC-159a is its rapid bactericidal activity against *S. pneumoniae* both in vitro and in vivo. The excellent MIC_{90}s translated to therapeutic efficacy against murine pneumonia with a good pharmacokinetic profile. However, against quinolone- and methicillin-resistant *S. aureus* strains, the MIC_{90} of DC-159a was 8 μg/mL and against *Enterococcus* spp., the MIC_{90} was 4–8 μg/mL suggesting limited utility against these organisms. DC-159a demonstrated a more balanced dual-targeting activity against DNA gyrase and topoisomerase IV in *S. pneumoniae* than gatifloxacin, moxifloxacin, and other quinolones tested. This potent inhibition of two essential targets results in a lower propensity for selecting first- and second-step resistant mutants. The drug entered Phase I clinical studies in the U.S. and Europe, but further development was discontinued for reasons not made public (Ryder 2010).

16.2.8 JNJ-Q2

JNJ-Q2 (**8**, Fig. 16.1) is an aminoethylidenylpiperidine fluoroquinolone originally reported by Johnson & Johnson and now under development by Furiex Pharmaceuticals. It has potent antibacterial activity against Gram-positive pathogens including MRSA (MIC_{90} = 0.25 μg/mL) and *S. pneumoniae* (MIC_{90} = 0.12 μg/mL) (Morrow et al. 2010). Activity against Gram-negative pathogens was generally equivalent to that of moxifloxacin. JNJ-Q2 also possesses desirable drug-like properties including acceptable solubility and lipophilicity (Morrow et al. 2010). Good efficacy, equivalent, or superior to that of comparator quinolones, was demonstrated in murine septicemia and skin infection models with MRSA as the pathogen and in a murine lung infection model with *S. pneumoniae* as the pathogen (Fernandez et al. 2011). The excellent antistaphylococcal activity was attributed to strong equipotent inhibition of both the gyrase and topoisomerase IV target enzymes (Morrow et al. 2011). JNJ-Q2 has completed a Phase II non-inferiority study in patients with ABSSSIs (Covington 2011). Patients were dosed with 250 mg twice a day (BID) and compared with treatment with linezolid, 600 mg BID. JNJ-Q2 was generally safe and well-tolerated and efficacy for early clinical response was observed. However, primary intent-to-treat analysis was unable to declare non-inferiority based on a 15 % criterion and additional clinical data will be required for further development (Covington 2011).

16.3 Quinolone-like Compounds

Compounds related to traditional quinolones, but with unique structural distinctions, have been described. The isothiazoloquinolones (ITQs) were first reported by Abbott over 20 years ago, but did not progress into clinical development (Chu et al. 1988). These compounds are essentially tricyclic quinolones containing an isothiazolone ring. Pfizer has more recently reported other quinolone-like analogs that possess the 3-aminoquinazoline-2,4-dione system (quinazolinediones or QDs) (Ellsworth et al. 2006). ITQs and QDs are structurally differentiated from the quinolones in that they do not possess the 3-carboxy substituent, which, in conjunction with the 4-oxo substituent, has been long considered essential for binding to the target enzymes DNA gyrase and topoisomerase IV. Both of these quinolone-like classes appear to offer some advantages in regard to activity against existing quinolone-resistant strains.

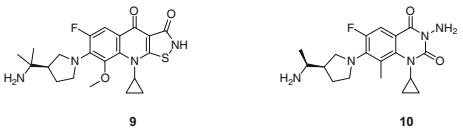


Fig. 16.2 Structures of quinolone-like compounds

16.3.1 ACH-702

ACH-702, reported by Achillion Pharmaceuticals (**9**, Fig. 16.2), is a representative of the ITQ compound class that has structural similarities to quinolones but differs due to the presence of an isothiazolone ring (Chu et al. 1988; Pucci et al. 2007; Wang et al. 2007). The ITQ compounds display potent, broad-spectrum antibacterial activity against a variety of important pathogens including fluoroquinolone-resistant isolates. Against recent MRSA clinical isolates, ACH-702 showed an MIC_{90} of 0.25 μg/mL including quinolone-resistant isolates (Pucci et al. 2011). Good antibacterial activity was also reported against other important Gram-positive pathogens including *S. epidermidis*, *S. pneumoniae*, and enterococci. Somewhat limited antibacterial activity was reported for Gram-negative pathogens with the exception of the respiratory Gram-negatives. In vivo efficacy was demonstrated against *S. aureus* in murine sepsis and thigh infection models with decreases in CFU/thigh equal to or greater than those observed for the vancomycin comparator (Pucci et al. 2011). Biochemical analyses indicated potent dual inhibition of the two antibacterial target enzymes, DNA gyrase, and topoisomerase IV. Rapid metabolism of the parent compound via extensive glucuronidation precluded systemic administration of ACH-702 and topical indications are currently under development.

16.3.2 Quinazolinediones

Novel bacterial gyrase and topoisomerase IV inhibitors, quinazolinediones (**10**, Fig. 16.2), were reported by Pfizer (Ellsworth et al. 2006). Two examples of this compound class, PD 0305970 and PD 0326448, were recently described by Huband et al. (2007). These compounds are structurally related to quinolones, but displayed excellent antibacterial activity against quinolone- and multidrug-resistant Gram-positive and fastidious organism groups. MIC_{90} values for PD 0305970 ranged from 0.125 to 0.5 μg/mL versus staphylococci, 0.03–0.06 μg/mL versus streptococci, 0.25–2 μg/mL versus enterococci, and 0.25–0.5 μg/mL versus *M. catarrhalis, H. influenzae, Listeria monocytogenes, Legionella pneumophila*, and *Neisseria* spp. PD 0305970 demonstrated excellent oral efficacy against pneumococcal pneumonia in a mouse infection model (Huband 2007). Selection of spontaneous mutants of both *S. aureus* and *S. pneumoniae* occurred at low frequencies and analyses of resistance mutations found that the primary targets for

these compounds were GyrB and ParE versus GyrA and ParC for quinolones (Pan et al. 2009). Mutations mapped to different regions of GyrB and ParE and these areas of the *gyrB* and *parE* genes were designated "dione resistance-determining regions" to differentiate these areas from the QRDRs critical to most quinolone resistant mutants. Potential mechanistic differences for QDs versus quinolones that could account for the differences in antibacterial activities were discussed by Pan et al. (2009).

16.4 Non-fluoroquinolone Bacterial Type II Topoisomerase Inhibitors

Compounds such as those described above along with previously marketed quinolones have demonstrated the value of DNA replication enzymes as antibacterial targets. In addition to the quinolone class of antibiotics, a number of other classical bacterial topoisomerase inhibitors have been explored over the years that include novobiocins, coumarins, and others (Bradbury and Pucci 2008; Black and Coleman 2009; Collin et al. 2011). These natural product derived compounds continue to be the subject of drug discovery research to the present day (Anderle et al. 2008; Angehrn et al. 2011; Oppegard et al. 2009; Heide 2009). Due to space limitations, however, we have omitted a discussion of these compounds and have chosen to highlight the newer synthetic small molecule compounds featured below.

Novel (non-fluoroquinolone) bacterial type II topoisomerase inhibitors (some authors have designated these compounds as NBTIs; Reck et al. 2011; Collin et al. 2011) have been described that are not impacted by the same target mutations that cause resistance to quinolones. The binding sites for representative compounds in the DNA-gyrase complex of *S. aureus* have been determined by crystallography (Bax et al. 2010) and do not appear to overlap with the two fluoroquinolone binding sites. Therefore, these compounds are of interest for the development of new antibacterial agents because they act on a clinically validated antibacterial target through a novel mechanism of inhibition and retain activity against quinolone-resistant isolates. Some recent examples of novel, non-quinolone compounds that also target bacterial DNA gyrase or topoisomerase IV are described below.

16.4.1 NXL-101

NXL-101 (Viquidacin; **11**, Fig. 16.3) is a quinoline bacterial topoisomerase inhibitor discovered at Aventis and further developed at Novexel S.A., which was acquired by AstraZeneca Pharmaceuticals. The goal was to develop it as an antibiotic for both parenteral and oral treatment of serious Gram-positive

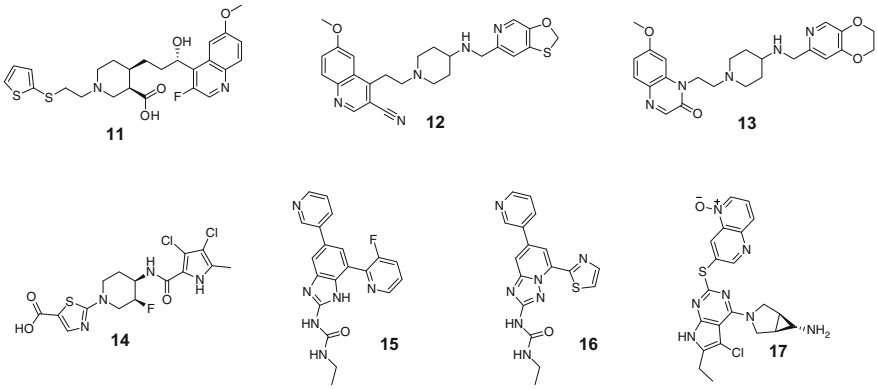


Fig. 16.3 Structures of novel bacterial topoisomerase inhibitors (NBTIs)

infections including CAP, ABSSSI, and endocarditis. This compound displayed potent antibacterial activity against Gram-positive pathogens including MRSA, VRE, and quinolone-resistant strains (Levasseur et al. 2005). In vivo efficacy was demonstrated in mouse bacteremia, pneumonia, and thigh infection models (Levasseur et al. 2007; Lowther et al. 2006). Interestingly, NXL-101 was found to possess a target specificity that was the converse of that of quinolones. In *E. coli*, topoisomerase was the preferred target while in *S. aureus* DNA gyrase was preferentially inhibited. These target inhibition differences were supported by mutation analyses and characterization of resistant mutants. The most common quinolone-resistant *S. aureus* mutants remained susceptible to NXL-101, while NXL-101 mutations were found to be distinct from those reported from selection with quinolone antibiotics and these mutant bacteria remained susceptible to quinolones (Black et al. 2008). One potential cautionary note is that single point mutations in the *gyrA* gene of *S. aureus* resulted in 16 to 128-fold increases in MIC. NXL-101 entered Phase I clinical trials and was found to achieve homogeneous and potent bactericidal concentrations in human volunteer plasma (Tarral et al. 2007). Further development was discontinued after QT interval prolongation was observed (Ryder 2010).

16.4.2 Piperidinylalkylquinolines

Another novel class of type II topoisomerase inhibitors (NBTIs), piperidinylalkylquinolines, represented by GSK299423 (**12**, Fig. 16.3), was reported by researchers at GlaxoSmithKline (Bax et al. 2010). These compounds are structurally and mechanistically distinct from fluoroquinolones, derived from a chemical series originating from an unbiased antibacterial screen. GSK299423 showed potent inhibition of supercoiling by DNA gyrases from *S. aureus* and *E. coli* with an IC_{50}s of 14 and 100 nM, respectively. The *S. aureus* value is more

than 2,000 times more potent than for ciprofloxacin. This compound was unaffected by the common *E. coli* quinolone resistance mutation GyrA(Ser83Leu) which would predict improved antibacterial activity against quinolone-resistant strains. In fact, GSK299423 displayed potent antibacterial activity against a broad spectrum of Gram-positive and Gram-negative bacterial pathogens, including quinolone-resistant isolates possessing DNA gyrase and topoisomerase IV mutations. High-level quinolone-resistant mutants in an isogenic *S. aureus* strain also did not affect GSK299423 susceptibility. Gyrase mutations selected by NBTIs mapped to positions that were distinct, but close to, mutations selected by quinolones suggesting, close but not identical, binding sites (Bax et al. 2010). A 2.1 Å crystal structure of the GSK299423-DNA-gyrase complex revealed that, unlike quinolones, the stabilized equilibrium state has uncleaved and single-stranded cleaved DNA. Therefore, these compounds appear to target a precleavage conformation that had not previously been exploited by drugs. Structural data support this proposed new mechanism of action against bacterial topoisomerases. The development status of these compounds is unknown.

16.4.3 Aminopiperidines

N-linked aminopiperidine inhibitors (**13**, Fig. 16.3) of bacterial type II topoisomerases were recently reported by AstraZeneca (Reck et al. 2011). These were derived from C-linked aminopiperidine leads from GlaxoSmithKline described in the patent literature (Axten et al. 2004). These compounds displayed potent broad-spectrum antibacterial activity against both Gram-positive and Gram-negative pathogens and one optimized analog demonstrated efficacy against *S. aureus* in a mouse thigh infection model with a one-log reduction of cfu at a dose of 24 mg/kg/day (Reck et al. 2011). N-linked aminopiperidines showed potent binding to bacterial topoisomerase IV from *E. coli* with low nanomolar IC_{50} values for the best compounds. The initial GSK leads carried considerable cardiovascular safety risk based on potent hERG inhibition with IC_{50} values of 3–4 μM. Optimization studies of aminopiperidines found that hERG inhibition correlated roughly with log D, with more polar N-linked compounds showing a significantly improved hERG profile ($IC_{50} = 31$ μM) over the C-linked lead compounds (Reck et al. 2011). However, further improvements in hERG inhibition are probably required to sufficiently de-risk these compounds from potential QT_c prolongation issues in humans.

16.4.4 Pyrrolamides

Another novel class of bacterial DNA gyrase inhibitors, the pyrrolamides (**14**, Fig. 16.3), was recently reported by researchers at AstraZeneca using a screening, structural, and chemical optimization approach (Sherer et al. 2011;

Eakin et al. 2012). These compounds were identified by a fragment-based lead generation approach using nuclear magnetic resonance (NMR) screening to identify low molecular weight compounds that bound to the ATP pocket of the *E. coli* GyrB enzyme. A screening library of ~1,000 diverse low molecular weight compounds along with fragments derived from known GyrB inhibitors was used to identify NMR hits that bound to GyrB. An initial pyrrole hit with a weak binding constant of 1 mM was used as a basis for an optimization effort that eventually led to a lead compound with an IC_{50} of 3 μM but insufficient antibacterial activity. Additional computational design and X-ray crystallography resulted in potent enzyme inhibitors with improved antibacterial activity and cellular mode of action through GyrB. One analog possessed broad-spectrum antibacterial activity against both Gram-positive and Gram-negative pathogens with MICs against *S. aureus* and *S. pneumoniae* of 0.5 μg/mL (Eakin et al. 2012). A representative compound from the pyrrolamide series demonstrated efficacy in a mouse lung infection model although at rather high oral doses of 160–320 mg/kg. Further improvements in potency and in vivo properties are required before this chemical series can yield novel antibacterial drugs.

16.4.5 Aminobenzimidazoles

Vertex Pharmaceuticals described VRT-752586 (**15**, Fig. 16.3), a member of a novel aminobenzimidazole class of bacterial topoisomerase inhibitors with potent dual target inhibition of GyrB and ParE (Charifson et al. 2008; Grossman et al. 2007). Potent antibacterial activity against staphylococci, enterococci, streptococci, and respiratory Gram-negative bacteria (*H. influenzae and M. catarrhalis*) including antibiotic-resistant strains indicated potential utility against a variety of nosocomial and community infections. MIC_{90}s for Gram-positive clinical isolates were ≤0.12 μg/mL (Mani et al. 2006). VRT-752586 was also reported to possess favorable pharmacokinetic properties in animals and showed efficacy in animal models of infection. However, MICs in the presence of 50 % human serum were shifted 16-fold higher suggesting a high degree of protein binding that could affect the amount of available free drug in vivo and, therefore, clinical efficacy. The current development status of these compounds is unknown.

16.4.6 Triazolopyridines

Prototype inhibitors based on imidazolo[1,2-*a*]pyridine and [1,2,4]triazolo[1,5-*a*]pyridine scaffolds that target the ATPase subunits of DNA gyrase (GyrB) and topoisomerase IV (ParE) (**16**, Fig. 16.3) were reported by Evotec Ltd. and Prolysis Ltd. (East et al. 2009) and are currently under development by Biota. A number of possible replacements for the benzimidazole core of a previously reported GyrB/

ParE inhibitor (Charifson et al. 2008) were explored and two chemotypes emerged, one with an imidazolopyridine scaffold and another with a triazolopyridine scaffold, with potential for further chemical optimization. The triazolopyridine scaffold (Fig. 16.3) was selected for SAR evaluation and compounds with good antibacterial activity, particularly against Gram-positive pathogens (MICs $\leq$ 1 μg/mL), were identified (East et al. 2009). Limited antibacterial activity against Gram-negative organisms was observed. Resistant mutants were difficult to obtain with the resistance frequency estimated to be $<1.8 \times 10^{-9}$, consistent with a dual-targeting inhibitor. Mammalian cytotoxicity for several analogs was evaluated in a HepG2 cell assay to test the effects on mitochondrial metabolism after 24 h exposures. The CC_{50}s for these compounds were all >64 μg/mL indicating good selectivity for antibacterial versus cytotoxic activity and suggesting that the compounds are not general, non-specific ATPase inhibitors. From initial evaluations, GyrB IC_{50}s of about 500 nM or lower correlated with single digit MICs (μg/mL). Additional study is required to further optimize and evaluate pharmacokinetics and in vivo efficacies.

16.4.7 Pyrrolopyrimidines

Pharmacophore-based fragment screening was used to discover new, dual targeting inhibitors of DNA gyrase B (GyrB), and topoisomerase IV (ParE) by Trius Pharmaceuticals (Tari et al. 2011a, b). Structural characterization of Gram-negative ParE ATP-binding domains from multiple organisms revealed unique structural and dynamic features compared to Gram-negative GyrB and the Gram-positive enzymes. This information allowed for the design of broad-spectrum, dual targeting inhibitors of GyrB/ParE. Using pharmacophore-based fragment screening and detailed characterization of differences in flexibility, shape, and composition between the active-site pockets of multiple GyrB and ParE orthologs, potent, dual-targeting inhibitors with broad spectrum potential were designed (Tari et al. 2011a, b). A pyrrolopyrimidine scaffold (**17**, Fig. 16.3) was identified with nanomolar potency across Gram-positive and Gram-negative GyrB and ParE enzymes from multiple bacterial species. Three compound examples were described with excellent antibacterial activity against Gram-positive pathogens with MICs of $\leq$0.13 μg/mL against *S. aureus* and 0.13–0.5 μg/mL against *S. pneumoniae* (Tari et al. 2011a, b). Antibacterial activity against Gram-negative pathogens was limited with MICs in the range of 2–64 μg/mL. However, MICs against an efflux-deficient *E. coli* strain ranged from 0.13 to 0.5 μg/mL suggesting that these compounds were substrates for efflux pumps. Similar MIC improvements were observed for a hyperpermeable *E. coli* strain indicating penetration issues. Optimization of the series is in progress to improve activity against bacterial drug efflux pumps and Gram-negative antibacterial activity in general.

16.5 Conclusions

DNA replication is an essential process in bacteria involving a number of enzymes that represent potential targets for therapeutic intervention. Bacterial topoisomerase inhibitors have been validated as important antibacterial agents over the past 50 years with quinolones continuing to represent one of the biggest selling and widely used classes of antibiotics in clinical use today. Due to this historical success, research and development of new topoisomerase inhibitors has also continued to the present day. These new compounds can be classified as quinolones, quinolone-like compounds, and non-quinolones (NBTIs) and recent examples of each have been described above. Just as new quinolones have been introduced over the years, there are expectations that quinolone-like derivatives and NBTIs will eventually reach the market. Such new compounds offer the advantage of inhibiting clinically validated targets while potentially overcoming existing antibiotic resistance.

References

Adam HJ, Laing NM, King CR, Lulashnyk B, Hoban DJ, Zhanel GG (2009) In vitro activity of nemonoxacin, a novel nonfluorinated quinolone, against 2,440 clinical isolates. Antimicrob Agents Chemother 53:4915–4920

Almer LS, Hoffrage JB, Keller EJ, Flamm RK, Shortridge VD (2004) In vitro and bactericidal activities of ABT-492, a novel fluoroquinolone, against Gram-positive and Gram-negative organisms. Antimicrob Agents Chemother 48:2771–2777

Anderle C, Stieger M, Burrell M, Reinelt S, Maxwell A, Page M, Heide L (2008) Biological activities of novel gyrase inhibitors of the aminocoumarin class. Antimicrob Agents Chemother 52:1982–1990

Angehrn P, Goetschi E, Gmuender H et al (2011) A new DNA gyrase inhibitor subclass of the cyclothialidine family based on a bicyclic dilactam-lactone scaffold synthesis and antibacterial properties. J Med Chem 54:2207–2224

Araake M, Hara T, Watabe H, Nishino T (2002) In vitro antibacterial activity of prulifloxacin, a new oral fluoroquinolone. Jpn J Antibiot 55:778–790

Axten JM, Brooks G, Brown P et al (2004) PCT Int Appl WO004058144

Bax BD, Chan PF, Eggleston DS et al (2010) Type IIA topoisomerase inhibition by a new class of antibacterial agents. Nature 466:935–940

Black MT, Coleman K (2009) New inhibitors of bacterial topoisomerase GyrA/ParC subunits. Curr Opin Investig Drugs 10:804–810

Black MT, Stachyra T, Platel D, Girard AM, Claudon M, Bruneau JM, Miossec C (2008) Mechanism of action of the antibiotic NXL101, a novel nonfluoroquinolone inhibitor of bacterial type II topoisomerases. Antimicrob Agents Chemother 52:3339–3349

Bradbury BJ, Pucci MJ (2008) Recent advances in bacterial topoisomerase inhibitors. Curr Opin Pharmacol 8:574–581

Cambau E, Matrat S, Pan XS, Bettoni RRD, Corbel C, Aubry A, Lascols C, Driot JY, Fisher LM (2009) Target specificity of the new fluoroquinolone besifloxacin in *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*. J Antimicrob Chemother 63:443–450

Charifson PS, Grillot AL, Grossman TH et al (2008) Novel dual-targeting benzimidazole urea inhibitors of DNA gyrase and topoisomerase IV possessing potent antibacterial activity:

intelligent design and evolution through the judicious use of structure-guided design and structure-activity relationships. J Med Chem 51:5243–5263
Chu DT, Fernandes PB, Claiborne AK, Shen L, Pernet AG (1988) Structure-activity relationships in quinolone antibacterials: design, synthesis and biological activities of novel isothiazoloquinolones. Drugs Exp Clin Res 14:379–383
Chung DT, Tsai TY, Chen SJ et al (2009) Multiple-dose safety, tolerability, and pharmacokinetics of oral nemonoxacin (TG-873870) in healthy volunteers. Antimicrob Agents Chemother 54:411–417
Collin F, Karkare S, Maxwell A (2011) Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. Appl Microbiol Biotechnol 92:479–497
Covington P, Davenport JM, Andrae D, O'Riordan W, Liverman L, McIntyre G, Almenoff J (2011) Randomized, double-blind, phase II, multicenter study evaluating the safety/tolerability and efficacy of JNJ-Q2, a novel fluoroquinolone, compared with linezolid for treatment of acute bacterial skin and skin structure infection. Antimicrob Agents Chemother 55:5790–5797
Domogala JM, Hagen SE (2003) Structure-activity relationships of the quinolone antibacterials in the new millennium: some things change and some do not. In: Hooper DC, Rubenstein E (eds) Quinolone antimicrobial agents, 3rd edn. ASM Press, Washington, DC
Drlica K, Hiasa H, Kerns R, Malik M, Mustaev A, Zhao X (2000) Quinolones action and resistance updated. Curr Top Med Chem 9:981–998
Drlica K, Malik M, Kerns RJ, Zhao X (2008) Quinolone-mediated bacterial death. Antimicrob Agents Chemother 52:385–392
Eakin AE, Green O, Hales N et al (2012) Pyrrolamide DNA gyrase inhibitors: fragment-based nuclear magnetic resonance screening to identify antibacterial agents. Antimicrob Agents Chemother 56:1240–1246
East SP, White CB, Barker O et al (2009) DNA gyrase (GyrB)/topoisomerase IV (ParE) inhibitors: synthesis and antibacterial activity. Bioor Med Chem Lett 19:894–899
Ellsworth EL, Tran TP, Showalter HDH et al (2006) 3-Aminoquinazolinediones as a new class of antibacterial agents demonstrating excellent antibacterial activity against wild-type and multidrug resistant organisms. J Med Chem 49:6435–6438
Emmerson AM, Jones AM (2003) The quinolones: decades of development and use. J Antimicrob Chemother 51(Suppl S1):13–20
Emrich NC, Heisig A, Stubbings W, Labischinski H, Heisig P (2010) Antibacterial activity of finafloxacin under different pH conditions against isogenic strains of *Escherichia coli* expressing combinations. J Antimicrob Chemother 65:2530–2533
Fernandez J, Hilliard JJ, Morrow BJ, Melton JL, Flamm RK, Barron AM, Lynch AS (2011) Efficacy of a new fluoroquinolone, JNJ-Q2, in murine models of *Staphylococcus aureus* and *Streptococcus pneumoniae* skin, respiratory, and systemic infections. Antimicrob Agents Chemother 55:5522–5528
Fritsche TR, Biedenbach DJ, Jones RN (2009) Antimicrobial activity of prulifloxacin tested against a worldwide collection of gastroenteritis-producing pathogens, including those causing traveler's diarrhea. Antimicrob Agents Chemother 53:1221–1224
Giannarini G, Tascini C, Selli C (2009) Prulifloxacin: clinical studies of a broad-spectrum quinolone agent. Future Microbiol 4:13–24
Grossman TH, Bartels DJ, Mullin S (2007) Dual targeting of GyrB and ParE by a novel aminobenzimidazole class of antibacterial compounds. Antimicrob Agents Chemother 5:657–666
Haas W, Pillar CM, Zurenko GM, Lee JC, Brunner LS, Morris TW (2009) Besifloxacin, a novel fluoroquinolone, has broad-spectrum in vitro activity against aerobic and anaerobic bacteria. Antimicrob Agents Chemother 53:3552–3560
Heide L (2009) Genetic engineering of antibiotic biosynthesis for the generation of new aminocoumarins. Biotechnol Adv 27:1006–1014

Higgins PG, Stubbings W, Wisplinghoff H, Seifert H (2010) Activity of the investigational fluoroquinolone finafloxacin against ciprofloxacin-sensitive and -resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother 54:1613–1615

Hoshino K, Inoue K, Murakami Y, Kurosaka Y, Namba K, Kashimoto Y, Uoyama S, Okumura R, Higuchi S, Otani T (2008) In vitro and in vivo antibacterial activities of DC-159a, a new fluoroquinolone. Antimicrob Agents Chemother 52:65–70

Huband MD, Cohen MA, Zurack M et al (2007) In vitro and in vivo activities of PD 0305970 and PD 0326448, new bacterial gyrase/topoisomerase inhibitors with potent antibacterial activities versus multidrug-resistant Gram-positive and fastidious organism groups. Antimicrob Agents Chemother 51:1191–1201

Jones RN, Fritsche TR, Sader HS (2008) Antimicrobial activity of DC-159a, a new fluoroquinolone, against 1,149 recently collected clinical isolates. Antimicrob Agents Chemother 52:3763–3775

Keam SJ, Perry CM (2004) Prulifloxacin. Drugs 64:2221–2234

Kim EJ, Shin WH, Kim KS, Han SS (2004) Safety pharmacology of DW-224a, a novel fluoroquinolone antibiotic agent. Drug Chem Toxicol 27:295–307

Kuramoto Y, Ohshita Y, Yoshida J, Yazaki A, Shiro M, Koike T (2003) A novel antibacterial 8-chloroquinolone with a distorted orientation of the N1-(5-amino-2,4-difluorophenyl) group. J Med Chem 46:1905–1917

Kwon AR, Min YH, Ryu JM, Choi DR, Shim MJ, Choi EC (2006) In vitro and in vivo activities of DW-224a, a novel fluoroquinolone antibiotic agent. J Antimicrob Chemother 56:684–688

Lawrence L, Benedict M, Hart J, Hawkins A, Li D, Medlock M, Hopkins S, Burak E (2011) Pharmacokinetics (PK) and safety of single doses of delafloxacin administered intravenously in healthy human subjects. In: 51st interscience conference on antimicrobial agents and chemotherapy, abstr A2-045a

Lesher GY, Froelic EJ, Gruett MD, Bailey JH, Brundage RP (1962) 1,8-Naphthyridine derivatives. A new class of therapeutic agents. J Med Chem 91:1063–1065

Levasseur, P, Delachaume C, Lowther J, Hodgson J (2005) Minimum inhibitory concentrations (MIC) and mutation prevention concentrations (MPC) of NXL101, a novel topoisomerase IV inhibitor, against *Staphylococcus aureus* including multi-resistant strains. In: 45th interscience conference on antimicrobial agents and chemotherapy, abstr F-507

Levasseur, P, Girard AM, Lowther J (2007) Efficacy of oral NXL101, a novel topoisomerase inhibitor, against fluoroquinolone-susceptible (FQS) and -resistant (FQR) *Streptococcus pneumoniae* (Sp) strains in a mouse pneumonia model. In: 47th interscience conference on antimicrobial agents and chemotherapy, abstr F1-2125

Lin L, Chang LW, Tsai CY, Hsu CH, Chung DT, Aronstein WS, Ajayi F, Kuzmak B, Lyon RA (2010) Dose escalation study of the safety, tolerability, and pharmacokinetics of nemonoxacin (TG-873870), a novel potent broad-spectrum nonfluorinated quinolone, in healthy volunteers. Antimicrob Agents Chemother 54:405–410

Lowther J, Levasseur P, Girard AM, Delachaume C, Borgonovi M, Hodgson J (2006) Efficacy of NXL101, a novel topoisomerase inhibitor, against multi-resistant *Staphylococcus aureus* in murine septicaemia and thigh muscle infections. In: 46th interscience conference on antimicrobial agents and chemotherapy, abstr F1-1999

Mani N, Gross CH et al (2006) In vitro characterization of the antibacterial spectrum of novel bacterial type II topoisomerase inhibitors of the aminobenzimidazole class. Antimicrob Agents Chemother 50:1228–1237

Matera MG (2006) Pharmacologic characteristics of prulifloxacin. Pulm Pharmacol Ther 19(Suppl 1):20–29

Mitscher LA (2005) Bacterial topoisomerase inhibitors: quinolone and pyridone antibacterial agents. Chem Rev 105:559–592

Morrow BJ, He W, Amsler KM, Foleno BD, Macielag MJ, Lynch AS, Bush K (2010) In vitro antibacterial activities of JNJ-Q2, a new broad-spectrum fluoroquinolone. Antimicrob Agents Chemother 54:1955–1964

Morrow BJ, Abbanat D, Baum EZ, Crespo-Carbone SM, Davies TA, He W, Shang W, Queenan AM, Lynch AS (2011) Antistaphylococcal activities of the new fluoroquinolone JNJ-Q2. Antimicrob Agents Chemother 55:5512–5521

Nilius AM, Shen LL, Hensey-Rudloff D, Almer LS, Beyer JM, Balli DJ, Cai Y, Flamm RK (2003) In vitro antibacterial potency and spectrum of ABT-492, a new fluoroquinolone. Antimicrob Agents Chemother 47:3260–3269

Oppegard LM, Hamann BL, Streck KR, Ellis KC, Fiedler HP, Khodursky AB, Hiasa H (2009) In vivo and in vitro patterns of the activity of simocyclinone D8, an angucyclinone antibiotic from *Streptomyces antibioticus*. Antimicrob Agents Chemother 53:2110–2119

Owens RC Jr, Ambrose PG (2000) Clinical use of the fluoroquinolones. Med Clin North Am 84:1447–1469

Owens RC Jr, Ambrose PG (2005) Antimicrobial safety: focus on quinolones. Clin Infect Dis 41(Suppl 2):S144–S157

Pan XS, Gould KA, Fisher LM (2009) Probing the differential interactions of quinazolinedione PD 0305970 and quinolones with gyrase and topoisomerase IV. Antimicrob Agents Chemother 53:3822–3831

Park HS, Kim HJ, Seol MJ, Choi DR, Choi EC, Kwak JH (2006) In vitro and in vivo antibacterial activities of DW-224a, a new fluoronaphthyridone. Antimicrob Agents Chemother 50:2261–2264

Pucci MJ, Cheng J, Podos SD, Thoma CL, Thanassi JA, Buechter DD, Mushtaq G, Vigliotti GA Jr, Bradbury BJ, Deshpande M (2007) In vitro and in vivo antibacterial activities of heteroaryl isothiazolones against resistant Gram-positive pathogens. Antimicrob Agents Chemother 51:1259–1267

Pucci MJ, Podos SD, Thanassi JA, Leggio MJ, Bradbury BJ, Deshpande M (2011) In vitro and in vivo profiles of ACH-702, an isothiazoloquinolone, against bacterial pathogens. Antimicrob Agents Chemother 55:2860–2871

Rafailidis PI, Polyzos KA, Sgouros K, Falagas ME (2011) Prulifloxacin: a review focusing on its use beyond respiratory and urinary tract infections. Int J Antimicrob Agents 37:283–290

Reck F, Alm R, Brassil P et al (2011) Novel N-linked aminopiperidine inhibitors of bacterial topoisomerase type II: broad-spectrum antibacterial agents with reduced hERG activity. J Med Chem 54:7834–7847

Rubenstein E (2001) History of quinolones and their side effects. Chemother 47(Suppl 3):3–8

Ryder NS (2010) Discontinued drugs in 2008: anti-infectives. Expert Opin Investig Drugs 19:1–21

Sherer BA et al (2011) Pyrrolamide DNA gyrase inhibitors: optimization of antibacterial activity and efficacy. Bioorg Med Chem Lett 21:7416–7420

Tari LW, Bensen D, Tzross M et al (2011a) The discovery of potent, dual targeting pyrrolopyrimidine inhibitors of bacterial DNA gyrase B and topoisomerase IV with broad spectrum antibacterial activity. In: 51st interscience conference on antimicrobial agents and chemotherapy, abstr F1-1837

Tari LW, Bensen D, Tzross M et al (2011b) The discovery of novel, dual targeting inhibitors of bacterial DNA gyrase B and topoisomerase IV using pharmacophore-based crystallographic fragment screening. In: 51st interscience conference on antimicrobial agents and chemotherapy, abstr F2-1872

Tarral A, Rangaraju M, Merdjan H, Girard AM, Delachaume C, Lowther J (2007) Plasma bactericidal activity of NXL101 in healthy volunteers after single intravenous administration of 50 to 700 mg. In: 47th interscience conference on antimicrobial agents and chemotherapy, abstr A808

Tepedino ME, Heller WH, Usner DW, Brunner LS, Morris TW, Haas W, Paterno MR, Comstock T (2009) Phase III efficacy and safety study of besifloxacin ophthalmic suspension 0.6% in the treatment of bacterial conjunctivitis. Curr Med Res Opin 25:1159–1169

Wang Q, Lucien E, Hashimoto A et al (2007) Isothiazoloquinolones with enhanced antistaphylococcal activities against multi-drug resistant strains: effects of structural modifications at the 6-, 7-, and 8-positions. J Med Chem 50:199–210

Wang X, Zhao X, Malik M, Drlica K (2010) Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. J Antimicrob Chemother 65:520–524

Wiles JA, Bradbury BJ, Pucci MJ (2010) New quinolone antibiotics: a survey of the literature from 2005 to 2010. Expert Opin Ther Patents 20:1295–1319

Chapter 17
Strategies to Discover Novel Antimicrobials to Cope with Emerging Medical Needs

Olga Genilloud and Francisca Vicente

Abstract The antibacterial screening strategies developed since the 1990s have seen an important evolution from the low throughput early phenotypic assays used to identify compounds targeting specific pathogens to target-based whole cell assays and structured-based design derived from *in silico* screening. Whereas empirical and target-based methods have been widely applied to screen for antibacterial agents, novel approaches such as structure-based drug design have proved to be a successful approach in other therapeutic areas, but they have not yet been widely applied to the development of antibacterial drugs. The objective of all these approaches has been the discovery of chemically novel leads that inhibit new molecular targets, or inhibit established targets by mechanisms distinct from those exploited by existing drugs. The focus of this chapter is to review a selection of different drug discovery paradigms that were developed to deliver novel antibacterial drugs as inhibitors of old and new essential bacterial targets proposed from single target to genome-wide initiatives. It also discusses some new trends in antibacterial discovery that have emerged with the rapid evolution and spread of antibiotic multi-resistances.

Abbreviations

AS-RNA	Antisense ribonucleic acid
AISS	Antisense-induced strain sensitivity
A-KDO	Keto-deoxyoctulosonate
ELT	Encoded library technology
FAS	Fatty acid synthesis
FBLD	Fragment-based lead discovery

O. Genilloud (✉) · F. Vicente
Fundación MEDINA, Parque Tecnológico de Ciencias de la Salud,
Avda. del Conocimiento 3 18016 Granada, Spain
e-mail: olga.genilloud@medinaandalucia.es

F. Vicente
e-mail: franscisca.vicente@medinaandalucia.es

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8_17,

GlmU	UDP-GlcNAc diphosphorylase/GlcNAc-1-P N-acetyltransferase
HTS	High throughput screening
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LPS	Lipopolysaccharide
LpxC	UDP-3-*O*-(R-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase
LtaS	Lipoteichoic acid synthase
MOA	Mode of action
MRSA	Methicillin-resistant *Staphylococcus aureus*
MDRSA	Multidrug-resistant *Staphylococcus aureus*
MIC	Minimal inhibitory concentration
4MUA	4-methylumbelliferyl acetate
NMR	Nuclear magnetic resonance
OD	Optical density
PDF	Peptide deformylase
PgdA	Peptidoglycan N-acetylglucosamine deacetylase A
pNPA	P-nitrophenyl acetate
RNR	Ribonucleotide reductase
SBDD	Structure-based drug discovery
SOS	Bacterial SOS response
UDP-gal	Uridine diphosphate galactose
UDP-glucose	Uridine diphosphate glucose
UPPS	Undecaprenyl diphosphate synthase

17.1 Introduction

The antibacterial discovery that took place intensively in the last century both in the pharmaceutical companies and the academic laboratories ensured the development of most of the antibiotics still in use today in the clinic. The discovery activity has seen a tremendous decline in the last decade due to the lack of return in terms of new leads from these screening programs, with a lack of a new classes being introduced after 1962 (Silver 2011). It is only after 2000 that four new classes of antibacterials were introduced in clinical use such as linezolid (see Zappia et al., this volume), daptomycin (see Baltz, this volume), retapamulin (see Kirst, this volume), and fidaxomicin (Fischbach and Walsh 2009; Monneret 2013; Novak 2012; Silver 2011; Venugopal and Johnson 2012; Yang and Keam 2008). In this period, the antibacterial research delivered clinical candidates, many of them analogs of old natural product scaffolds, to fight the increasing sophisticated mechanisms of resistance in bacteria against a particular family of antibiotics (telavancin, dalbavancin, tigecycline, and omadacycline, ACHN-490 or GSK1322322 among others) from which some of them have already reached the

clinic (Butler and Cooper 2011; Donadio et al. 2010; Kirst 2012; see as well Genilloud and Vicente, Kirst, Kirst and Marinelli, Leemans et al., Pucci and Wiles, this volume).

The strategies developed since the 1990s have seen an important evolution from the low throughput early phenotypic assays used to identify compounds targeting specific pathogens without any clue about their potential mode of action to target-based whole cell assays and structured-based design derived from *in silico* screening (Payne et al. 2007; Silver 2012).

Empirical methods or phenotypic assays utilize both intact bacteria and isolated biochemical targets for high throughput screening (HTS) of natural product or chemical libraries to detect inhibitor leads. Structure-based methods for drug design are based upon understanding the molecular architecture of the active site in an appropriate target molecule and include both virtual high-throughput screening and fragment-based drug discovery, both of which are dependent upon high-resolution structural data for the target. The objective of these approaches has been the discovery of chemically novel leads that inhibit new molecular targets, or inhibit established targets by mechanisms distinct from those exploited by existing drugs (Gwynn et al. 2010; Payne et al. 2007; Wimberly 2009).

Empirical methods have been widely applied to screen for antibacterial agents and the introduction of combinatorial methods for the synthesis of chemical libraries considerably expanded the potential of these screening methods. In contrast, novel approaches such as structure-based drug design have not yet been widely applied to the development of antibacterial drugs, although it has proved to be a successful approach in other therapeutic areas.

Recently, the research community and the pharmaceutical industry undertook an expensive experience trying to develop new antibiotics using genomics approaches (Chan et al. 2004; Payne et al. 2007). The sequencing of bacterial genomes assisted both empirical and structure-based approaches by identifying new and essential bacterial genes whose products might become the targets of new agents with selective antibacterial activity.

Despite these efforts, the main classes of antibacterial drugs developed in the last couple of decades have been shown to inhibit mostly classical bacterial functions: DNA replication, cell-wall biosynthesis, and protein biosynthesis. Most of these families of antibiotics have been well reviewed in other chapters of this book (see Baltz, Genilloud and Vicente, Kirst, Leemans et al., Marcone and Marinelli, Marinelli and Kirst, Pucci and Wiles, this volume). Recommended further reading should refer to relevant antibiotic drug discovery reviews specially focused on targets and technologies approaches (Donadio et al. 2010; Fabbretti et al. 2011; Genilloud 2012; Kirst 2012, 2013; Silver 2011, 2012; Singh et al. 2011), as well as recent reviews on some of latest compounds in the market, such as the RNA polymerase inhibitor fidaxomicin, that were not covered as individual chapter in this volume (Mullane and Gorbach 2011; Srivastava et al. 2011; Venugopal and Johnson 2012).

The scope of this chapter is to review a selection of different drug discovery paradigms that was developed to deliver novel antibacterial drugs as inhibitors of

old and new essential bacterial targets proposed from genome-wide initiatives, with special focus on targets associated to DNA replication, cell wall and protein synthesis, and discusses new trends in antibacterial discovery that have emerged with the evolution and spread of multi-resistance problem.

17.2 Bacterial DNA Synthesis and Cell division Screens

The discovery of DNA gyrase as target for fluoroquinolones in the 1980s led to the proposal of additional DNA replication enzymes as potential antibacterial targets and the development of many screens that were not published at that time (Silver and Bostian 1990; Silver 2012). Modulation of chromosomal supercoiling through topoisomerase-catalyzed strand breakage and rejoining reactions was found to be not only required for DNA synthesis, but as well for mRNA transcription and cell division (Wang 2002). Cell division has been identified as a crucial process that requires to be coordinated with other cellular events such as chromosome replication and nucleotide segregation. Nevertheless, bacterial cell division inhibitors have not been developed so far in the clinic (Table 17.1).

17.2.1 DNA Filamentation

Different approaches have been tested to identify novel DNA synthesis inhibitors (Singh et al. 2011) (Table 17.2). Bacterial DNA filamentation has been one of the earliest technologies used and was based on the filamentation observed in *Escherichia coli* cells harboring conditionally lethal mutations in genes involving DNA synthesis when DNA synthesis is prevented (Konrad 1977; Grompe et al. 1991). This phenotype is observed for several hours after DNA synthesis is arrested and cells continue to synthesize RNA and proteins, and they form long filaments, 4 to 32 fold longer than normal cells. It is assumed that antibiotics that inhibit the enzymes involved in DNA replication would result in the same phenotype. Filamenting strains, whether in the presence or absence of DNA synthesis, continue to increase in optical density (OD) over the course of several hours (Bernander et al. 1995). A microtiter-based liquid whole cell screen was designed at Merck to detect the specific inhibition of DNA synthesis in bacteria in the absence of inhibition of either RNA or protein synthesis. The assay easily measured the OD as an indication of continued synthesis of protein in the absence of DNA synthesis. The antibiotics identified by this screen, in addition to the known natural products of the coumermycin family (Hooper 1982), which inhibit the function of the β subunit of DNA gyrase, would mimic the response of DNA replication mutants. Several DNA gyrase inhibitors (norfloxacin, nalidixic acid, and novobiocin, see Pucci and Wiles, this volume) that cause some filamentation of *E. coli* were used, as well as DNA replication mutants, in the development of the whole cell liquid

Table 17.1 Type of assays and compounds

Target category	Compounds	Target / Type of screen	References
DNA synthesis			
DNA replication	Coelomycin, Kibdelomycin	Whole-cell empiric combined test against the whole genome *S. aureus* Fitness Test (anti-sense technology)	Goetz et al. (2010); Phillips et al. (2011)
SOS response	Quinolones	Promoter-reporter fusion (luciferase)	Freiberg et al. (2005)
RecA protein	Drug-like small molecules	ATPase (colorimetric, fluorescence polarization)	Wigle et al. (2009); Peterson et al. (2012)
Ribonucleotide reductase	Nucleotide analogs	PCR-based HTS method	Tholander and Sjoberg (2012)
Cell division (Septum formation)	Viriditoxin	Ftsz polymerization (cell-free flurescein)	Wang et al. (2003)
	PC190723	Fragment-based approach	Haydon et al. (2008)
Cell wall synthesis			
Empirical screens	Cephamycin C, thienamycin, fosfomycin, teicoplanin, ramoplanin	Spheroplasts, L-forms (light microscopy)	Donadio (2002); Silver (2011)
Peptidoglycan synthesis	Tunicamycin, ramoplanin,	Translocase and transferase (radiolabel assay)	Singh et al. (2011) Silver (2011)
	Bambermycin, ophiobolin A, coleophomone A & B	Transglycosylase (radiolabel assay)	Singh et al. (2011)
	Thielavins (thielavin B)	Transglycosylase (rescue of vancomycin-dependent strain, reporter gene-induction beta-galactosidase)	Silver (2011); Wilson (2000)
	AC98	AmpC (envA- strain), secondary labeling assay	Mani et al. (1998) DeCenzo (2002)
	Beta-lactams, glycopeptides	Transpeptidase (two strains, wild and PBP5 overproductor mutant) (agar diffusion assay)	Singh et al. (2011)
	Synthetic compounds, rhodanine analogs	Mur pathway "one-pot" in vitro screen MurA to MurF, A Mur pathway screen	Mansour et al. (2007); Tomasic and Masic (2009)
	Ramoplanin analogs	Stage II peptidoglycan synthesis induce LiaRS (whole-cell reporter, lacZ gene, assay), lipoteichoic acid synthase (LiaS)	Mascher et al. (2004); Grundling and Schneewind (2007)

(continued)

Table 17.1 (continued)

Target category	Compounds	Target / Type of screen	References
Peptidoglycan deacetylation	Two NCI compounds selected by virtual screening	N-acetylglucosamine deacetylase A (PgdA) chromogenic substrates p-nitrophenyl acetate or 4-methylumbelliferyl acetate (virtual screening)	Bui et al. (2012)
Intracellular processes peptidoglycan biosynthesis	Pleurotin, epigallocathecin	D-Ala-D-Ala dipeptide synthetic pathway and MurF (cell-free HTS)	Murakami et al. (2009)
Lipid 1	AA896	AmpC (envA- strain), secondary labeling assay	DeCenzo (2002)
Lipid A of LPS	L-573,656, L-161,240 LpxC inhibitors from libraries arrays	Galactose epimerase (*gal*E) mutants (radiolabel assay) LpxC label free high throughput mass spectrometry assay (binding assay)	Onishi et al. (1996); Barb and Zhou (2008) Langsdorf et al. (2010)
UDP-N-Acetylglucosamine synthase	Arylsulfonamide series	Acetyl transferase GlmU	Oluyinka et al. (2012); Oldfield (2010)
Isoprenoid biosynthesis	UPPS inhibitor analogs	Undecaprenyl diphosphate synthase UPPS (binding assay)	Zhu et al. (2013)
Lipid metabolism			
Fatty acid synthesis	Thiolactomycin, thiotetromycin,Phomallenic acids A-C, platensimycin platencin, compound 4	FabF/H antisense assay (two-plate agar diffusion assay) Fab I (enzymatic inhibition assay)	Wang et al. (2006, 2007); Payne et al. (2002)
Protein synthesis inhibitors			
Aminoacyl-tRNA	Pseudomicin acid, borrelidin, furanomycin,	Glutamyl-tRNA, isoleucyl-tRNA, Lysyl-tRNA (radiolabel assay)	Schimmel et al. (1998)
RNA transcription	Thienamycin; philipimycin	Bind 23S rRNA ribosomal subunit at protein L11 (resistant-sensitive strains assay)	Zhang et al. (2008)
	Lucensimycins A-B, C, and D-G, coniothyrione, pleosperone, okilactomycin	Ribosome protein S4 antisense assay (two-plate agar diffusion assay)	Singh et al. (2011)
Protein secretion	Pannomycin CJ21058	SecA protein translocase, antisense assay (two-plate agar diffusion assay) Cell-free protein translation assay	Parish et al. (2009) Sugie et al. (2002)
Peptide deformylase	Actinonin,VRC3375, BB3497.	Peptide deformylase, PDF (enzymatic assay)	Chen et al. (2000), (2004); Jain et al. (2005)

screen. Any new DNA inhibitor would cause an increase in optical density of a culture (over a limited time) with little or no increase in the DNA content of the cells, evaluated by measuring the fluorescence of the DNA binding dye Hoechst #33258 (Weisblum and Haenssler 1974).

17.2.2 Lethal Overproduction

Another technology developed to look for specific DNA inhibitors was designed to select for inhibitors of DnaA and DnaC, two essential proteins of the initiation of bacterial DNA replication. The assay was based on the concept of lethal overproduction that can be rescued in the presence of an inhibitor of such proteins (Table 17.2). In practice, the approach involved a whole cell agar diffusion assay where the samples were dispensed onto seeded plates of two independent strains of *E. coli* in which DnaA and DnaC were overproduced by IPTG induction and lethal to the cells. Rescued cells were recognized as a zone of exhibition (growth) around

Table 17.2 Screening strategies to search for antimicrobials using whole cell and cell-free enzymatic assays

Whole cell assays		**Enzymatic assays**	
Strategy	**Technology**	**Strategy**	**Technology**
Whole cell empiric phenotypic screens	Light microscopy, liquid growth inhibition or agar diffusion	Cell-free assays: Specific or multiple enzymes Inhibition or induction	Radiolabel Fluorescence and colorimetric assays
Selective testing for specific mode of action against known target	Liquid growth inhibition or agar diffusion	Substrate binding or depletion	High resolution mass spectrometry (MS) assays
Differential sensitivity test using two strains (including hypersensitive, resistant, or overproducing strains)	Liquid growth differential inhibition, or agar diffusion	Virtual screening Fragment based, structure-based drug discovery	*In silico* screening Fragment libraries as starting points Ligand or protein-based
Reporter-based screening	Beta-galactosidase, luciferase, GFP protein: promoter reporter fusions	Encoded library technology	Combinatorial libraries with double-stranded DNA molecular tags
Antisense reporting strains	Two or single plates Agar diffusion or liquid growth	Nuclear magnetic resonance (NMR)-based screening	Characterization of ligand binding
Genome-wide target-based	*S. aureus* fitness test		

the sample, and were selected as active hits (Carl 1970; Hirota et al. 1970; Schaus et al. 1981). Despite the different natural product collections that were screened for inhibitors of each protein, no inhibitors are known as positive controls and only some active hits are still under investigation.

17.2.3 Cellular Antibiotic Biosensors

Cellular antibiotic biosensors were developed at Bayer as an alternative approach to diagnose the interference with major bacterial biosynthetic pathways. In the case of DNA synthesis inhibition, one of the targeted genes was *yor*B. This gene belongs to the *lexA* regulon, which comprises a group of genes regulating the *Bacillus subtilis* SOS response triggered by DNA damage. A construction involving the fusion of the promoter region of the gene *yorB* from *B. subtilis* to the luciferase gene was established to monitor from its high induction DNA damaging events (Urban et al. 2007). These biosensors were used in 384-well microtiter plate format assays and were validated with a panel of known antibiotics to confirm the mode of action (MOA)-specific induction of the biomarkers and a set of 14,000 pure natural products. The use of the *yor*B promoter-reporter fusion permitted us to identify in addition to quinolones, other compounds interfering with DNA replication by covalent DNA binding and strand breaking, or by inhibition of gyrase and topoisomerase IV. These *B. subtilis* reporter strains were developed by the group of Freiberg with an optimized set of promoters from major biosynthetic pathways in bacteria. These tools that permitted us to show MOA-specific transcriptional activation patterns represent an efficient high-throughput screening approach to diagnose bioactive compounds interfering with major biosynthetic pathways in bacteria.

17.2.4 Prospective Targets: RecA and Ribonucleotide Reductase

The bacterial RecA protein has been proposed as another novel prospective target for antibacterial discovery as it plays an essential role in the repair of DNA damage and stalled replication and is well conserved in bacterial pathogens (Wigle et al. 2009). Small molecule inhibitors of RecA may sensitize bacteria to established antibacterial agents. RecA functions require the formation of a complex formed by multiple adenosine 5′-O-triphosphate (ATP)-bound RecA monomers on single stranded DNA where RecA hydrolyzes ATP. The screening of a collection of 35,780 drug-like small molecules for the inhibition of RecA using a colorimetric high-throughput ATPase assay permitted the identification of different chemical classes inhibiting the ATPase activity of recA *in vitro*. One of these

classes of compounds inhibits RecA's ssDNA-dependent ATPase activity *in vitro* and RecA's DNA strand exchange activity *in vitro*, and attenuates the ciprofloxacin-induced SOS gene expression in live *E. coli* cells (Wigle et al. 2009). More recently was reported a new high-throughput assay involving the evaluation of RecA ATPase activity with lower concentrations of ATP and RecA assay and using the commercial TranscreenerR adenosine 5′-O-diphosphate [ADP]2 fluorescence polarization assay. The screening of more than 110,000 small molecules against purified RecA protein permitted the identification of hits corresponding to previously unidentified scaffolds for RecA with activity both in biochemical and bacteriological assays (Peterson et al. 2012).

Ribonucleotide reductase (RNR) is an essential enzyme for *de novo* synthesis of DNA and a rate-limiting enzyme in DNA synthesis. RNR is a well-established target for the treatment of cancer, but current labor-intensive screening methods due to the experimental limitation to work with nucleotides have limited the number of samples to be processed. In consequence, the identification of novel RNR inhibitors has been restricted to nucleotide analogy and chemical properties. Tholander and Sjoberg (2012) developed a PCR-based HTS method to screen for inhibitors of RNR. This screen was tested on class I RNR from the pathogen *Pseudomonas aeruginosa* with a diversity library of compounds from which potent inhibitors were found with IC_{50} values between 0.2 and 34 μM. Four of these compounds with distinctly different structures inhibited the growth of *P. aeruginosa*, and three compounds were at least as bactericidal against *P. aeruginosa*, supporting the potential of this approach to identify novel antibiotic leads with a known intracellular target (Tholander and Sjoberg 2012).

17.2.5 Bacterial Cell Division

FtsZ, a tubulin-like GTPase, plays a central role in bacterial division, forming a structural element at the division site. FtsZ has been identified in diverse bacteria (almost all eubacteria and archaea), with high functional as well as sequence conservation between species whereas no FtsZ-like protein has been identified in any higher organism. Both FtsZ and tubulin are cytoskeletal proteins which require GTP for assembly, but is limited homology and functional similarity, and tubulin inhibitors have no effect on FtsZ assembly. During cell division, FtsZ polymerizes in the presence of GTP recruiting other division proteins to make the cell division apparatus and its inhibition would prevent cells from dividing, leading to cell death. All these characteristics have prompted to propose FtsZ as target for new antibacterials (Kapoor and Panda 2009).

A polymerization inhibition cell-free assay including wild-type FtsZ and 65Cys-fluorescein FtsZ was designed by Trusca and Bramhill (2002) to monitor FtsZ polymerization (Table 17.2). Polymers were trapped onto a filter membrane with 0.2 μm size pores while monomers pass through upon centrifugation, and fluorescence was used as read out. The SulA protein, induced as part of the SOS

response in *E. coli*, directly inhibiting FtsZ polymerization, was used as a positive control for inhibition of polymerization. A screening of more than 100,000 microbial and plant extracts getting gave a hit rate of 0.043 %. All the active extracts were tested secondarily for filament morphology and also in an SOS induction assay (Trusca and Bramhill 2002) to further refine the quality of the actives providing only eight promising hits. Bioassay-guided purification of one of the most promising active extracts led to the isolation of viriditoxin from an *Aspergillus* sp (Fig. 17.1). This compound blocks FtsZ polymerization with an IC_{50} of 8.2 μg/ml and concomitant GTPase inhibition with an IC_{50} of 7.0 μg/ml. The mode of action of viriditoxin via inhibition of FtsZ was confirmed after observation of its effects on cell morphology, macromolecular synthesis, DNA-damage response and increased minimum inhibitory concentration as a result of an increase in the expression of the FtsZ protein (Wang et al. 2003).

This target has also been addressed at Prolysis using a fragment-based approach to antibacterial drug discovery focused on the FtsZ ligand 3-methoxybenzamide. A medicinal chemistry program was undertaken to determine the structure-activity relationships of 3-methoxybenzamide, and to explore and extend the methoxy group. The antibacterial activity and the microscopic cell morphology determined by each derivative was measured to direct the iterative rounds of synthetic chemistry. Analysis of more than 500 analogs yielded a compound, PC190723 (Fig. 17.1), that inhibited cell division with improved potency and had potent antibacterial activity against all tested species of staphylococci that were tested (MRSA and MDRSA isolates) (Haydon et al. 2008).

17.3 Cell Wall Synthesis Screens

Enzymes and structures involved in cell-wall biosynthesis have proved to be excellent targets for antibacterial agents as they are conserved among bacterial pathogens and absent from mammalian cells. Inhibitors of several steps in cell-wall biosynthesis have been identified in some but not in all of these targets, and more than 40 cell wall active agents have been approved for clinical use in few of these steps (Silver 2006; Wong and Pompliano 1998, see Carter and McDonald, Cortes, Leemans et al., Marcone and Marinelli, this volume). Stages I and II of cell wall synthesis take place in the cytoplasm and inner face of the cytoplasm, whereas stage III reactions occur on the extracytoplasmic side of the membrane (Silver 2006, 2012).

17.3.1 Cell Wall Empirical Screens

One of the oldest methodologies used to identify cell wall synthesis inhibitors was the classical phenotypic screening using a *spheroplasting* assay, in which

individual or groups of spheroplasts (Lederberg 1956) were observed after overnight incubation in both liquid and agar diffusion assays with the tested samples (Gadebush et al. 1992). Normal growth and cell death were scored as negative activity and a positive response meant that the spheroplasts have been formed. Important cell wall agents, including known inhibitors of transpeptidase (cephamycin C and thienamycin), transglycosylase (moenomycin), and MurA (fosfomycin), have been discovered using this technology. Different modifications of the procedure were carried out for selecting diverse types of antibiotics and increasing the possibilities of indentifying molecules of larger molecular sizes. This included the use of a mutant *env*A *E. coli* strain, more permeable and allowing larger molecules as vancomycin to penetrate into the cell. Another methodology to detect inhibitors of any step in the cell wall pathway was used at Lepetit Research Center. It involved a L-form assay that identified agents able to differentially inhibit growth of wild-type bacterial cells but not L-forms lacking cell walls. Teicoplanin, actagardin, and ramoplanin, inhibitors of Lipid II and therefore interfering with membrane localized pathway steps were discovered using this assay (Payne et al. 2007; Silver 2006; Donadio et al. 2002, see Cortés, Marcone and Marinelli, this volume).

17.3.2 Peptidoglycan Biosynthesis

Whereas a major part of drugs in the market target the synthesis of peptidoglycan inhibiting mostly the extracellular stages of its synthesis, past and recent studies were focused on identifying inhibitors targeting early intracellular processes determining peptidoglycan biosynthesis.

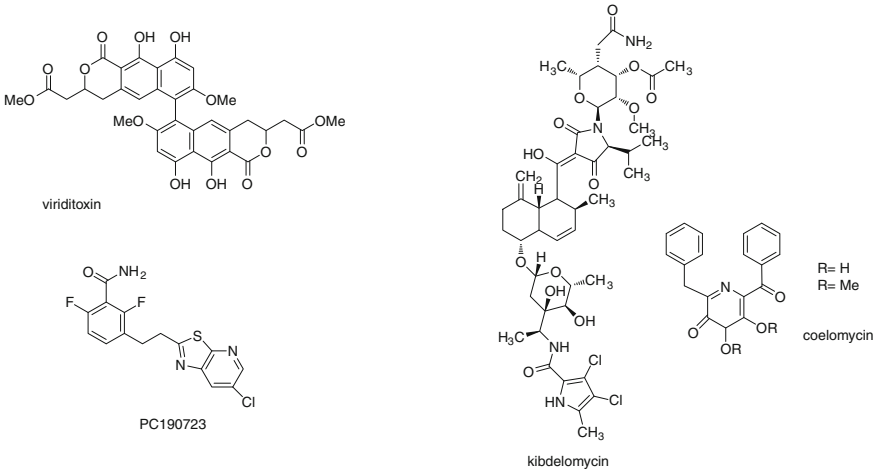


Fig. 17.1 Cell division and DNA replication inhibitors: Viroditoxin (Wang et al. 2003); PC19900723 (Haydon et al. 2008); kibdelomycin (Phillips et al. 2011); coelomycin (Goetz et al. 2010)

17.3.2.1 The Mur Pathway

The Mur intracellular enzymes are ATP-dependent ligases involved in the early stages of peptidoglycan synthesis in bacteria. Since MurC to MurF share similarities in structure and function it was initially considered that all four enzymes might be amenable to multiple inhibition by a single inhibitor. A "one-pot" *in vitro* screen for inhibitors of MurA to MurF was reported by Merck and Versicor but no inhibitors arising from them have been reported (Silver 2011, 2012).

A Mur pathway screen was undertaken at Wyeth to find inhibitors of multiple enzymatic steps in this pathway, with the idea that hitting multiple targets would reduce resistance selection (Mansour et al. 2007). These screening campaigns yielded sets of naphthylfuranone compounds with various spectra of target inhibition, from those inhibiting a single target (from MurA to MurF) to others inhibiting multiple sets of enzymes (Fig. 17.2). With a similar goal, a group at the University of Ljubljana discovered rhodanine derivatives that were balanced inhibitors of MurD, -E, and -F, one of which had extremely weak antibacterial activity (Tomasic and Masic 2009) (Fig. 17.2).

Murakami et al. (2009) describes as well the development of a new cell-free HTS to detect inhibitors of the D-Ala-D-Ala dipeptide synthetic pathway and MurF. From a screening of a large collection of microbial products, they identified the translocase I inhibitors reported earlier (capuramycins, mureidomycins, liposidomycins, and tunicamycins), the inhibitor of the D-Ala–D-Ala pathway, F-11334A1, and (−)-epigallocatechin gallate and pleurotin as MurF inhibitors (Murakami et al. 2009) (Fig. 17.2).

17.3.2.2 Peptidoglycoan Synthesis Stages II and III

A radiolabel assay to identify inhibitors of stages II and III of peptidoglycan synthesis was developed at Merck by measuring the radioactivity incorporated in the hot-SDS insoluble fraction of permeabilized *E. coli* cells when they were incubated in the presence of the precursors (UDP-NAc-(^{14}C)-glucosamine and UDP-MurNAc-pentapeptide). Several known inhibitors of both steps in stage II (**translocase**, tunicamycin and **transferase**, and ramoplanin) and two types of stage III inhibitors (**transglycosylase**, bambermycin, and ophiobolin A) were identified with this assay. In addition, coleophomone A and B, two structurally novel natural products that inhibit transglycosylase, an enzyme in bacterial cell wall synthesis that polymerizes disaccharide–pentapeptide units from Lipid II to form uncrosslinked peptidoglycan were discovered by the above screening (Wilson et al. 2000).

However, no inhibitors of the transpeptidase step could be detected. A dual mutant strains assay was then developed to identify inhibitors of the transpeptidase step. The penicillin high resistant mutant *Enterococccus hirae* R40 contains a deletion in the gene coding for the PBP5 synthesis repressor (*psr*) and overproduces PBP5, a penicillin binding protein with very low affinity for ß-lactam

antibiotics. In the presence of a ß-lactam antibiotic peptidoglycan synthesis in this mutant is due exclusively to PBP5 (Ligozzi et al. 1993). A second mutant *E. hirae* Rev14 hypersensitive to penicillin is unable to produce PBP5 due to a nucleotide substitution in the PBP5 structural gene (Ligozzi et al. 1993). The assay consists of two strains agar diffusion assay of *E. hirae*: a PBP5 overproductor mutant Rev 40 and a PBP5 nonproducer mutant Rev14. Both *E. hirae* mutants are equally sensitive to all types of peptidoglycan synthesis inhibitors, except those that inhibit the transpeptidation reaction and therefore are well suited for the identification of transpeptidase inhibitors (beta-lactams and glycopeptides). The two mutants are also equally sensitive to polymyxin B, rifampicin and several inhibitors of protein synthesis.

Moreover, Millennium Pharmaceuticals developed cell-based screens to detect inhibitors of transglycosylation by two approaches. One approach focused on the rescue of a vancomycin-dependent mutant of *Enterococcus* screening for inducers that promote growth at low concentrations, but inhibit cell growth at high concentrations. These vancomycin-dependent mutants can be isolated from the intestinal tract of patients who were treated for extended periods with high levels of vancomycin. The second approach utilized a reporter gene fused to the *van*H promoter, whose induction of *β*-galactosidase can be measured in the presence of transglycosylation inhibitors. Following the screening of fungal natural products extracts, Millennium Pharmaceuticals identified several strains that produced a new family of compounds, the thielavins. Thielavin B (Fig. 17.2) inhibited the formation of peptidoglycan in an *in vitro* assay, suggesting that these screening systems can detect compounds that interfere with cell wall transglycosylation (Mani et al. 1998). Another screening system was described by researchers at Wyeth and Millennium Pharmaceuticals that can detect and confirm inhibitors of either early or late steps of cell-wall biosynthesis (Sun et al. 2002). The primary HTS monitors induction of *β*-lactamase following exposure to samples, in an *E.coli env*A strain that carries on a plasmid the *ampC β*-lactamase gene from *Citrobacter freundii*. Positive samples were detected from both synthetic compounds and natural products libraries, and from fractions of natural products crude preparations that were confirmed with a secondary labeling assay. Two novel compound classes derived from natural products were identified (AA896 and AC98), one of which inhibits the formation of Lipid I and the other which prevents transglycosylation (DeCenzo et al. 2002) (Fig. 17.2).

A new reporter system based on the *lia* promoter responding strongly and specifically to antibiotics that interfere with the Lipid II cycle was developed at Cornell University. The LiaR-dependent, antibiotic-responsive promoter of the *lia*IHGFSR locus in *B. subtilis* autoregulates the *lia*IHGFSR operon upon addition of inhibitors of stage II peptidoglycan synthesis (Mascher et al. 2004). This system provides a tool for the detection and classification of antimicrobial compounds, using whole-cell reporter strains for parallel screening where the lacZ gene has been fused to the promoter for *lia*RS. Inhibitors of stage II synthesis induce LiaRS, an induction detected by the hydrolysis of X-gal by the *β*-galactosidase which is

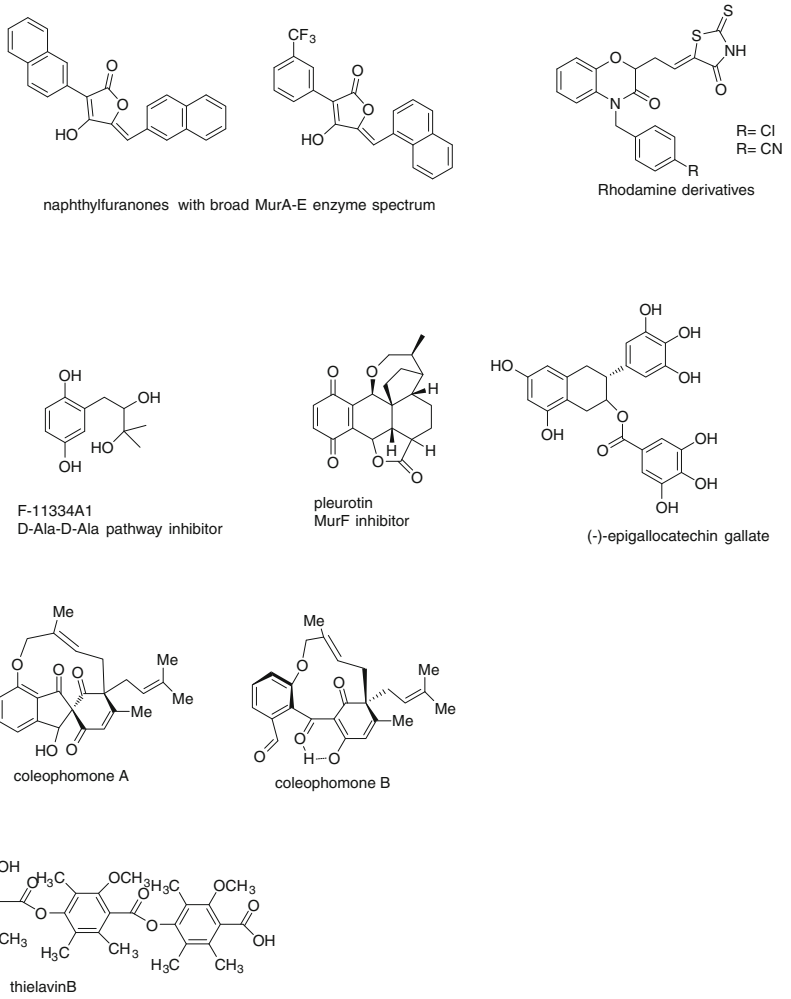


Fig. 17.2 Peptidoglycan synthesis inhibitors: Naphthylfuranones with broad MurA-E enzyme spectrum (Mansour et al. 2007); rhodamine derivatives (Tomasic and Masic 2009); F-11334A1, pleurotin, and (−)-epigallocatechin gallate (Murakami et al. 2009); coleophomone A and B (Wilson et al. 2000); thielavin B (Mani et al. 1998)

produced in response to the presence of the inhibitors. A positive response is described by a zone of inhibition surrounded by a dense, blue ring.

A natural product screening program developed at Merck to detect inhibitors that interfere with stage II peptidoglycan biosynthesis based on this methodology identified several actinomycete strains with antibacterial activity and producing new ramoplanin analogs (De la Cruz et al. 2006, 2011).

17.3.3 Other Cell Wall Targets

Peptidoglycan deacetylation in *Streptococcus pneumoniae* determines the modification of the glycan chains shortly after their synthesis. This deacetylation is catalyzed by the peptidoglycan N-acetylglucosamine deacetylase A (PgdA), an activity that contributes to the bacterial resistance to lyzozyme and is considered a virulence factor. The *pgd*A gene was first identified in *S. pneumoniae* and has been reported to be present in many other species (Bui et al. 2012). The elucidation of the crystal structure of *S. pneumoniae* PgdA has permitted us to identify the catalytic mechanism of peptidoglycan deacetylation and the kinetic parameters of the enzyme. A simple microtiter-based assay based on the acetate release of the chromogenic substrates p-nitrophenyl acetate (pNPA) or 4-methylumbelliferyl acetate (4MUA) was developed to screen for potential PgdA inhibitors previously selected by computational virtual-screening on a National Cancer Institute (NCI, Nancy) library of 280,000 compounds. Two of the identified inhibitors were confirmed in the *in vitro* assay but with IC_{50} values in the micromolar range (584 and 130 μM) (Bui et al. 2012).

In the case of Gram-positive bacteria, teichoic acid is another essential component of the cell wall that can be found linked to the peptidoglycan or bound to membrane lipids. Earlier studies in *S.aureus* and *B. subtilis* have shown that mutations in the synthetic pathways of wall teichoic acid and lipoteichoic acid are lethal for the cell, and that *S. aureus* lacking wall teichoic acid are defective in colonization and virulence in animal infection models (Weidenmaier et al. 2005; D'Elia et al. 2009). Whereas the presence of lipoteichoic acid has been extensively studied, the discovery of lipoteichoic acid synthase (LtaS) has been recently reported and its physiological role studied in *S. aureus* (Grundling and Schneewind 2007). The limited expression of LtaS determines a cellular growth arrest and defective cell division, opening the possibility to use targeted inhibition of LtaS as an antibiotic therapy for *S. aureus* infections.

Lipid A of lipopolysaccharide (LPS) is another essential molecule and the major lipid found in the outer membrane. Two observations suggest that the synthesis of lipid A is a target for antibiotic activity against Gram-negative bacteria. First, inhibition of the synthesis of the lipid A-KDO (keto-deoxyoctulosonate) region of lipopolysaccharide (LPS) causes inhibition of growth and second, the Lipid A portion of LPS contains two structural features which are unique to Gram-negative bacteria: a β-1,6 linkage between the glucosamine subunits of Lipid A and the presence of 3-hydroxymyristic acid residues. A radiolabel assay was developed at Merck based on galactose epimerase (*gal*E) mutants, which were unable to convert UDP-gal to UDP-glucose. In this context, galactose taken up from the medium is not catabolized and is incorporated only into LPS. In *gal*E mutants, the rate of incorporation of ^{3}H-gal is a measurement of the rate of LPS synthesis. Since LPS is synthesized sequentially, the incorporation of ^{3}H-gal into the core region is dependent on the synthesis of lipid A (Onishi et al. 1996). This assay helped to discover an inhibitor of the second step in the pathway

for Lipid A synthesis (L-573,656) that inhibited LPS accumulation. L-573,656 was tested against all nine enzymes of Lipid A biosynthesis and shown to specifically inhibit LpxC activity. Analogs of L-573,656 were synthesized and the most potent compound, L-161,240, was found to be a competitive inhibitor (Fig. 17.3) (Barb and Zhou 2008). LpxC (UDP-3-*O*-(R-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase), catalyzes the committed reaction step in the biosynthesis of lipid A, the membrane anchor of LPS. This enzyme has also been targeted for the search for new specific inhibitors as it is conserved among many Gram-negative pathogens. CHIR-90 is a potent LpxC inhibitor with activity against *P. aeruginosa* with broad spectrum activity against Gram-negative pathogens that targets the active site zinc within the deacetylase although no clinical candidates were reported (McClerren et al. 2005; Barb et al. 2007) (Fig. 17.3). Previous assays with LpxC involved radiolabeled substrates with TLC separations or complex fluorescence-based methods that were not suitable for HTS. A new label free high throughput mass spectrometry assay developed later at Schering-Plough permitted the use of the native ligand and the study of the entire ligand-binding pocket (Langsdorf et al. 2010). Inhibitors have been reported from libraries arrays of more than 700,000 small molecules from which some hit compounds showed whole cell antimicrobial activity against a hypersensitive strain of *E. coli* with reduced LpxC activity (Langsdorf et al. 2010).

Pereira et al. (2009) have described a new assay that targets for inhibitors of all three surface molecules targeting the acetyltransferase GlmU that catalyzes the formation of UDP-*N*-acetylglucosamine, a common precursor of bacterial peptidoglycan, lipopolysccharide and wall teichoic acid. The C-terminal acetyltransferase domain of GlmU has been shown to present structural and mechanistic features unique to bacterial UDP-*N*-acetylglucosamine synthases, characteristics that were used as a target for antibacterial discovery. The assay was used to screen a library of 50,000 drug-like compounds for inhibitors of GlmU from which potential novel scaffolds were described as binding the acetyltransferase active site and competitive with the substrate acetyl coenzyme A. Novel arylsulfonamide-containing series of compounds were discovered at Astra Zeneca by HTS. These molecules inhibit the acetyltransferase domain of GlmU what led to improvement in *in-vitro* potency against Gram-negative and Gram-positive bacterial enzymes (Oluyinka et al. 2012) (Fig. 17.3).

Isoprenoid biosynthesis is a potentially important route for antibiotic discovery as isoprenoids are involved in the early steps of bacterial cell-wall biosynthesis. Zhu et al. (2013) have proposed to target the bacterial undecaprenyl diphosphate synthase (UPPS), an essential enzyme that catalyses the formation of undecaprenyl diphosphate involved in cell-wall biosynthesis. UPPS inhibitors are expected to synergize with the more-conventional cell-wall biosynthesis inhibitors, potentially reducing the toxicity of drugs by decreasing dosage, or restoring drug sensitivity. So far several UPPS inhibitors analogs of benzoic, phosphonic, and diketoacids have been reported with *in vitro* activity against UPPS opening new routes for antibiotic therapy.

17.4 Fatty Acid Biosynthesis Screens

The fatty acid biosynthetic pathway is an essential metabolic process in bacteria and presents several novel targets for antibiotic development. FabH and FabF are two essential enzymes in type II fatty acid synthesis that were used as targets for antibacterial drug discovery. The technologies for reducing expression of targets and identifying bacterial inhibitors were applied to identify inhibitors of these specific targets. A rapid shotgun antisense procedure for the comprehensive identification of *S. aureus* genes essential for growth was developed at Elitra Pharmaceuticals. The generation of antisense RNA to selectively decrease the production of intracellular gene products involved a xylose inducible promoter very useful for primary screening (Forsyth et al. 2002). In this method, short sequences of selectively expressed mRNA strand bind to a complementary gene sequence, reducing transcription of that targeted gene and translation of the corresponding essential gene product. This methodology is based on differential sensitization of strains to drug targets by manipulation of target protein levels through induction of antisense RNA. The mechanism of silencing by inducible antisense RNA in this system is probably due to the degradation of the antisense-targeted mRNA. By expressing the antisense to essential genes, the cell becomes hypersensitive to inhibitors of that target. This information was used in the determination of true minimal genome sets and to create conditional lethal strains to be used in the identification of antimicrobial compounds against important pathogens. In this sense, these antisense screening strategies in *S. aureus* have been shown to have the advantage of being unique discovery platforms, which have recently been used to successfully identify known and novel antibacterials (Jayasuriya et al. 2007; Ondeyka et al. 2007; Young et al. 2006; Singh et al. 2006; Wang et al. 2006, 2007).

The *S. aureus* antisense target-based whole cell screens reported by Elitra were developed in liquid format (Forsyth et al. 2002). Although they served well for testing compounds, this method required titration of each and every compound and extract for both antisense and control strains. Therefore, this methodology was neither cost effective nor practical for high throughput primary screening on an industrial scale. These *S. aureus* antisense target-based assays were transferred at Merck to an agar diffusion whole cell screening assay format, to be used as

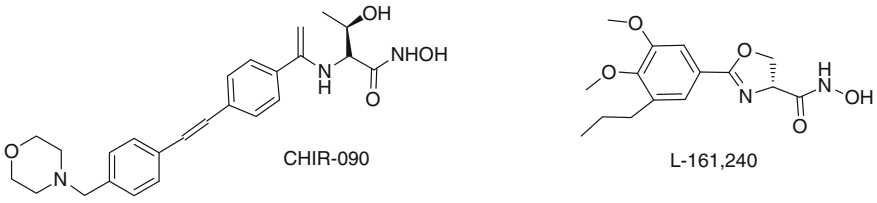


Fig. 17.3 Lipid A synthesis inhibitors: CHIR-090 (McClerren et al. 2005); L-161,240 (Barb and Zhou 2008)

primary screening (Young et al. 2006). The two-plate antisense assay was optimized as a differential sensitivity assay in which one plate contained *S. aureus* cells expressing AS-RNA and the other one *S. aureus* cells lacking AS-RNA expression (Singh et al. 2007) This technique presents several advantages, being applied as a differential whole cell screening.

These technologies were focused at Merck on different essential genes selected from diverse pathways, and screens were developed over several years with microbial natural products libraries. In each case, the two-plate assay specific for each gene was validated using a large number of reference antibiotics with known modes of action. The antibiotics that inhibited the specific target in each assay showed a differential inhibition zone in the assay whereas the antibiotics that inhibited other targets or pathways did not show this effect. These antisense assays determined the hypersensitivity of the antisense strains to the target-based inhibitors, permitting the detection of active compounds even when they were present in relatively low concentrations or low titers including mixtures. The screening approach was rapidly validated with the identification of known compounds acting on the selected targets and more importantly with the discovery of novel important compounds with specific activity against these targets. Some targets used were especially prolific at selecting novel compounds with selective antibacterial activity.

Young et al. (2006) reported for the first time the combined use of the antisense and the agar-diffusion technologies, an agar-diffusion two-plate differential sensitivity assay (two-plate assay) using as target the fatty acid synthesis (FAS) pathway, which is essential for the viability of bacteria (Young et al. 2006). The screening of 250,000 natural products extracts with the two-plate FabF/H antisense assay allowed us to detect the previously known inhibitors of condensing enzymes cerulenin, thiolactomycin, and thiotetromycin (Young et al. 2006) and the analog of thiotetromycin Tu3010. Phomallenic acids A-C are acetylenic acid versions of fatty acids also found in this assay (Fig. 17.4) (Ondeyka et al. 2006). But most importantly, this screening approach led to the discovery of the novel antibiotics platensimycin and platencin, two specific inhibitors of condensing enzymes (Fig. 17.4) (Genilloud et al. 2011) Platensimycin and platencin have a complete novel mode of action, by inhibition of the acyl enzyme intermediate of the fatty acid condensing enzyme but they differ in their specificity as determined in the cell-free enzyme FASII assay (Jayasuriya et al. 2007). Both compounds exhibited excellent in vivo efficacy in an *S. aureus* mouse model of infection when infused intravenously.

Following a similar sensitization approach, GSK identified a novel but weak inhibitor with no detectable antibacterial activity against *S. aureus* by a HTS of the *S. aureus* FabI enzyme. Iterative medicinal chemistry and X-ray crystal structure-based design led to the identification of the compound which is 350-fold more potent than the original lead compound obtained by high-throughput screening in the FabI inhibition assay (Payne et al. 2002) (Fig. 17.4).

17.5 Protein Synthesis Screens

Protein synthesis is one of the best validated targets with a large number of clinically useful antibiotic classes in the market but targeting mostly RNA transcription (see Kirst and Marinelli, Kirst, Zappia et al., Genilloud and Vicente, East, this volume). Novel approaches have been recently introduced targeting new essential genes identified after whole genome analysis as well as alternative steps of protein synthesis not totally explored in the past.

17.5.1 tRNA Aminoacylation

One of the key intermediates in protein biosynthesis are aminoacyl-tRNAs, and the inhibition of their synthesis is validated by inhibitors such as mupirocin, widely used as topical antibiotic against MRSA, which selectively inactivates bacterial isoleucyl-tRNA synthetase (IleRS) (Hurdle et al. 2005). Radiolabel screens for specific aminoacyl-tRNA synthesis have been developed where the aminoacylation of tRNA with glutamic acid catalyzed by the glutamyl-tRNA synthetase from *S. aureus* is quantitated by measuring the incorporation of ^{3}H-glutamic acid into trichloroacetic acid-precipitable ^{3}H-glutamyl-tRNA (Schimmel et al. 1998). This methodology has been shown to be useful to detected inhibitors for many aminoacyl-tRNAs synthetases such as indolmycin and chuangxinmycin (which target TrpRS), borrelidin (which targets ThrRS), granaticin (which targets LeuRS), furanomycin (which targets IleRS), ochratoxin A (which targets PheRS), and cispentacin (which targets ProRS). These inhibitors have not seen any further development as chemotherapeutic agents since they either exhibit poor antibacterial activity or lack of specificity for the bacterial target (Hurdle et al. 2005) (Fig. 17.5).

17.5.2 RNA Translation Inhibitors

Another approach for discovering new inhibitors of protein synthesis is the screening using a pair of drug-sensitive and single drug-resistant organisms (Table 17.2).

It has been shown that thiazolyl peptide antibiotics such as thiazomycin and thiostrepton (see Kirst, this volume) bind the 23S rRNA in the large (50S) ribosomal subunit at the site where ribosomal protein L11 and the pentameric complex L10 $(L12)_4$ interact, the same site where the ternary complex of EF-G (EF-Tu), GTP and charged tRNA binds. It has been proposed that the binding of thiazolyl peptide antibiotics blocks the ternary complex from binding and prevents the peptide chain elongation. Studies on this class of antibiotics shown that the

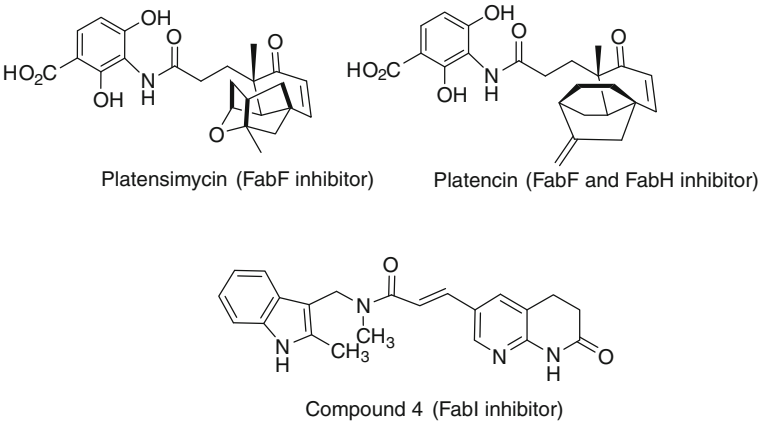


Fig. 17.4 Fatty acid synthesis inhibitors. Platensimycin and platencin (Wang et al. 2006, 2007); Compound 4 (Payne et al. 2002)

resistance to them was developed at low frequency (10^{-9}) and that most of the mutations mapped in protein L11, with no cross resistance to other protein synthesis inhibitors. These facts made this target very attractive for the discovery of new antibiotics and an assay was developed based on the differential response of two strains, sensitive and resistant to thiazomycin respectively to an unknown antibiotic acting on the same target as that of thiazomycin. This sensitive-resistance pair screening approach was designed as a two-plate agar-based assay to screen for inhibitors that exert their activity by binding at the thiazomycin binding site of L11 protein. The screening of microbial extracts as part of a screening program ran at Merck led to the identification of a new thiazolyl peptide, philipimycin, active on *S. aureus* and mechanistically similar to thiazomycin (Darst 2001; Zhang et al. 2008) (Fig. 17.6).

The bacterial ribosome protein S4 encoded by the rpsD gene is an essential component of the 30S ribosomal subunit, as shown in the antisense genome wide strategy used to identify essential genes in *S. aureus,* and conserved across bacterial species (Forsyth et al. 2002). RpsD was selected as a broad spectrum target in Merck screening and a two-plate assay with a reduced expression of the rpsD gene by antisense and leading to hypersensitivity against RpsdD inhibitors, was implemented for the screening of natural product libraries. The screening of about 138,000 microbial extracts in a rpsD two-plate antisense whole-cell assay has led to the isolation of a large diversity of new compounds including the lucensimycins A-B, C, and D-G (seven related natural products polyketides), coniothyrione (related to coniochaetone B and remisporines A and B), pleosperone (tetrahydrotetrahydroxy anthraquinone), phaenosphenone (new dimeric compound, consisting on an anthraquinone and an octahydro anthraquinone), okilactomycin (okilactomycin, old known molecule, and four congeners okilactomycin A, B, C, and D) and glabramycins A-C (new bicyclic macrolactones) (Fig. 17.6) that were never developed further (Singh et al. 2011) (Table 17.2).

17.5.3 Protein Secretion

Bacterial protein secretion is highly conserved among a wide range of species and it has been considered by many as an attractive target for antibacterial agents given the differences with the eukaryotic machinery and the potential little toxicity. The sec-dependent pathway is responsible for protein translocation across the cytoplasmic membrane and a multicomponent system of at least seven proteins, five of which are essential (Mori and Ito 2001). Homologues of these proteins have been identified in both Gram-negative and Gram-positive bacteria. Whereas the SecYEG heterotrimer forms an integral membrane channel, SecA is an ATPase with high affinity for secYEG with a central role played in the protein translocase pathway, making it an attractive target for the inhibition of secretion.

The two-plate assay based on the sensitization of the cell by a secA antisense was used to screen a collection of 115,000 natural products extracts. From this screening campaign was discovered the novel natural product pannomycin (Fig. 17.7), a cis-decalin natural product structurally similar to equisetin and CJ-21,058, both known inhibitors of SecA previously identified from a cell-free protein translation assay (Parish et al. 2009; Sugie et al. 2002) (Fig. 17.7).

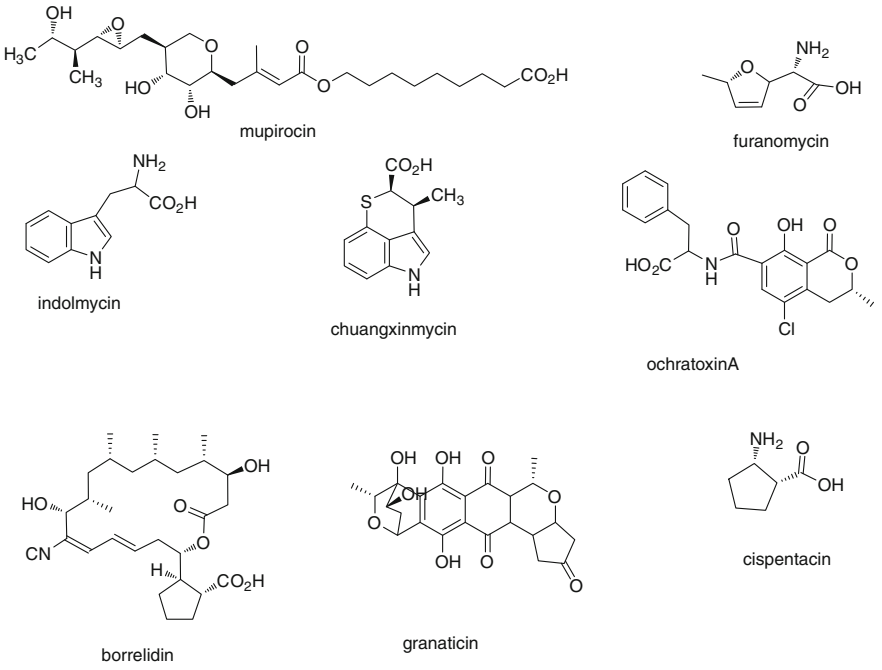


Fig. 17.5 tRNA aminoacylation inhibitors: Mupirocin, indolmycin, borrelidin, granaticin, furanomycin, ochratoxin A, cispentatin (Hurdle et al. 2005)

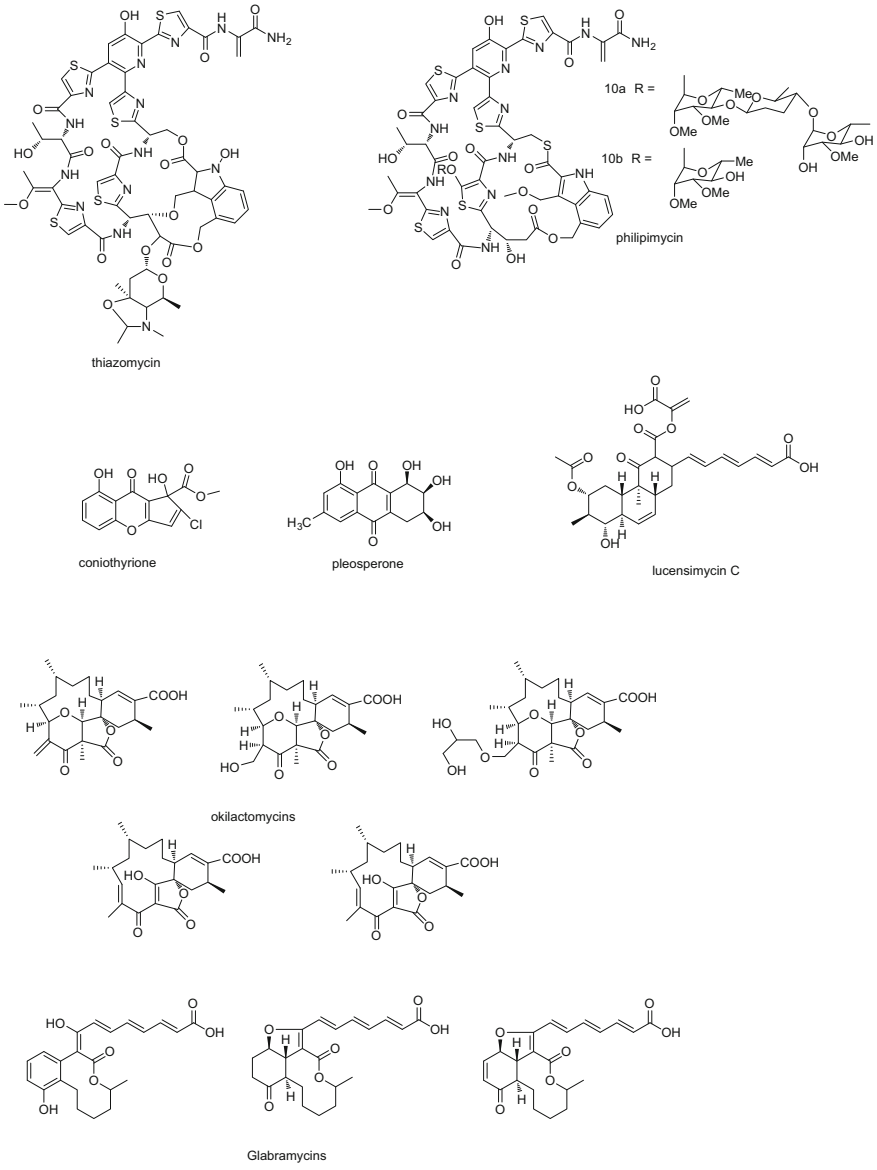


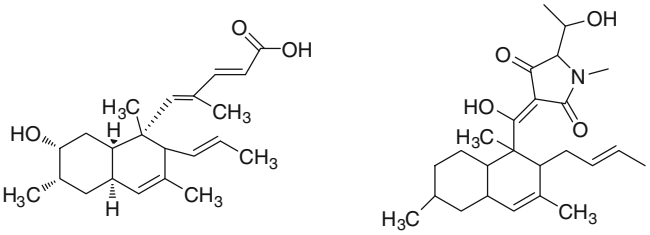
Fig. 17.6 RNA translation inhibitors: Thiazomycin, philipimycin, lucensimycin C, coniothyorione, pleosperone, okilactomycins, glabramycins (Singh et al. 2011)

17.5.4 Peptide Deformylase

The deformylation of the initiating methionine on nascent peptides in bacteria is produced by peptide deformylase, an enzyme only identified in eubacteria (see

Kirst, East, this volume). This deformylation occurs after formylation of methionine following recognition by $tRNA_{fmet}$ catalyzed by methionyl-tRNA formyl transferase (Yuan et al. 2001). The discovery of actinonin as a potent inhibitor of peptide deformylase was the origin of a search for new inhibitors of peptide deformylase as a target (Chen et al. 2000) (Fig. 17.8). The gene encoding PDF (*def*) was first identified as an essential gene in *E. coli* (Meinnel et al. 1993), a gene initially not found in the genomes of eukaryotes such as *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Mazel et al. 1994). Later sequencing data revealed *def* homologs in the nuclear genomes of higher eukaryotes, including humans raising concern about the poor selectivity of PDF inhibitors. (Guilloteau et al. 2002). PDF is a metalloprotease and the natural instability of the ferrous ion-containing PDF has limited its use in the screening for novel inhibitors. The finding of a nickel containing PDF that was oxygen insensitive and catalytically as active as the native ferrous ion-containing PDF determined the availability of an enzyme, stable under typical assay conditions, that provided a reliable form of enzyme for biochemical screening.

The screening of PDF has ensured the generation of potent inhibitors with good antibacterial activity against medically important pathogens although their physiological mechanism is still poorly understood (Jain et al. 2005). Although all these compounds inhibit PDF activity, most of them do not show antibacterial activity, presumably because of the weak potency against PDF or the inability to penetrate the bacterial cell. From three classes of inhibitors that were shown to exhibit antibacterial activity, all contained chelating groups (i.e. hydroxamate, N-formyl hydroxyl, or thiol) (Chen et al. 2000). The validation of PDF as an *in vivo* target was provided recently by several synthetic PDF inhibitors VRC3375 and BB3497, and the progress to the clinic of LBM415 and BB83698 (Chen 2004; Jain et al. 2005) (Fig. 17.8).



Pannomycin CJ-21,058

Fig. 17.7 Protein secretion inhibitors: Pannomycin (Parish et al. 2009); CJ21058 (Sugie et al. 2002)

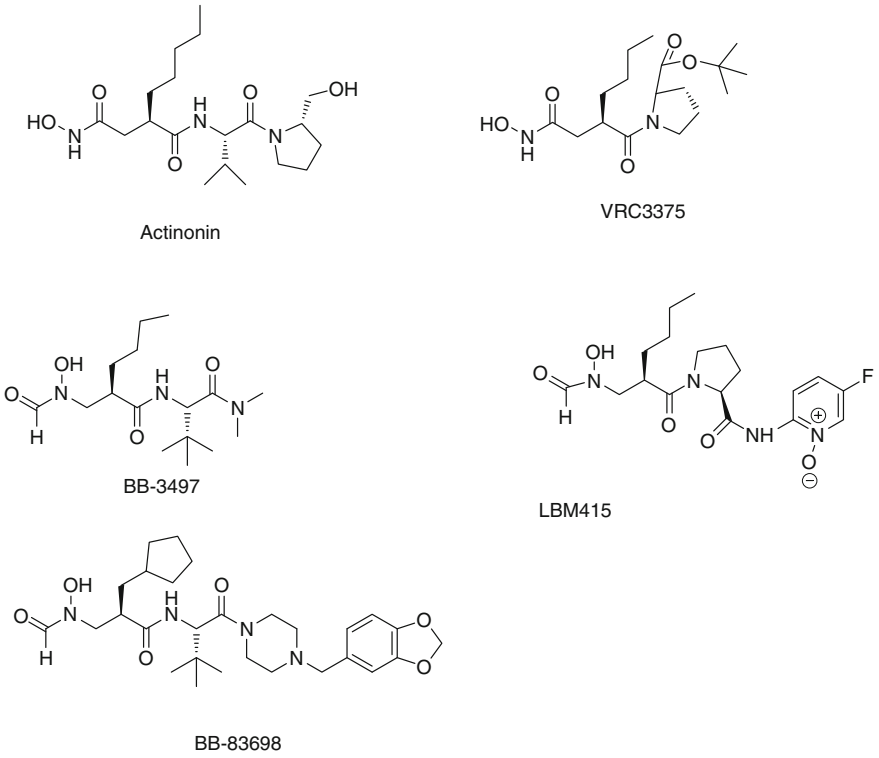


Fig. 17.8 Peptide deformylase inhibitors: Actinonin, BB3497 and BB83698; LBM415 and VRC3375 (Chen et al. 2004; Jain et al. 2005)

17.6 Whole-cell Empiric and Genome-wide Screens: Genome-wide Fitness Test

The Genome-wide Fitness Test is a mode of action screening approach that was developed by Merck in collaboration with Elitra Pharmaceuticals on the basis of the anti-sense technology. The *S. aureus* fitness test consists of a collection of 245 inducible *S. aureus* antisense RNA strains engineered for reduced expression of a single target that correspond to essential genes for which xylose-inducible anti-sense RNA expression imparts a growth phenotype. When pooled, Fitness Test strains are grown together for approximately 20 population doublings in the presence of test compounds with antibacterial activity. The strains which are rendered sensitive to the biological effects of the compound by their targeted antisense RNA preferentially drop out of the population. Multiplex PCR, capillary electrophoresis, and gene fragment analysis are used to compare the abundance of

the strains at the end of the experiment with mock treated controls. The antisense induced strain sensitivity (AISS) profiles reflect the mechanistic selectivity of a structurally diverse set of reference antibiotics for a variety of known targets including the cell wall, the protein synthesis, nucleic acid biosynthesis, and several metabolic pathways. This type of competitive growth assay serves to generate a profile of strain sensitivities that are specific for the mechanism of action (MOA) of the compound being tested. This method was validated by mechanistically profiling a diverse set of 59 antibacterial compounds and is capable of analyzing the compound MOA either by direct inference from the AISS profile or by comparison to a database of AISS profiles of known inhibitors (Donald et al. 2009). The platform has broad applications in the field of antibacterial drug discovery, including MOA determination for newly discovered antibacterial agents, the identification of novel drug targets and the detection of reporter strains whose chemically induced hypersensitivity correlates with specific antibacterial modes of action (Phillips et al. 2011).

A new strategy was used at Merck applying an empiric screening to look for activity against a target microorganism, followed by the examination of the mode of action using a simultaneous antisense screen for all essential bacterial gene targets (Donald et al. 2009). This new paradigm pursued crude natural product extracts and rapidly differentiated extracts that contained novel activities from those containing known compounds/activities. The screening of more than 450,000 acetone extracts followed by mass spectrometry dereplication of an average of 18,000 hits against proprietary LCMS-UV databases of known antibiotics permitted the early identification of almost all major known classes of antibiotics distributed across active extracts with characteristic depletion profiles (Genilloud et al. 2011). Only extracts that showed a low minimal inhibitory concentration (MIC) against *S. aureus* were subjected to the *S. aureus* fitness test for AISS profiling (Goetz et al. 2010) to discover new antibacterial agents working through target-based MOAs and prioritization for natural products chemistry.

The application of a *S. aureus* whole-cell empiric test combined to the whole genome *S. aureus* Fitness Test as a new screening technology has represented a major shift in the way the antimicrobial discovery was approached from natural products crude extracts. In addition to the validation of the mode of action of many known compounds, novel active chemical scaffolds such as the highly substituted 2,6-dioxo-pyrazine coelomycin (Goetz et al. 2010) and the novel class of bacterial gyrase inhibitor kibdelomycin have been identified with both potent *in vitro* and whole-cell antibacterial activity against *S. aureus* (Fig. 17.1). Kibdelomycin is the first bacterial gyrase inhibitor to be discovered from natural product sources since the 1950s and it represents a new chemistry, with apparently a new binding mode, that could help respond to issues of resistance and toxicity as shown in the class of the coumarin antibiotics (Phillips et al. 2011).

17.7 New Trends in Strategies to Discover Novel Antimicrobials

The determination of complete bacterial genome sequences and the parallel development of proteomics techniques inspired a new genomics-based approach to drug discovery since the mid-1990s with a huge investment in antibacterial drug discovery (Payne et al. 2007). However, the success rate of the concerted genomic and HTS target-based screening has been extremely low, and few genomics-derived compounds are currently in clinical development or in later preclinical stages (Oberbye and Barrett 2005; Silver 2011). In addition, the rapid widespread of resistances to most antibiotics in the clinic urgently require to consider new strategies to antibacterial-drug discovery and to develop the next generation of antibiotics.

The information derived from the rapid access to the genome of many microbial pathogens has been providing new routes to antibiotic discovery, and made bioassay-based screening efforts more effective. Recent knowledge and discoveries related with the structural basis of many of these molecular targets and resistance mechanisms are opening the door to new strategies amenable to be applied in coordination with more classical approaches to the field of antibiotic discovery in order to identify the new classes of antibacterial drugs that will be required to fight infection in the twenty-first century and beyond.

The potential of structure-based drug discovery (SBDD) in antibacterial-drug discovery has yet to be fully realized, but many validated molecular targets are already available. The growing number of validated targets for which structural information has been obtained makes this approach increasingly attractive (Schmid 2006). The starting point for all structure-based design work, whether ligand- or protein-based, is choosing a suitable target. Tools for prioritizing targets on the basis of their predicted suitability for SBDD are emerging. A recent study detected a correlation between the characteristics of protein-binding pockets and the frequency of the binding ligands identified by nuclear magnetic resonance (NMR)-based screening (Halgren 2009). This has led to the creation of an algorithm that can predict the suitability of the binding pocket on the basis of the characteristics identified from high-resolution protein structures, such as the rigidity of the binding site and its hydrophobic character. Such algorithms will allow future researchers to focus their drug discovery efforts on proteins that are more likely to yield high-affinity ligands (Halgren 2009; Villoutreix et al. 2007; Cheng et al. 2007). Many studies emphasize the importance of allowing for protein and ligand flexibility when performing SBDD (Carlson and McCammon 2000; Jain 2007; Zhao and Sanner 2007). A good example of the large degree of conformational flexibility seen during substrate binding is provided by MurA, which is involved in the synthesis of bacterial peptidoglycan. High-resolution crystal structures of MurA have been solved in the ‘apo’ form and also with bound substrate and substrate analogs. The inhibitor T6361 binds to MurA and blocks the conformational change that is normally induced by substrate binding. The crystal structure

of the MurA–T6361 complex reveals that the protein adopts a substantially different conformation from that seen in the structure of the MurA–substrate crystal. This example demonstrates the challenge of predicting the conformation of the ligand-binding pocket, even where knowledge of the dynamic motion of the protein is available. This situation is not unusual and creates a challenge for both 'virtual HTS' and *in silico* SBDD. Several methods for overcoming these difficulties have emerged but have yet to be completely evaluated (Deweese-Scott and Moult 2004). Approaches to producing these models range from methods based only on physical and chemical principles, to models based on sequence and structural information (Yang et al. 2007).

Different main methods have been described to assist in the identification of new putative ligands on the basis of structural information. The design can be inspired on a substrate or known inhibitor of a particular target enzyme to modify these structures to become inhibitors by maximizing complementary interactions in the target site (Dorsey et al. 1994; Chan et al. 2001). The virtual screening approach uses databases containing the structures of small molecules that are docked into a region of interest *in silico* and scored according to their predicted interactions in the target site. In the de novo design of inhibitor scaffolds, fragments of molecules are positioned in chosen sites in the target protein and are then linked *in silico* to give complete molecules. SBDD is still a very new technology, but it may provide more successful attempts in the future to discover the new classes of antibacterial drugs.

GSK has recently begun to use a new method for synthesizing and selecting molecule combinatorial libraries through the use of double-stranded DNA molecular tags and called, encoded library technology (ELT). This approach enables to screen billions of small compounds against very small quantities of target proteins. Since each compound carries a unique DNA tag, sequencing the associated tag enables to identify those that bind to the target. Compounds of interest are then tested without the tag for further evaluation in functional assays. This new screening paradigm is enabling to revisit a large number of bacterial targets that were previously identified from genomics research (Benowitz et al. 2010).

Another strategy recently used to identify new antibacterials is the fragment-based lead discovery (FBLD), an approach that both large and small pharmaceutical companies are quickly adopting. The hits it generates from fragment libraries typically of fewer than 20,000 compounds are only starting points that require considerable follow-up development. These fragments have low complexity, and therefore, it is critical to have detailed structural information, such as protein-fragment X-ray or NMR data, combined with a robust molecular modeling effort to enable further development. Nonetheless, this low-molecular weight, low-lipophilic chemical space is ideally suited to antibacterial hit and lead generation (Benowitz et al. 2010).

Efflux mechanisms responsible for the decreased effectiveness of common antibiotics also account for the resistance to new, recently described antimicrobial agents such as a peptide deformylase inhibitor, plectasin, and platensimycin. This

strongly supports the need for research and development of compounds able to circumvent or block efflux pumps and to restore/preserve antibacterial potency of older as well as newer antibiotics. Different strategies to reach this objective can include by-passing efflux activity by improving the molecular design of old antibiotics to reduce their efflux, directly act on the permeability of the bacterial cell envelope by decreasing the efficacy of the membrane barrier or blocking the efflux capacity of bacterial cell by altering the pump function. To date, no efflux pump inhibitor has been licensed for use in the treatment of bacterial infections in human or veterinary medicine, and it is clear that this gap in the antimicrobial armamentarium should stimulate research that leads to the development of new efflux pump inhibitor molecules (Mahamoud et al. 2007).

In addition to the discovery of new antibiotic compounds and inhibitors of resistance mechanisms, new strategies to prolong the effectiveness of the current arsenal of antibiotic drugs (antibiotic adjuvants, including novel compound combinations) as well as agents that interfere with bacterial virulence and block infection are being explored. Significant progress in bacterial pathogenesis has made possible to consider as alternative to disarm bacteria by targeting bacterial virulence. Anti-virulence drugs blocking adhesion, invasion, protein secretion, protein translocation, capsule formation, exotoxin, and endotoxin activity could be identified and used as novel anti-infective agents. New data have shown that bacterial pathogens have co-evolved with their hosts, establishing refined molecular mechanisms and specialized biochemical reactions between the microbe and the human target cells. This knowledge may help in the near future to develop new strategies to identify novel antibacterial compounds that inactivate virulence mechanisms in the pathogen and intercellular communication (Keyser et al. 2008; Wright and Sutherland 2007).

17.8 Conclusions

The new technologies and innovative strategies discussed above have the potential to answer the challenge of multidrug-resistant bacteria in the coming years. In summary, there is still good reason to believe that an integrated strategy combining the traditional and genomics-based approach, together with the battery of available novel *in silico* and fragment-based and target-directed techniques, will result in the discovery of new antibacterial classes that are so urgently needed to cope with bacterial resistance development (Freiberg and Brötz-Oesterhelt 2005). Within this new paradigm, it may also be expected that natural products, dramatically abandoned by most discovery programs in the last decade, will see a come-back to become part of most screening initiatives as continued unique inspiration sources of new chemical diversity.

References

Barb AW, Zhou P (2008) Mechanism and Inhibition of LpxC: an essential zinc-dependent deacetylase of bacterial lipid A synthesis. Curr Pharm Biotechnol 9:9–15

Barb A, Jiang L, Raetz C, Zhou P (2007) Structure of the deacetylase LpxC bound to the antibiotic CHIR-090: time-dependent inhibition and specificity in ligand binding. Proc Natl Acad Sci U S A 104:18433–18438

Benowitz AB, Hoover JL, David J, Payne DJ (2010) Antibacterial drug discovery in the age of resistance. Microbe 5:390–396

Bernander R, Akerlund T, Nordström K (1995) Inhibition and restart of initiation chromosome replication: effects on exponentially growing *Escherichia coli* cells. J Bacteriol 177:1670–1682

Bui NK, Eberhardt A, Vollmer D, Kern T, Bougault C, Tomasz A, Simorre JP, Vollmer W (2012) Isolation and analysis of cell wall components from *Streptococcus pneumoniae*. Anal Biochem 421:657–666

Butler MS, Cooper MA (2011) Antibiotics in the clinical pipeline in 2011. J Antibiot 64:413–425

Carl PL (1970) Escherichia coli mutants with temperature-sensitive synthesis of DNA. Mol Gen Genet 109:107–122

Carlson HA, McCammon JA (2000) Accommodating protein flexibility in computational drug design. Mol Pharmacol 57:213–218

Chan DC, Laughton CA, Queener SF, Stevens MF (2001) Structural studies on bioactive compounds. 34. Design, synthesis, and biological evaluation of triazenyl-substituted pyrimethamine inhibitors of Pneumocystis carinii dihydrofolate reductase. J Med Chem 44:2555–2564

Chan PF, Holmes DJ, Payne DJ (2004) Finding the gems using genomic discovery: antibacterial drug discovery strategies—the successes and the challenges. Drug Discov Today 1:519–527

Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC, Margolis PS, Wu C, Ni ZJ, Trias J, White RJ, Yuan Z (2000) Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. Biochemistry 39:1256–1262

Chen D, Hackbarth C, Ni ZJ, Wu C, Wang W, Jain R, He Y, Bracken K, Weidmann B, Patel DV, Trias J, White RJ, Yuan Z (2004) Peptide deformylase inhibitors as antibacterial agents: identification of VRC3375, a proline-3-alkylsuccinyl hydroxamate derivative, by using an integrated combinatorial and medicinal chemistry approach. Antimicrob Agents Chemother 48:250–261

Cheng AC, Coleman RG, Smyth KT, Cao Q, Soulard P, Caffrey DR, Salzberg AC, Huang E (2007) Structure-based maximal affinity model predicts small-molecule druggability. Nat Biotechnol 25:71–75

D'Elia M, Henderson J, Beveridge T, Heinrichs D, Brown E (2009) The N-acetylmannosamine transferase catalyzes the first committed step in teichoic acid assembly in Bacillus subtilis and *Staphylococcus aureus*. J Bacteriol 191:4030–4034

Darst SA (2001) Bacterial RNA polymerase. Curr Opin Struct Biol 11:155–162

De la Cruz M, Robles A, González I, Salazar O, Feliz J, Cercenado MI, Martín J, González del Val A, Tormo JR, Hernández P, Onishi R, Parish C, Zink DL, Cully D, Genilloud O, Díez MT, Peláez F, Vicente MF (2006) Ramoplanin-related components produced by Actinomycetes. The 2nd FEMS Congreso of European Microbiologists, Madrid, Spain

De la Cruz M, El Aouad N, Jiménez E, Palomo S, González I, Tormo R, Martín J, Genilloud O, Reyes F, Vicente F (2011) Estudio preliminar de la caracterización biológica y química de análogos de ramoplanina aislados de Actinomicetos. XXIII Congreso de la Sociedad Española de Microbiología. SEM2011. Salamanca, Spain

DeCenzo M, Kuranda M, Cohen S, Babiak J, Jiang ZD, Su D, Hickey M, Sancheti P, Bradford PA, Youngman P, Projan S, Rothstein DM (2002) Identification of compounds that inhibit late steps of peptidoglycan synthesis in bacteria. J Antibiot 55:288–295

DeWeese-Scott C, Moult J (2004) Molecular modeling of protein function regions. Proteins 55:942–961

Donadio S, Monciardini P, Alduina R, Mazza P, Chiocchini C, Cavaletti L, Sosio M, Puglia AM (2002) Microbial technologies for the discovery of novel bioactive metabolites. J Biotechnol 99:187–198

Donadio S, Maffioli S, Monciardini P, Sosio M, Jabes D (2010) Antibiotic discovery in the twenty-first century: current trends and future perspectives. J Antibiot 63:423–430

Donald RG, Skwish S, Forsyth RA, Anderson JW, Zhong T, Burns C, Lee S, Meng X, LoCastro L, Jarantow LW, Martin J, Lee SH, Taylor I, Robbins D, Malone C, Wang L, Zamudio CS, Youngman PJ, Phillips JW (2009) A Staphylococcus aureus fitness test platform for mechanism-based profiling of antibacterial compounds. Chem Biol 16:826–836

Dorsey BD, Levin RB, McDaniel SL, Vacca JP, Guare JP, Darke PL, Zugay JA, Emini EA, Schleif WA, Quintero JC et al (1994) L-735,524: the design of a potent and orally bioavailable HIV protease inhibitor. J Med Chem 37:3443–3451

Fabbretti A, Gualerzi CO, Brandi L (2011) How to cope with the quest for new antibiotics. FEBS Lett 585(11):1673–1681

Fischbach MA, Walsh CT (2009) Antibiotics for emerging pathogens. Science 325:1089–1093

Freiberg C, Brötz-Oesterhelt H (2005) Functional genomics in antibacterial drug discovery. Drug Discov Today 10:927–935

Forsyth RA, Haselbeck RJ, Ohlsen KL, Yamamoto RT, Xu H, Trawick JD, Wall D, Wang L, Brown-Driver V, Froelich JM, C KG, King P, McCarthy M, Malone C, Misiner B, Robbins D, Tan Z, Zhu Zy ZY, Carr G, Mosca DA, Zamudio C, Foulkes JG, Zyskind JW (2002). A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. Mol Microbiol 43:1387–1400

Gadebush HH, Stapley EO, Zimmerman SB (1992) The discovery of cell wall active antibacterial antibiotics. Crit Rev Biotechnol 12:225–243

Genilloud O (2012) Current challenges in the discovery of novel antibacterials from microbial natural products. Recent Pat Antiinfect Drug Discov 7:189–204

Genilloud O, González I, Salazar O, Martín J, Tormo JR, Vicente F (2011) Current approaches to exploit actinomycetes as a source of novel natural products. J Ind Microbiol Biotechnol 38:375–389

Goetz MA, Zhang C, Zink DL, Arocho M, Vicente F, Bills GF, Polishook J, Dorso K, Onishi R, Gill C, Hickey E, Lee S, Ball R, Skwish S, Donald RG, Phillips JW, Singh SB (2010) Coelomycin, a highly substituted 2,6-dioxo-pyrazine fungal metabolite antibacterial agent discovered by Staphylococcus aureus fitness test profiling. J Antibiot 63:512–518

Grompe M, Versalovic J, Koeuth T, Lupski JR (1991) Mutations in the *Escherichia coli dna*G gene suggest coupling between DNA replication and chromosome partitioning. J Bacteriol 173:1268–1278

Grundling A, Schneewind O (2007) Synthesis of glycerol phosphate lipoteichoic acid in Staphylococcus aureus. Proc Natl Acad Sci U S A 104:8478–8483

Guilloteau JP, Mathieu M, Giglione C, Blanc V, Dupuy A, Chevrier M, Gil P, Famechon A, Meinnel T, Mikol V (2002) The crystal structures of four peptide deformylases bound to the antibiotic actinonin reveal two distinct types: a platform for the structure-based design of antibacterial agents. J Mol Biol 320:951–962

Gwynn MN, Portnoy A, Rittenhouse SF, Payne DJ (2010) Challenges of antibacterial discovery revisited. Ann N Y Acad Sci 1213:5–19

Halgren TA (2009) Identifying and characterizing binding sites and assessing druggability. J Chem Inf Model 49:377–389

Haydon DJ, Stokes NR, Ure R, Galbraith G, Bennett JM, Brown DR, Baker PJ, Barynin VV, Rice DW, Sedelnikova SE, Heal JR, Sheridan JM, Aiwale ST, Chauhan PK, Srivastava A, Taneja A, Collins I, Errington J, Czaplewski LG (2008) An inhibitor of FtsZ with potent and selective anti-staphylococcal activity. Science 321:1673–1675

Hirota Y, Mordoh J, Jacob F (1970) On the process of cellular division in Escherichia coli. 3. Thermosensitive mutants of *Escherichia coli* altered in the process of DNA initiation. J Mol Biol 53:369–387

Hooper DC, Wolfson JS, McHugh GL, Winters MB, Swartz MN (1982) Effects of novobiocin, coumermycin A1, clorobiocin, and their analogs on *Escherichia coli* DNA gyrase and bacterial growth. Antimicrob Agents Chemother 22:662–671

Hurdle JG, O'Neill AJ, Chopra I (2005) Prospects for Aminoacyl-tRNA synthetase inhibitors as new antimicrobial agents. Antimicrob Agents Chemother 49:4821–4833

Jain AN (2007) Surflex-Dock 2.1: robust performance from ligand energetic modeling, ring flexibility, and knowledge-based search (2013). J Comput Aided Mol Des 21:281–306

Jain R, Chen D, White RJ, Patel DV, Yuan Z (2005) Bacterial peptide deformylase inhibitors: a new class of antibacterial agents. Curr Med Chem 12:1607–1621

Jayasuriya H, Herath KB, Zhang C, Zink DL, Basilio A, Genilloud O, Díez MT, Vicente F, González I, Salazar O, Peláez F, Cummings R, Ha S, Wang J, Singh SB (2007) Isolation and structure of platencin: a FabH and FabF dual inhibitor with potent broad-spectrum antibiotic activity. Angew Chem Int Ed Engl 46:4684–4688

Kapoor S, Panda D (2009) Targeting FtsZ for antibacterial therapy: a promising avenue. Expert Opin Ther Targets 13:1037–1051

Keyser P, Elofsson M, Rosell S, Wolf-Watz H (2008) Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. J Intern Med 264:17–29

Kirst HA (2012) Recent derivatives from smaller classes of fermentation-derived antibacterials. Expert Opin Ther Patents 22:15–35

Kirst HA (2013) Developing new antibacterials through natural product research. Expert Opin Drug Discov 8:479–493

Konrad EB (1977) Method for the isolation of duplications between chromosomal enhanced recombination *Escherichia coli* mutants with. J Bacteriol 130:167–172

Langsdorf EF, Malikzay A, Lamarr WA, Daubaras D, Kravec C, Zhang R, Hart R, Monsma F, Black T, Ozbal CC, Miesel L, Lunn CA (2010) Screening for antibacterial inhibitors of the UDP-3-*O*-(R-3-Hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC) using a high-throughput mass spectrometry assay. J Biomol Screen 15:52–61

Lederberg J (1956) Bacterial protoplasts Induced by penicillin. Proc Natl Acad Sci U S A. 42:574–577

Ligozzi M, Pittaluga F, Fontana R (1993) Identification of a genetic element (*psr*) which negatively controls expression of *Enterococcus hirae* penicillin-binding protein 5. J Bacteriol 175:2046–2051

Mahamoud A, Chevalier J, Alibert-Franco S, Kern WV, Pagès JM (2007) Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. J Antimicrob Chemother 59:1223–1229

Mani N, Sancheti P, Jiang ZD, McNaney C, DeCenzo M, Knight B, Stankis M, Kuranda M, Rothstein DM (1998) Screening systems for detecting inhibitors of cell wall transglycosylation in *Enterococcus*. Cell wall transglycosylation inhibitors in *Enterococcus*. J Antibiot 51:471–479

Mansour TS, Caufield CE, Rasmussen B, Chopra R, Krishnamurthy K, Morris M, Svenson K, Bard J, Smeltzer C, Naughton S, Antane S, Yang Y, Severin A, Quagliato D, Petersen PJ, Singh G (2007) Naphthyl tetronic acids as multi-target inhibitors of bacterial peptidoglycan biosynthesis. Chem Med Chem 2:1414–1417

Mascher T, Zimmer SL, Smith TA, Helmann JD (2004) Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. Antimicrob Agents Chemother 48:2888–2896

Mazel D, Pochet S, Marliere P (1994) Genetic characterization of polypeptide deformylase, a distinctive enzyme of eubacterial translation. EMBO J 13:914–923

McClerren A, Endsley S, Bowman J, Andersen N, Guan Z, Rudolph J, Raetz C (2005) A slow, tight-binding inhibitor of the zinc-dependent deacetylase LpxC of lipid A biosynthesis with antibiotic activity comparable to ciprofloxacin. Biochemistry 44:16574–16583

Meinnel T, Blanquet S (1993) Evidence that peptide deformylase and methionyl-tRNA(fMet) formyltransferase are encoded within the same operon in Escherichia coli. J Bacteriol 175:7737–7740

Monneret C (2013) Four new drugs on the market: abiraterone, belatacept, vandetanib and fidaxomicin. Ann Pharm 71:95–103

Mori H, Ito K (2001) The Sec protein-translocation pathway. Trends Microbiol 9:494–500

Mullane KM, Gorbach S (2011) Fidaxomicin: first-in-class macrocyclic antibiotic. Expert Rev Anti Infect Ther 9:767–777

Murakami R, Muramatsu Y, Minami E et al (2009) A novel assay of bacterial peptidoglycan synthesis for natural product screening. J Antibiot 62:153–158

Novak R (2012) Retapamulin: a first-in-class pleuromutilin antibiotic. In: Genilloud O, Vicente F (eds) Drug discovery from natural products. RSC Publishing, London

Oldfield E (2010) Targeting isoprenoid biosynthesis for drug discovery: bench to bedside. Acc Chem Res 43:1216–1226

Oluyinka MG, McKenziea AR, Shapiroa AB, Otterbeinb L, Nic H, Pattena A, Stokesa S, Alberta R, Kawatkara S, Breedd J (2012) Inhibitors of acetyltransferase domain of N-acetylglucosamine-1- phosphate-uridyltransferase/glucosamine-1-phosphateacetyltransferase (GlmU). Part 1: Hit to lead evaluation of a novel arylsulfonamide series. Bioorg Med Chem Lett 22:1510–1519

Ondeyka JG, Zink DL, Young K, Painter R, Kodali S, Galgoci A, Collado J, Tormo JR, Basilio A, Vicente F, Wang J, Singh SB (2006) Discovery of bacterial fatty acid synthase inhibitors from a *Phoma* species as antimicrobial agents using a new antisense-based strategy. J Nat Prod 69:377–380

Ondeyka JG, Zink D, Basilio A, Vicente F, Bills G, Diez MT, Motyl M, Dezeny G, Byrne K, Singh SB (2007) Coniothyrione, a chlorocyclopentandienyl benzopyrone as a bacterial protein synthesis inhibitor discovered by antisense technology. J Nat Prod 70:668–670

Onishi HR, Pelak BA, Gerckens LS, Silver LL, Kahan FM, Chen MH, Patchett AA, Galloway SM, Hyland SA, Anderson MS, Raetz CRH (1996) Antibacterial agents that inhibit lipid A biosynthesis. Science 274:980–982

Overbye KM, Barrett JF (2005) Antibiotics: where did we go wrong? Drug Discov Today. 10:45–52

Parish CA, de la Cruz M, Smith SK, Zink D, Baxter J, Tucker-Samaras S, Collado J, Platas G, Bills G, Díez MT, Vicente F, Peláez F, Wilson K (2009) Antisense-guided isolation and structure elucidation of pannomycin, a substituted cis-decalin from Geomyces pannorum. J Nat Prod 72:59–62

Payne DJ, Miller WH, Berry V, Brosky J, Burgess WJ, Chen E, DeWolf WE Jr, Fosberry AP Jr, Greenwood R, Head MS, Heerding DA, Janson CA, Jaworski DD, Keller PM, Manley PJ, Moore TD, Newlander KA, Pearson S, Polizzi BJ, Qiu X, Rittenhouse SF, Slater-Radosti C, Salyers KL, Seefeld MA, Smyth MG, Takata DT, Uzinskas IN, Vaidya K, Wallis NG, Winram SB, Yuan CC, Huffman WF (2002) Discovery of a novel and potent class of FabI-directed antibacterial agents. Antimicrob Agents Chemother 46:3118–3124

Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov 6:29–40

Pereira MP, Blanchard JE, Murphy C, Roderick SL, Brown ED (2009) High-throughput screening identifies novel inhibitors of the acetyltransferase activity of *Escherichia coli* GlmU. Antimicrob Agents Chemother 53:2306–2311

Peterson EJR, Janzen WP, Kireev D, Singleton SF (2012) High-throughput screening for RecA inhibitors using a Transcreener Adenosine 5-O-diphosphate assay. Assay Drug Dev Technol 10:260–268

Phillips JW, Goetz MA, Smith SK, Zink DL, Polishook J, Onishi R, Salowe S, Wiltsie J, Allocco J, Sigmund J, Dorso K, Lee S, Skwish S, de la Cruz M, Martín J, Vicente F, Genilloud O, Lu

J, Painter RE, Young K, Overbye K, Donald RG, Singh SB (2011) Discovery of kibdelomycin, a potent new class of bacterial type II topoisomerase inhibitor by chemical-genetic profiling in *Staphylococcus aureus*. Chem Biol 18:955–965

Schaus N, O'Day K, Peters W, Wright A (1981) Isolation and characterization of amber mutations in gene *dna*A of *Escherichia coli* K-12. J Bacteriol 145:904–913

Schimmel P, Tao J, Hill J (1998) Aminoacyl tRNA synthetases as targets for new anti-infective. FASEB J 12:1599–1609

Schmid MB (2006) Crystallizing new approaches for antimicrobial drug discovery. Biochem Pharmacol 71:1048–1056

Silver LL (2006) Does the cell wall of bacteria remain a viable source of targets for novel antibiotics? Biochem Pharmacol 71:996–1005

Silver LL (2011) Challenges of antibacterial discovery. Clin Microbiol Rev 24:71–109

Silver LL (2012) Rational approaches to antibacterial discovery: pre-genomic directed and phenotypic screening. In: Dougherty TJ, Pucci MJ (eds) Antibiotic discovery and development. Springer Science, New York

Silver L, Bostian K (1990) Screening of natural products for antimicrobial agents. Eur J Clin Microbiol Infect Dis 9:455–461

Singh SB, Zink DL, Huber J, Genilloud O, Salazar O, Diez MT, Basilio A, Vicente F, Byrne KM (2006) Discovery of lucensimycins A and B from *Streptomyces lucensis* MA7349 using an antisense strategy. Organic Lett 8:5449–5452

Singh SB, Phillips JW, Wang J (2007) Highly sensitive target-based whole-cell antibacterial discovery strategy by antisense RNA silencing. Curr Opin Drug Discov Devel 10:160–166

Singh SB, Young K, Miesel L (2011) Screening strategies for discovery of antibacterial natural products. Expert Rev Anti Infect Ther 9:589–613

Srivastava A, Talaue M, Liu S, Degen D, Ebright RY, Sineva E, Chakraborty A, Druzhinin SY, Chatterjee S, Mukhopadhyay J, Ebright YW, Zozula A, Shen J, Sengupta S, Niedfeldt RR, Xin C, Kaneko T, Irschik H, Jansen R, Donadio S, Connell N, Ebright RH (2011) New target for inhibition of bacterial RNA polymerase: 'switch region'. Curr Opin Microbiol 14:532–543

Sugie Y, Inagaki S, Kato Y, Nishida H, Pang CH, Saito T, Sakemi S, Dib-Hajj F, Mueller JP, Sutcliffe J, Kojima Y (2002) CJ-21,058, a new SecA inhibitor isolated from a fungus. J Antibiot 55:25–29

Sun D, Cohen S, Mani N, Murphy C, Rothstein DM (2002) A pathway-specific cell based screening system to detect bacterial cell wall inhibitors. J. Antibiotics 55:279–287

Tholander F, Sjöberg BM (2012) Discovery of antimicrobial ribonucleotide reductase inhibitors by screening in microwell format. Proc Natl Acad Sci U S A 109:9798–9803

Tomasić T, Masic LP (2009) Rhodanine as a privileged scaffold in drug discovery. Curr Med Chem 16:1596–1629

Trusca D, Bramhill D (2002) Fluorescent assay for polymerization of purified bacterial FtsZ cell-division protein. Anal Biochem 307:322–329

Urban A, Eckermann S, Fast B, Metzger S, Gehling M, Ziegelbauer K, Rübsamen-Waigmann H, Freiberg C (2007) Novel whole-cell antibiotic biosensors for compound discovery. Appl Environ Microbiol 73:6436–6443

Venugopal AA, Johnson S (2012) Fidaxomicin: a novel macrocyclic antibiotic approved for treatment of *Clostridium difficile* infection. Clin Infect Dis 54:568–574

Villoutreix BO, Renault N, Lagorce D, Sperandio O, Montes M, Miteva MA (2007) Free resources to assist structure-based virtual ligand screening experiments. Curr Protein Pept Sci 8:381–411

Wang J (2002) Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 3:430–440

Wang J, Galgoci A, Kodali S, Herath KB, Jayasuriya H, Dorso K, Vicente F, González A, Cully D, Bramhill D, Singh S (2003) Discovery of a small molecule that inhibits cell division by blocking FtsZ, a novel therapeutic target of antibiotics. J Biol Chem 278:44424–44428

Wang J, Soisson SM, Young K, Shoop W, Kodali S, Galgoci A, Painter R, Parthasarathy G, Tang YS, Cummings R, Ha S, Dorso K, Motyl M, Jayasuriya H, Ondeyka J, Herath K, Zhang CW, Hernandez L, Allocco J, Basilio A, Tormo JR, Genilloud O, Vicente F, Pelaez F, Colwell L, Lee SH, Michael B, Felcetto T, Gill C, Silver LL, Hermes JD, Bartizal K, Barrett J, Schmatz D, Becker JW, Cully D, Singh SB (2006) Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature 441:358–361

Wang J, Kodali S, Lee SH, Galgoci A, Painter R, Dorso K, Racine F, Motyl M, Hernandez L, Tinney E, Colletti SL, Herath K, Cummings R, Salazar O, González I, Basilio A, Vicente F, Genilloud O, Peláez F, Jayasuriya H, Young K, Cully DF, Singh SB (2007) Discovery of platencin, a dual FabF and FabH inhibitor with *in vivo* antibiotic properties. Proc Natl Acad Sci USA 104:7612–7616

Weidenmaier C, Peschel A, Xiong Y, Kristian S, Dietz K, Yeaman M, Bayer A (2005) Lack of wall teichoic acids in Staphylococcus aureus leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. J Infect Dis 191:1771–1777

Weisblum B, Haenssler E (1974) Fluorometric properties of the bibenzimidazole derivative Hoechst 33258, a fluorescent probe specific for AT concentration in chromosomal DNA. Chromosoma 46:255–260

Wigle TJ, Sexton JZ, Gromova AV, Hadimani MB, MA, GR, Yeh L-A, Singleton SF (2009) Inhibitors of RecA activity discovered by high-throughput screening: cell-permeable small molecules attenuate the SOS response in *Escherichia coli*. J Biomol Screen 14:1092–1101

Wilson KE, Tsou NN, Guan Z, Ruby CL, Pelaez F, Gorrochategui J, Vicente F, Onishi HR (2000) Isolation and structure elucidation of coleophomones A and B, novel inhibitors of bacterial cell wall transglycosylase. Tetrahedron Lett 41:8705–8709

Wimberly BT (2009) The use of ribosomal crystal structures in antibiotic drug design Curr Opin Investig Drugs 10:750–65

Wong KK, Pompliano DL (1998) Peptidoglycan biosynthesis. Unexploited antibacterial targets within a familiar pathway. Adv Exp Med Biol 456:197–217

Wright GD, Sutherland AD (2007) New strategies for combating multidrug-resistant bacteria. Trends Mol Med 13:260–267

Yang LP, Keam SJ (2008) Retapamulin: a review of its use in the management of impetigo and other uncomplicated superficial skin infections. Drugs 68:855–873

Yang JS, Chen WW, Skolnick J, Shakhnovich EI (2007) All-atom ab initio folding of a diverse set of proteins. Structure 15:53–63

Young K, Jayasuriya H, Ondeyka JG, Herath K, Zhang CW, Kodali S, Galgoci A, Painter R, Brown-Driver V, Yamamoto R, Silver LL, Zheng YC, Ventura JI, Sigmund J, Ha S, Basilio A, Vicente F, Tormo JR, Pelaez F, Youngman P, Cully D, Barrett JF, Schmatz D, Singh SB, Wang J (2006) Discovery of FabH/FabF inhibitors from natural products. Antimicrob Agents Chemother 50:519–526

Yuan Z, Trias J, White RJ (2001) Deformylase as a novel antibacterial target. Drug Discov Today 6:954–961

Zhang C, Occi J, Masurekar P, Barrett JF, Zink DL, Smith S, Onishi R, Ha S, Salazar O, Genilloud O, Basilio A, Vicente F, Gill C, Hickey EJ, Dorso K, Motyl M, Singh SB (2008) Isolation, structure, and antibacterial activity of philipimycin, a thiazolyl peptide discovered from Actinoplanes philippinensis MA7347. J Am Chem Soc 130:12102–12110

Zhao Y, Sanner MF (2007) FLIPDock: docking flexible ligands into flexible receptors. Proteins 68:726–737

Zhu W, Zhang Y, Sinko W, Hensler ME, Olson J, Molohon KJ, Lindert S, Cao R, Li K, Wang K, Wang Y, Liu YL, Sankovsky A, de Oliveira CA, F, Mitchell DA, Nizete V, McCammon JA, Oldfielda E (2013) Antibacterial drug leads targeting isoprenoid biosynthesis. Proc Natl Acad Sci U S A 110:123–128

Index

F. Marinelli and O. Genilloud (eds.), *Antimicrobials*,
DOI: 10.1007/978-3-642-39968-8, © Springer-Verlag Berlin Heidelberg 2014