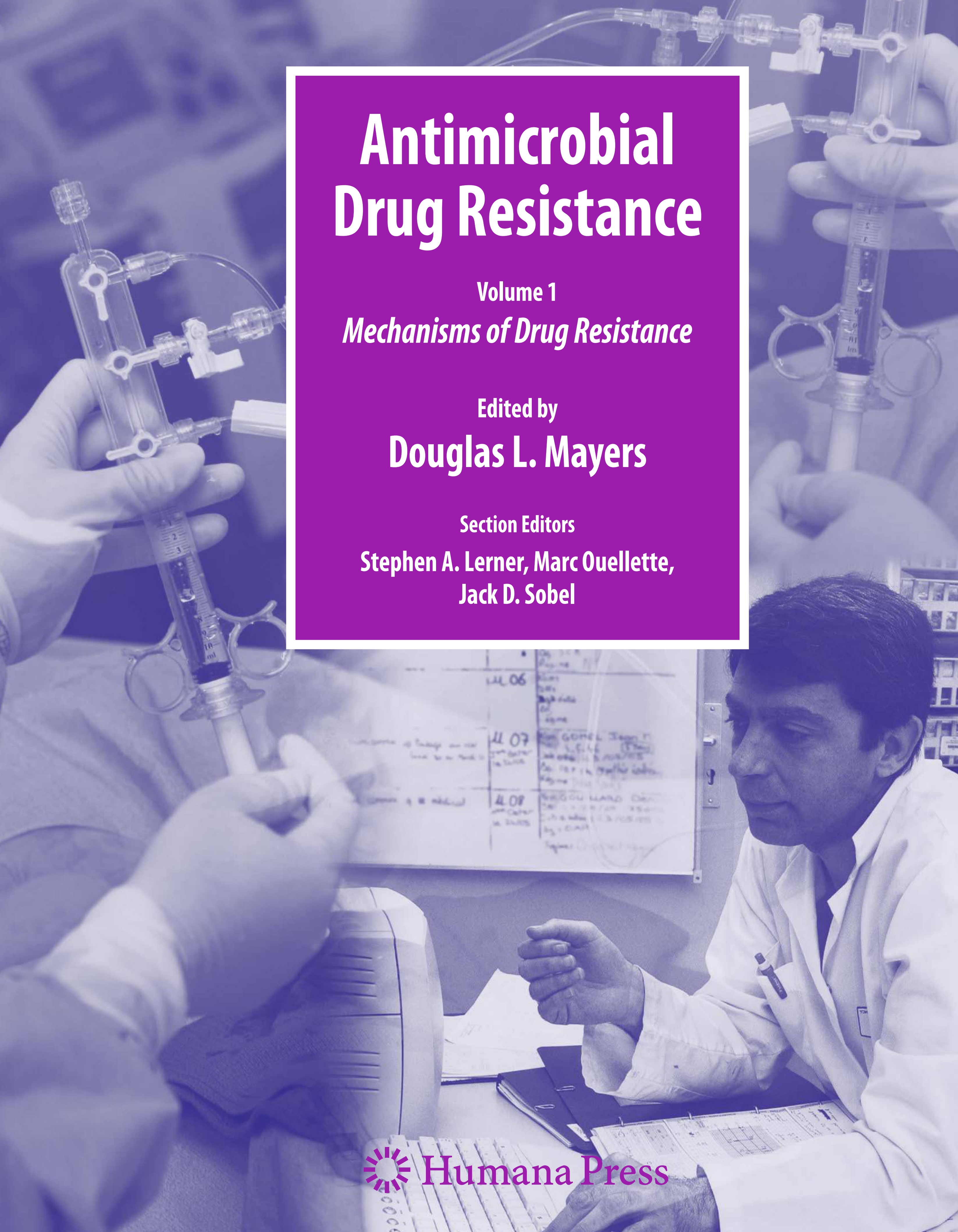
Antimicrobial
Drug Resistance
Volume 1
Mechanisms of Drug Resistance
Edited by
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Section Editors
Stephen A. Lerner, Marc Ouellette,
Jack D. Sobel
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Volume 1

Mechanisms of Drug Resistance

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Preface

This first edition of *Antimicrobial Drug Resistance* grew out of a desire by the editors and authors to have a comprehensive resource of information on antimicrobial drug resistance that encompassed the current information available for bacteria, fungi, protozoa and viruses. We believe that this information will be of value to clinicians, epidemiologists, microbiologists, virologists, parasitologists, public health authorities, medical students and fellows in training. We have endeavored to provide this information in a style which would be accessible to the broad community of persons who are concerned with the impact of drug resistance in our clinics and across the broader global communities.

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

We are very grateful to the 175 international experts who have contributed to this textbook for their patience and support as the work came together. The editors would like to especially thank Shelley Crim for her administrative assistance in pulling the book together. The staff at Humana and Springer including Renata Hutter, Kathleen Lyons, Jenny Wolkowicki, and Harvey Kane have provided exceptional support and encouragement to the editors over several years required to develop this textbook. Finally, the book would never have been completed without the patience and support of our wives and families.

Douglas L. Mayers, M.D.
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Marc Ouellette Ph.D.
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Section A
General Overview

Chapter 1
History of Drug-Resistant Microbes

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

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

An early concern was whether acquired resistance represented an adaptive response to the drug, which persisted for many generations after the drug was removed, or a selection from the initial population of rare preexisting resistant mutants. The adaptation hypothesis was championed by Hinshelwood who argued that, if a culture was grown in the presence of an inhibitor, the concentration of the substrate for the blocked reaction would accumulate and reverse the inhibition. Serial culturing in successively higher concentrations of a drug was interpreted, thus, as "training" the culture to tolerate the inhibition (4). The issue was settled in favor of mutation by demonstrations that resistance could emerge in the absence of an antibiotic and by the transfer of resistance with DNA. For example, the Lederbergs showed by replica plating that streptomycin-resistant colonies of *Escherichia coli* were present in a culture never exposed to the drug (5), while Hotchkiss demonstrated that penicillin resistance could be transferred to a susceptible pneumococcus by the DNA of a resistant one (6). Adaptation returned later, however, in the form of adaptive mutations, i.e. mutations that are formed in response to the environment in which the mutants are selected (7). Such mutants occur in nondividing or slowly dividing cells and are specific for events that allow growth in that environment, as, for example, the emergence of ciprofloxacin-resistant mutants in nondividing cultures of *E. coli* exposed for a week to ciprofloxacin in agar (8).

Until penicillin became available, sulfonamides were widely used for both treatment and prophylaxis, and before long resistance began to appear in several pathogens. Daily administration of sulfadiazine to prevent upper respiratory infections at military bases during World War II was followed by the emergence of resistant β-hemolytic streptococci. The question was whether the resistance was acquired or preexisting. Since the resistant organisms mainly belonged to only a few serotypes, selection of naturally resistant strains was favored, although the possibility that only particular serotypes could readily acquire resistance seems not to have been considered (9, 10). Use of sulfonamides for treatment of gonorrhea

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was followed by increasing failure rates and the proliferation of sulfonamide-resistant strains of *Neisseria gonorrhoeae* (11). Increasing sulfonamide resistance was also noted in *Neisseria meningitidis* with corresponding clinical failure (12). Whether the neisseria truly acquired resistance was unclear since sulfonamide-resistant strains were discovered in cultures of *N. gonorrhoeae* or *N. meningitidis* from the pre-sulfonamide era (12, 13). Sulfonamide treatment of bacillary dysentery became complicated as well by the isolation of resistant strains, especially of resistant *Shigella sonnei* (14). Isolated instances were also reported of sulfadiazine resistance in pneumococci recovered after therapy of either pneumococcal pneumonia (15) or pneumococcal meningitis (16). Knowledge of bacterial biochemistry and metabolism had advanced after the empirical discovery of sulfonamides so that in 1940 *p*-aminobenzoic acid (PABA) was discovered to block the action of sulfonamide. PABA was proposed to be an essential metabolite for bacteria. Sulfonamide was hypothesized to mimic the chemical structure of PABA and to impede bacterial growth by competing with PABA to prevent its utilization (17). Extracts of resistant pneumococci were soon found to contain increased amounts of a sulfonamide inhibitor (18), which was identified as PABA in extracts of other sulfonamide-resistant bacteria (19), so all seemed consistent with resistance as a result of PABA overproduction. The story took another twist, however, when sulfonamide-resistant *E. coli* were found to make not excess PABA but a sulfonamide-resistant enzyme that utilizes PABA in an early step of folic acid biosynthesis (20). Such target enzyme insensitivity is now thought to be the main, if not the sole, mechanism for sulfonamide resistance (21).

The major mechanism for resistance to penicillin was identified much more quickly. The dramatic increase in penicillin resistance in *Staphylococcus aureus* that took place in the first decade of the antibiotic's use resulted from the selective advantage provided by an enzyme that inactivated penicillin, which was present initially in only a few isolates. The enzyme, penicillinase, was first described, not in *S. aureus*, but in *E. coli*, in 1940, and in the same year clinical studies with penicillin began (22). By 1942 increased resistance was reported in *S. aureus* from patients receiving penicillin (23), and in 1944 penicillinase was extracted from resistant strains of *S. aureus* obtained from patients who had not even been exposed to the drug (24). At Hammersmith Hospital in London the fraction of *S. aureus* isolates that were penicillin resistant increased rapidly from 14% in 1946, to 38% in 1947, and to 59% in 1948 (25) eventually stabilizing at the 90% resistance seen today and inspiring the development of semisynthetic β-lactamase-resistant penicillins, which were the first antibiotics specifically designed to overcome a characterized resistance mechanism (26). Unfortunately, methicillin-resistant *S. aureus* appeared within a few years and were found to make not a methicillin-degrading enzyme but rather a novel methicillin-resistant protein involved in cell wall biosynthesis (27, 28). The battle between bacteria and pharmaceutical chemists synthesizing improved β-lactam antibiotics had been joined and would continue (29).

The basis of resistance to streptomycin remained a puzzle for a long time. Streptomycin-resistant mutations arose at low frequency in many kinds of bacteria, including, unfortunately, *Mycobacterium tuberculosis* when the agent was used alone for treatment. Mutation produced not only high-level resistance but also bacteria dependent on streptomycin for growth, a curious type that could even be recovered from patients treated with the drug (30). A variety of biochemical changes followed exposure to streptomycin, including damage to the cell membrane (31), but it was the observation that the growth of a streptomycin-dependent mutant of *E. coli* in a suboptimal concentration of streptomycin resulted in decreased concentrations of protein and increased amounts of RNA led Spotts and Stanier to propose that streptomycin blocked protein synthesis in susceptible cells but was required for proper mRNA attachment to the ribosome in dependent ones (32). Direct demonstration that streptomycin impaired amino acid incorporation in a cell-free system soon followed (33). Streptomycin at a concentration as low as 10^{-6} M could inhibit polyuridylate-directed incorporation of phenylalanine, but a 1,000-fold higher concentration was required if the cell-free system was derived from a streptomycin-resistant organism. Furthermore, streptomycin was found to cause misreading of the genetic code, so that in its presence, polyuridylate catalyzed the misincorporation of isoleucine and other amino acids (34). So much was learned in studying the interaction of streptomycin and other drugs with the bacterial ribosome (35) that it came as something of a surprise that clinical isolates resistant to streptomycin relied on quite a different strategy, namely modification by adenylation, phosphorylation, and, for other aminoglycosides, acetylation as well (36). The lesson that resistance selected in the laboratory could be different from that selected in the clinic had to be learned.

Resistance to other antimicrobial agents emerged and was studied, but the next major conceptual advance was the appreciation of the importance of R-plasmids, which led not only to a better understanding of resistance acquisition and dissemination but ultimately to recombinant DNA and the biotechnology revolution. The demonstration of transferable resistance in Japan dated from 1959 but took several more years to attract attention and be accepted (37, 38). An explosion of discoveries followed. R-plasmids were found around the world not only in *Enterobacteriaceae* but also in pseudomonas, acinetobacter, staphylococci, enterococci, bacteroides, clostridia, and in virtually every bacterial species examined. Some had remarkably wide host ranges, while others were limited to Gram-positive, Gram-negative, anaerobic, or even smaller bacterial subsets. Techniques were developed

for plasmid transfer, isolation, and classification (39, 40). Transposons that allowed resistance genes to jump from one DNA site to another were discovered (41), as were integrons that allowed resistance gene cassettes to be captured on plasmids and efficiently expressed (42). Restriction enzymes, often plasmid mediated, facilitated analysis of plasmid structure and permitted DNA cloning. The genetics of antibiotic resistance became as tractable as its biochemistry and contributed much to the emerging discipline of molecular biology.

The finding that a β-lactamase (designated TEM) from a clinical isolate of *E. coli* was carried on an R-plasmid (43) led to the realization that this resistance mechanism could spread, not only to other *E. coli* but also to other genera. Before long, TEM β-lactamase was found in ampicillin-resistant *Haemophilus influenzae* (44) and in penicillin-resistant *N. gonorrhoeae* (45). Enzymes more active on cephalosporins than penicillins were discovered, functional classification of the growing body of β-lactamases began (46), the technique of isoelectric focusing was added to the repertoire of β-lactamase biochemists (47), introduction of cefamandole led to the recognition that β-lactamase derepression could provide resistance in some organisms (48), and clinical use of expanded-spectrum cephalosporins was followed by an explosion of extended-spectrum and other β-lactamases (29, 49).

Plasmids carry genes for resistance to many other antimicrobial agents. Some genes code for enzymes that modify or inactivate the agents, others for enzymes that alter drug targets in the cell or provide alternate biosynthetic pathways. Genes for antibiotic efflux (chloramphenicol, tetracycline) were also found to be plasmid determined, but efflux-mediated resistance occurred also from chromosomal mutations that altered control circuits involved in expression of outer membrane proteins that form porin channels for antibiotic uptake. Study of bacteria collected in the preantibiotic era indicated that the plasmids that organize, express, and transmit resistance predated the clinical use of antibiotics (50). R-plasmids resulted from the insertion of resistance genes into previously existing plasmids. The resistance genes themselves probably had a diverse origin. Some could have come from organisms producing antibiotics since those organisms needed a mechanism for self-protection (51, 52). Others may have originally had another function in the cell that could be adapted for antibiotic protection. Given the degree of horizontal gene exchange occurring between bacteria, the donor could be a quite distant relative.

Plasmids are not the only vehicle for such a gene transfer. Naturally transformable pathogens such as *Streptococcus pneumoniae*, *N. meningitidis*, *N. gonorrhoeae*, and *H. influenzae* were found to exchange chromosomal genes with members of closely related species, including genes for penicillin-binding proteins and topoisomerases that provide resistance to penicillin or quinolones (53–55). Mutation plays an important role in resistance to some antimicrobial agents usually by altering enzyme specificity or reducing binding to a lethal target. The notion that resistance was based on infrequent mutational events also led to the concept that resistance could be prevented by simultaneous administration of two drugs since the product of the likelihood of resistance emerging to each would be greater than the size of any possible infecting inoculum, a thesis best justified by the success of multidrug treatment of tuberculosis. An increased mutation rate eventually exerts a fitness cost, but limited rate increases have been found in organisms with resistance attributable to an altered target (quinolone resistance from *gyrA* mutations) (56) or modified enzyme (expanded-spectrum β-lactam resistance due to extended-spectrum β-lactamases) (57).

Antibiotic resistance has come to be accepted as an inevitable consequence of antibiotic use. The ubiquity of the phenomenon has been amply illustrated with emerging resistance to antiviral and antiparasitic agents as well. On the positive side understanding the mechanisms of antibiotic resistance has often provided important insights into how antibiotics work. Knowledge about R-factors has unfortunately not made a direct attack on the genetic basis of resistance possible, but insight into resistance mechanisms has guided the development of expanded-spectrum β-lactams (cefepime, cefotaxime, ceftazidime, ceftriaxone, aztreonam, and others), aminoglycosides (amikacin), and tetracyclines (tigecycline) as well as such resistance inhibitors as clavulanic acid, sulbactam, and tazobactam. A number of enigmas remain. Some organisms, such as *S. aureus* and *Pseudomonas aeruginosa*, seem particularly adept at acquiring resistance, while others are puzzlingly reluctant with certain drugs. *Treponema pallidum* and *Streptococcus pyogenes*, for example, remain fully susceptible to penicillin G despite decades of exposure to the drug, while other organisms have become progressively more resistant. The tempo at which resistance develops is also remarkably variable (Table 1). Resistance may appear

Table 1 Timetable of Antibiotic Discovery and Resistance

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

soon after a drug is introduced or only after many years. Methicillin-resistant *S. aureus* were isolated in the UK within a few years of the drug being introduced (58, 59), but 20 years elapsed before pneumococci with reduced susceptibility to penicillin were isolated and another 20 years before resistance was recognized as a worldwide problem (60). Vancomycin resistance took even longer to appear (61). The equilibrium level at which resistance becomes stabilized is also curiously variable. β-Lactamase production has reached 10–30% in the gonococcus, 15–35% in *H. influenzae*, 30–40% in *E. coli*, 75% in *Moraxella catarrhalis*, and 90% in *S. aureus*, but what determines these levels is poorly understood. Once it has been acquired, however, resistance is slow to decline (62), and there are few examples of reduced antibiotic use associated with diminished resistance (63) so that prevention of resistance by prudent antibiotic use remains the keystone to control. Appropriate use applies to nonhuman applications as well with restraining antibiotics in animal feed as a prominent example.

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Chapter 2
Evolutionary Biology of Drug Resistance

Fernando Baquero and Rafael Cantón

1 Introduction

It is widely upheld that evolution is the result of two essential forces: variability (chance) and selection (necessity). This assumption is confirmed by a number of simple phenomena in antibiotic resistance. Variability is created by random mutation, and some of these variants (for instance those with a mutation in the antibiotic target) become resistant. These variants are selected by antibiotic use and consequently they increase the frequency of resistance. If we increase variability (as in a hyper-mutable strain) or the intensity of selection (antibiotic hyper-consumption), the result is more resistance. This is true, but not the whole truth. Most determinants of antibiotic resistance are not based on simple mutations, but rather on sophisticated systems frequently involving several genes and sequences; moreover, resistance mutations are seldom transmitted by lateral gene transfer. The acquisition of any type of resistance produces a change. In biology, any change is not only an opportunity, but is also a risk for evolution. Bacterial organisms are highly integrated functional structures, exquisitely tuned by evolutionary forces to fit with their environments. Beyond the threshold of the normal compliance of these functions, changes are expected to disturb the equilibrium. Therefore, the acquisition of resistance is not sufficient to survive; evolution should also shape and refine the way of managing the resistance determinants.

Indeed the field of research in drug resistance is becoming more and more complex, and constitutes a growing discipline. More than 20 years ago, Yves A. Chabbert (a brilliant pioneer in research about resistance) and one of us (F.B.), asked the pharmacologist John Kosmidis to coin the right Greek expression to describe "the science of studying resistance", and he immediately produced the word "antochology" (from Αντοχυ, resistance). To our knowledge, this has never been used. In this chapter, we will examine the two essential processes that shape microbial evolution of drug resistance; first, "variability", the *substrate of evolution*, the process providing material in the evolutionary processes and second, "selection", the *mechanism of evolution* (1), the process by which evolution is able to adapt genetic innovation to environmental needs in the bacterial world.

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2 Variability: The Substrate of Evolution of Drug Resistance

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

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

Recent studies of gene expression suggest that a number of cellular functions (some of them increasing fitness) are modified when bacteria are exposed to sub-inhibitory concentrations of antibiotics (2). Sub-inhibitory concentrations of aminoglycoside antibiotics induce biofilm formation in

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Pseudomonas aeruginosa and *Escherichia coli*. In *P. aeruginosa*, the aminoglycoside response regulator gene (*arr*) is essential for this induction and has contributed to biofilm-specific aminoglycoside resistance (3). These results support the notion that antibiotics in nature are not only bacterial weapons for fighting competitors, but they are also signalling molecules that may regulate the homeostasis of microbial communities. Competition, in microbial communities, is seldom a permanent effect; competitors might just be sufficiently aggressive to control the size of their populations, in order to avoid dominance of a single genotype. Diversity, rather than dominance of a particular group, is the landmark of evolutionary success. Indeed the major aim of evolution is to survive, to persist in time; finally, the gain in space or in cell numbers only serves to assure persistence in time (4). This view about an ecological role of antibiotics, serving as both weapons and signals (the classic armament-ornament duality) should immediately influence our view about the evolution of resistance traits (2). If antibiotics act as weapons in nature, antibiotic resistance develops not only to prevent suicide in the producer organisms, but also to protect the diversity of the coexisting microbial communities. If the weapons are intended to be just sublethal, just to modulate the growth rate or to alter the gene expression profile of microbes sharing the same habitat, resistance traits are modifiers or back-modulators of these effects. Indeed we should be open to consider that the emergence and evolution of resistance not only applies for high-level, clinically relevant resistance, but also for resistance protecting the modulation of microbial interactions. If these interactions are important to maintain the bacterial lifestyle, resistance will develop even at very low "signalling" concentrations. In short, there is a multiplicity of the effects of antibiotics in bacteria; consequently, there are many levels on which antibiotic resistance is exerted, from very specific to very general ones (Table 1).

Table 1 Levels of specificity in antibiotic resistance

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

2.1.1 Adaptation without Change: Redundancy and Degeneracy of Bacterial Systems

Even though antibiotics might exert a number of effects on the bacterial cell even at low antibiotic concentrations, a number of cells within a population will be essentially non-affected and could restore the original population (see also Sect. 2.1.2). At the level of a biological system, this is an example of environmental *canalization* defined as the property of a biological system to maintain the normal standard phenotype despite environmental perturbations. This *robustness* or inertia to perturbation depends in part on the redundancy and degeneracy of the biological system. *Redundancy* means that multiple identical units perform the same or very similar functions inside the system. For instance, by assuring high reproductive rates, which results in high cell densities, the negative effects of variation on the entire population is diluted. Indeed small populations have a high risk of extinction by deleterious variation. Interestingly, bacteria tend to increase their replication rate at concentrations of growth-inhibiting substances that are only slightly lower than those that prevent multiplication, but the adaptive interest of this phenomenon has been scarcely explored as yet.

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

2.1.2 Phenotypic Tolerance

Non-inherited antibiotic resistance (no-susceptibility) illustrates the flexibility of bacterial populations to adapt to antibiotic challenges. As stated in the previous paragraph, fully susceptible bacteria from the genetic point of view (that is, lacking specific mechanisms of resistance) might exhibit phenotypic tolerance to antibiotics, that is, they are able to persist at concentrations in which the majority of the population is

dying. Cells regrown from these refractory bacteria remain as susceptible to the antibiotic as the original population. Although canalization, redundancy, and degeneracy probably contribute to this phenomenon, it is the changes in the physiological state of the bacterial organisms along the cell cycle that are probably critical. In practical terms, the main trait of the phenotype is slow growth. Recent experiments have shown that when growing bacteria are exposed to bactericidal concentrations of antibiotics, the sensitivity of the bacteria to the antibiotic commonly decreases with time and substantial fractions of the bacteria survive, without developing any inheritable genetic change (5). Interestingly, these tolerant subpopulations generated by exposure to one concentration of an antibiotic are also tolerant to higher concentrations of the same antibiotic and can be tolerant to other types of antibiotics. It is possible that in any bacterial population, a certain spontaneous switch might occur between normal and persister cells, and it has been proposed that the frequency of such a switch might be responsive to environmental changes (6). In fact, we could designate as "persistence" the result of such a switch, and phenotypic tolerance or indifference to drugs as the physiological status of any cell to become refractory to drugs. However, in our opinion such distinctions are not always clear. Mathematical modelling and computer simulations suggest that phenotypic tolerance or persistence might extend the need of antibiotic therapy, cause treatment failure of eradication, and promote the generation and ascent of inherited, specific resistance to antibiotics (7).

2.2 The Source of Antibiotic-Resistance Genes

Genes currently involved in antibiotic-resistance may have evolved for purposes other than antibiotic resistance (Table 2). From this point of view, resistance should be considered as a chance product, determined by the interaction of an antibiotic and a particular genotype. This is not incompatible with the idea of a gradual modification of some genes of pre-existing cellular machinery to finally "convert" into resistance genes. Some genes which may be neutral or almost neutral in the prevailing non-antibiotic environment may possess a latent potential for selection that can only be expressed under the appropriate conditions of antibiotic selection. In this case we are probably facing a *pre-adaptation* (8, 9), in the sense of assumption of a new function without interference with the original function via a small number of mutations, or gene combinations. In a later paragraph we will see in detail, the possible origin of enzymes hydrolyzing beta-lactam antibiotics (beta-lactamases) as an alteration of the tridimensional structure of the active site of cell wall biosynthetic enzymes (transglycosilases–transpeptidases). In other cases, the mere amplification of genes with small activity for the purposes of resistance may also result in a resistant phenotype (10). Finally, we can have an *exaptation* (11) if the genetic conditions which exist for a function are equally well adapted to serve for antibiotic resistance.

Cryptic tetracycline-resistance determinants are present in the chromosomes of susceptible *Bacillus*, *Bacteroides* or *E. coli* strains. Cryptic beta-lactamase-mediated resistance to carbapenems is present in intestinal *Bacteroides*, or in *Listeria* (12, 13, Pérez-Díaz, personal communication). Chromosomally mediated beta-lactamases are usually found in Gram-negative organisms. Resistance mediated by drug-efflux pumps constitutes an excellent example of exaptation. For instance, a blast search for proteins similar to the macrolide-resistant Mef protein of *Streptococcus* reveals hundreds of hits of similar sequences encompassing all microorganisms, including *Neisseria, Bacteroides, Legionella, Enterococcus, Desulfitobacterium, Lactococcus, Lactobacillus, Ralstonia, Bacillus, Geobacter, Thermologa,* or *Streptomyces*. Recently, the possibility that genetic variants of the aminoglycoside-inactivating enzyme *aac*(6′)-*Ib* gene might reduce the susceptibility to quinolones was reported (14). A number of these enzymes are normal chromosomal genes in a number of species, such as members of enterococci,

Table 2 Examples of resistance mechanisms in clinical strains that evolved from natural functions in non-clinical organisms

Antimicrobial group	Mechanisms	Related natural protein	Natural reservoirs
Aminoglycosides	Acetylation	Histone-acetylases	*Streptomyces*
	Phosphorylation	Protein kinases	
Tetracyclines	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	*Streptomyces*
Chloramphenicol	Acetylation	Acetylases	*Streptomyces*
	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	
Macrolides	Target site modification	rRNA methylases	*Streptomyces*
β-lactams (methicillin)	PBP2a	Homologous PBP2a	*Staphylococcus sciuri*
β-lactams (cefotaxime)	CTX-M-3 beta-lactamase	Homologous beta-lactamases	*Kluyvera ascorbata*
Glycopeptides (vancomycin)	Target site modification: D-ala-D-ala replacement (Van operon)	Van operon homologous genes	*Paenibacillus, Streptomyces, Amycolatopsis*
Fluoroquinolones	Topoisomerase protection	Qnr-like protein	*Shewanella algae*

where they can contribute to the so-called "natural resistance" to aminoglycosides and quinolones. The evolution of vancomycin-resistance determinants is particularly intriguing. They are found in a limited number of limited complex operons-clusters. However these clusters are composed of genes from different sources, and almost certainly originated from a genus other than *Enterococcus*, such as *Paenibacillus, Streptomyces*, *Amycolatopsis*, or from strict anaerobic bacteria from the bowel flora. The classic "*eye evolution problem*" applies here. It is difficult to conceive how such a complicated mechanism of defence against glycopeptidic antibiotics might have evolved, as apparently all its intricate functions are required for the vancomycin-resistance phenotype. In the case of the many different elements that are needed to "construct" an eye, a principal component should emerge first (in the eye, the starting point is the existence of light-sensitive cells). Some small degree of glycopeptide resistance must have evolved first (probably mediated by D-Ala:D-Lac ligases) and this must have been selected and eventually refined by further evolutionary steps. It is likely that unsuccessful combinations have been produced along time, and probably a number of different "solutions" have arisen. Indeed photoreceptors or eyes have also independently evolved more than forty times in the animal kingdom. This example illustrates how Nature evolves in many parallel ways, and the same occurs for drug resistance. The high diversity in determinants of resistance strongly suggests that many of them have evolved to the current function from "pre-resistance" molecules originated from different evolutionary lineages. Indeed we know about dozens of aminoglycoside-modifying enzymes, hundreds of beta-lactamases, many of them redundantly inactivating the same antibiotic substrates.

This panorama helps to visualize the almost unlimited number and variety of potential antibiotic-resistance determinants in the microbial world. Obviously most of the genes involved in actual or potential mechanisms of resistance are located in the environmental bacteria. In a particular location, the ensemble of all these resistance genes constitutes the local *resistome* (15). The size of the resistome is difficult to ascertain because of the huge diversity of microbial species, and also because most microorganisms have never been cultured. In fact only few hundred microbial genomes have been sequenced. Recent bioinformatic approaches for data mining and metagenomics needs to be implemented to reach the desirable goal of describing resistomes. For instance, a recently published work analyzes the presence of metallo-beta-lactamases (MBLs) in the genomes of 12 different *Rhizobiales* (16). Fifty-seven open reading frames were classified as potential MBLs. Four of them were functionally analyzed and one was demonstrated to be a functional MBL. This work showed how bioinformatic tools linked to functional analysis constitute a powerful methodology for exploring the presence of resistance genes in sequenced bacterial genomes.

Clearly, *antibiotic-producing microorganisms* might be considered as a major source of highly efficient resistance determinants. It can be presumed that both antibiotic biosynthetic pathways and the mechanisms of resistance avoiding self-damage may be the result of a co-evolutionary process. In fact, resistance can be viewed as a pre-condition for significant antibiotic production. The benefit associated with antibiotic production (probably preventing habitat invasion by sensitive competitors) (17) probably also selected the producer strains harbouring the more efficient resistance strategies. As previously stated, these resistance mechanisms may in their turn have originated in housekeeping genes (for instance, sugar kinases or acetyl-transferases for aminoglycoside resistance) (18, 19) (Table 1).

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

The origin and function of beta-lactamases in nature are still a matter of debate. Current knowledge upholds that PBPs and beta-lactamases are related to each other from a structural and an evolutionary point of view and that these proteins might have common ancestors in primitive antibiotic-producing bacteria (20). It has been traditionally postulated that antibiotic-producing bacteria need to produce their own antidote to avoid committing suicide and that beta-lactam and beta-lactamase production in these organisms could be coregulated. The filamentous soil bacteria such as *Streptomyces, Nocardia*, and *Actinomadura* produce, among others, beta-lactam antibiotics and beta-lactamases and soil fungi such as *Penicillium* are also able to produce beta-lactam antibiotics. Some of the genes participating in the biosynthesis of beta-lactams, such as *cef* or *pcb* gene variants, share similar sequences in different species of antibiotic producers, including *Cephalosporium*, *Streptomyces,* and *Penicillium*. Amino acid sequence, alignment and bioinformatic analysis led to the proposal that all these genes have evolved from an ancestral gene cluster, which has been later mobilized from ancient bacteria to pathogenic organisms. Horizontal gene transfer must have taken place in the soil about 370 million years ago and multiple gene transfer events occurred from bacteria to bacteria or from bacteria to fungi (21). Beta-lactam gene clusters participating in antibiotic biosynthesis also often include genes for beta-lactamases and PBPs. The beta-lactamase gene products have been shown to participate in part in the regulation of the production of these antibiotics such as cephamycins in *Nocardia lactamdurans* or cephalosporin C in *Streptomyces clavuligerus*. The latter also produces a potent inhibitor of class A beta-lactamase, probably to protect itself from formed antibiotics.

Beta-lactamases and PBPs also share issues other than potential common ancestors, gene sequences, or potential involvement in antibiotic biosynthesis regulation. Both of

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

Amino acid sequences analysis of PBPs and beta-lactamase argue in favour of a common origin of these proteins. Both proteins are members of a single superfamily of active-serine enzymes that are distinct from the classical serine proteases. The amino acid alignments of the main PBPs and different beta-lactamases reveal the presence of conserved boxes with strict identities or homologous amino acids. Moreover, site-directed mutagenesis in the residues essential for the catalytic activity of PBP in *E. coli* and the counterpart residues in class A beta-lactamases has shown similar features in these positions. In essence, the same structural motifs that bind penicillin in PBPs can be used to hydrolyze beta-lactams for beta-lactamases (22).

Structural evidence also supports the proposal that beta-lactamases descend from the PBP cell wall biosynthesis enzymes (23). PBPs are ancient proteins, as bacteria, and came into existence approximately 3.8 billion years ago, but the development of beta-lactamases is a relatively new event, which must have taken place after the evolution of the first biosynthetic pathway in beta-lactamase-producing organisms. It has been argued that this process has been reproduced several times to generate the different class A, C, and D beta-lactamases. Beta-lactamases have had to undergo structural alterations to become effective as antibiotic resistance enzymes, avoiding the interaction with the peptidoglycan or peptidoglycan precursors, which are the substrates for PBPs. This has been disclosed in X-ray interaction models with cephalosporin derivatives and AmpC beta-lactamase variants from *E. coli*. These models revealed not only three-dimensional structural similarities but also that the surface for interaction with the strand of peptidoglycan that acylates the active site, which is present in PBPs, is absent in the beta-lactamase active site.

Alternative hypotheses of the origin and function of beta-lactamases have also been postulated. Antibiotics are known to be secondary metabolite compounds that are normally released in the early stationary growth phase. For this reason, it has been hypothesized that beta-lactamases may also play a role in catalyzing the hydrolysis of beta-lactam nucleus to reutilize carbon and nitrogen as an energy source in adverse conditions and they may act as nutrients for potential growing bacteria (24). Some environmental organisms, including some *Burkholderia cepacia* genomovars and *Pseudomonas fluorescens* have been shown to grow in the presence of penicillin as a sole carbon and nitrogen source and to stimulate the synthesis of beta-lactamase under this condition. From an evolutionary point of view the beta-lactamase-producing bacteria have had advantages over non-beta-lactamase producing-organisms, particularly in soil communities. The former have been able not only to avoid the action of natural beta-lactam products secreted by these antibiotic producers but also to simultaneously use beta-lactams as nutrients.

2.3 Global Stress Regulation and Antibiotic Resistance

In most cases, antibiotic resistance requires time to be expressed in a particular bacterial cell. The best example is when this expression occurs as a consequence of antibiotic exposure (antibiotic-mediated-induction). Only bacteria able to survive during the time required for full induction of resistance mechanisms will be able to resist antibiotic effects and consequently be selected. This "need-to-resist-to-become-resistant" paradox deserves some explanation. Antibiotic action, even at sub-inhibitory conditions, results in alterations of the bacterial physiological network. Physiological networking and signalling mechanisms increase (amplify) any cell disturbance, just as a cobweb increases small oscillations, and immediately provoke unspecific mechanisms of global adaptation. Phenotypic tolerance or formation of "persister cells" might be among this type of response (see above). Mechanisms might involve sigma factors, key components of the translation cell machinery that are responsive to different types of stress (25, 26). Sigma-S defective strains are more susceptible to antimicrobial agents (27). Sigma-regulons are induced by beta-lactam agents, fosfomycin, teicoplanin, rifampicin, or polymyxins (28–30). Probably heat-shock proteins also contribute to unspecific antibiotic defence (31). Of course that means that the excitement of global stress responses by factors other than antibiotics might unspecifically reduce the antibiotic potency. SOS adaptive response might also be unspecifically triggered by antibiotics. For instance, beta-lactam-mediated PBP-3 inhibition results in the induction of the SOS machinery in *E. coli* through the DpiBA two-component signal transduction system (32, 33). Among the immediate consequences of such as early antibiotic sublethal effect is that bacteria might reduce their growth rate, eventually entering in some degree of phenotypic tolerance to drugs, and also that some other adaptive responses are triggered (33).

2.4 Genetic Variation: Mutation

2.4.1 Mutation Frequency and Mutation Rate

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

At the mechanistic level, mutation essentially depends on the error rate of replication that is set by the accuracy of DNA polymerases and various DNA repair systems. In most DNA-based microbes the base-pair substitution mutation rate is in the range of 10^{-10} to 10^{-9}/cell/generation depending on the specific substitution, the gene and the organism. This number is around ten times lower than the typical frequency of mutation (10^{-8} for *E. coli*). It is likely that the lower limits for mutation rates are set by the costs of maintaining high accuracy DNA polymerases and repair systems. Furthermore, the particular environmental characteristics influence selection of the optimal amount of genetic variation for a given organism with a specific population structure.

2.4.2 Hyper-Mutation

If the environment changes rapidly in time, includes stressful conditions and bottlenecks, and particularly if it is highly compartmentalized, variants with increased mutation rates (mutators) tend to be selected since they have an increased probability of forming beneficial mutations. Hyper-mutation is frequently due to the impairment of the mismatch repair system, and more particularly involving alterations in *mutS* gene, but also in *mutL*, or *mutH*. Note that in an asexually reproducing organism, a mutator allele (for instance the *mutS* allele that hyper-generates mutation) and the beneficial mutations are physically and genetically associated in the same chromosome. As a result the mutator allele will hitchhike to increased frequency in the population together with the beneficial mutation.

The lungs of cystic fibrosis patients are chronically infected for years by one or a few lineages of *P. aeruginosa*. These bacterial populations adapt to the highly compartmentalized and anatomically deteriorating lung environment of cystic fibrosis patients, as well as to the challenges of the immune defences and long-term antibiotic therapy. These selective conditions are precisely those mentioned before which increase the rate of mutational variation. Determination of spontaneous mutation rates in *P. aeruginosa* isolates from cystic fibrosis patients revealed that 36% of the patients were colonized by a hypermutable (mutator, mostly *mutS* deficient) strain (exceeding by 10–1,000× the normal mutation frequency, 10^{-8}) that persisted for years in most patients. Mutator strains were not found in a control group of non-cystic fibrosis patients acutely infected with *P. aeruginosa*. This investigation also revealed a link between high mutation rates in vivo and high rates of antibiotic resistance (34). An analogous rise in the proportion of hyper-mutable strains in cystic fibrosis patients has been documented for other organisms, including *Streptococcus*, *Haemophilus*, *Staphylococcus*, and *Stenotrophomonas*, and for analogous clinical conditions, as chronic obstructive pulmonary disease (35–37)

About 1% of the *E. coli* strains have at least 100× the modal mutation frequency of 10^{-8} (strong mutators) and a very high proportion of strains, between 11 and 38% in the different series, had frequencies exceeding by 4–40 times this modal value (weak mutators) (38) (Fig. 1). These proportions are obviously far higher than could be expected by random mutation of the genes that stringently maintain the normal mutation frequency. Moreover, increased mutation frequency may result in a loss of fitness for the bacterial population in the gut (39) as random deleterious mutations are much more frequent than the advantageous ones. Therefore the abundance of strains

with increased frequency of mutation ought to be maintained by positive selection for the hyper-mutable organisms. As hyper-mutability is not an advantage by itself, these strains are likely to be selected by the acquisition (hitchhiking) of an advantageous mutation (40). *E. coli* clones are frequently circulating among different hosts (particularly in the hospital), they are therefore likely to be exposed to heterogeneous environments, which could maintain a continuous selection for hyper-mutable bacteria, particularly weak mutators. Possibly the fitness cost in terms of deleterious mutations is lower in a weak mutator and this allows their raising to higher frequencies in the population. This outcome is expected to occur only in those bacterial populations reaching a considerable size, as in the case of *E. coli*, and not in small populations. Indeed mutators are fixed in competition with non-mutators when they reach a frequency equal or higher than the product of their population size and mutation rate (41). In populations of sufficient size, advantageous mutations tend to appear in weak mutators, and the selective process will therefore enrich low-mutating organisms. The adaptive success of weak mutators may indeed prevent further fixation of strong mutators (41).

Striking differences have been found in the frequency of hyper-mutable *E. coli* strains depending on the origin: faecal samples of healthy volunteers, urinary tract infections, or bloodstream infections. *E. coli* strains from blood cultures are typically isolated from hospitalized patients and are therefore expected to have been submitted to a longer exposure to different hosts and antibiotic challenges. For instance, the frequency of hyper-mutable *E. coli* strains is higher among *E. coli* strains producing extended-spectrum beta-lactamases (ESBLs) (42). In summary, mutation rates show a certain degree of polymorphism, and differences between isolates might reflect the degree of unexpected variation of the environment in which they are located (34, 43–46).

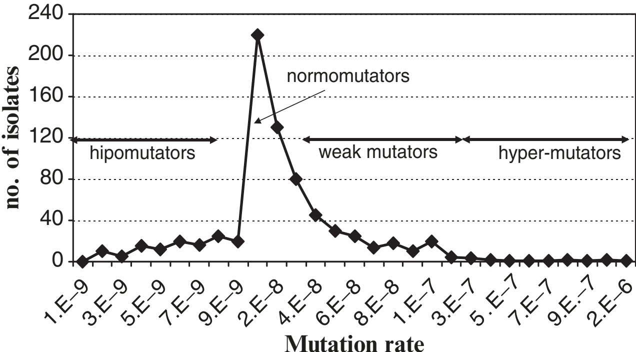

Fig. 1 Distribution of mutation frequencies for rifampicin-resistance in a large international series of *Escherichia coli* isolates recovered from patients and healthy volunteers. Hyper-mutators only account for 1% of the strains, but weak mutators are frequently found in clinical strains, and rare among healthy volunteers (38)

2.4.3 Antibiotics Inducing Mutations

A number of antibiotics induce adaptive responses to their own action, frequently – but not exclusively – by induction of the SOS repair system. SOS induction might be mediated by the SOS repair systems, not only those acting on DNA, but also on the cell wall, as previously stated. One of the non-SOS effects (LexA/RecA independent) is related with PBP3-inhibition cell-wall damage response is the induction of *dinB* transcription, resulting in the synthesis of an error-prone DNA polymerase IV (47). The consequence of this is an increase in the number of transcriptional mistakes, which might result in the emergence of adaptive mutations producing resistance to the challenging agents (46, 48). Antibiotics that produce mistranslation, as aminoglycosides, induce translational stress-induced mutagenesis (non-inheritable!) (49). Many antibiotics induce the SOS repair system, resulting in mutational increase, not only of DNA-damaging agents, as fluoroquinolones (50), but also of beta-lactam agents (51). The reason for mutational increase is the SOS-mediated induction of alternative error-prone DNA polymerases PolII, PolIV and PolV.

2.5 Genetic Variation: Gene Recombination

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

On the contrary, gene recombination might assure spread of mutations associated with antibiotic-resistance phenotypes. This might occur inside the same bacterial cell (intragenomic recombination) or between cells; in the latter case, horizontal genetic transfer is required. Intragenomic recombination facilitates spread of homologous repeated genetic sequences. Gene conversion assures non-reciprocal transfer of information between homologous sequences inside the same genome. This might lead to minimizing the costs associated with the acquisition of a particular mutation (replacing the mutated sequence), or, on the contrary, to maximizing the benefits of mutations that confer a weak advantage when present as a single member (spreading copies of the mutated sequence) (52). Various reports of the latter can be found to explain

how single-mutated rRNAs easily produce antibiotic resistance to aminoglycosides (and probably this is the case for other antibiotics) when the rest of the copies of rRNA sequences remain unchanged: the advantageous mutation spread by gene conversion (53).

The possibility of gene recombination between bacterial organisms is highly dependent on the availability of horizontal gene-transfer mechanisms and the acceptance by the recipient cell of the foreign DNA. For instance, DNA uptake in *Neisseria meningitidis* or *Haemophilus influenzae* is highly sequence-specific. Transformation with *Streptococcus pneumoniae* DNA is exceptional outside this genus. In these very human-adapted organisms, intrageneric transfer facilitates the required variability in the surface proteins needed for colonization of mucosal surfaces in the human host, but the same strategy has been applied for optimizing mechanisms of antibiotic resistance. A variety of mosaic (hybrid) genes, encoding antibiotic-resistant variants of the target-proteins for beta-lactam antibiotics, have appeared in those organisms which are under antibiotic pressure. In these cases, this type of genetic exchange appears to be (as in plants or animals) a force preventing population divergence. In most bacterial organisms, homologous recombination may occur between genes of very divergent sequence.

2.6 Genetic Variation: Modularization

Modularization is a process by which variability is produced as a consequence of the building-up of different combinations among modular genetic elements, creating alternative genetic orders. Genomes of bacterial communities, species, and plasmids, and transposons, and integrons, frequently harbour or are constituted by modular genetic units. Genetic modules are any kind of repeated, conserved cohesive genetic entities that are loosely coupled (25, 54). Common or highly related genetic sequences (from small to very large ones) encoding resistance traits or associated with resistance genes have been found among different bacterial organisms, frequently belonging to different species and phylogenetic groups. The commonality of these sequences can be explained by a common phylogeny, by convergent evolution, or, probably more frequently, by lateral transmission of modular units, in a kind of reticulate evolutionary process. Incremental modularization, the addition of new "resistance" modules to a particular region might occur because there is a "module-recruiting" module (for instance a recombinase), or by duplication of a pre-existing module, or by insertion of an incoming module. As the incoming modules or multi-modular structures frequently provide new interactive sequences, module accretion increases the local possibilities of recruitment of new modules. As this process of modularization occurs at particular genetic regions, these tend to become highly recombinogenic and module-promiscuous (high-plasticity zones). The cumulative collection of antibiotic resistance traits within particular multi-modular structures (integrons, transposons, plasmids) results from this type of nested evolution. The assemblage of modular components occurs by transposition, homologous recombination, and illegitimate recombinational events. Insertion sequences (ISs) are frequently involved in modularization. For instance, IS*26* mediates the mobilization of bla_{SHV} genes encoding ESBLs. The success of a plasmid containing one given $bla_{CTX\text{-}M}$ gene, as is the case of $bla_{CTX\text{-}M\text{-}15}$, also assures the spread of several IS*26* copies which might be involved in further modularization processes leading to multiresistance (55).

The best beautiful recent example of capturing the efficiency of IS modules is the ability of the IS*Ecp1*B element to capture a wild beta-lactamase CTX-M-2 gene from the environmental organism *Kluyvera ascorbata* and mobilizing it into *E. coli*, that has now become resistant to third-generation cephalosporins (56). This recruiting module is involved in the expression and mobilization of many ESBLs (57). Interestingly, the capturing ability of the IS*Ecp1*B module is dependent on a malfunctioning of this insertion sequence for excising itself in a precise way, and so integrating in the excising module sequences adjacent to the point of insertion. It has indeed been proposed that "imprecision" favours DNA arrangements and modularization. Other highly efficient IS module capturing and transposing not only ESBLs, but also metallo-beta-lactamases or cotrimoxazol, aminoglycoside, chloramphenicol, and even fluoroquinolone resistance and large chromosomal modules (genomic islands) are ISCR-type modules (58). ISCR, IS with CR (common region), is a designation that implicitly reflects the modular structure of the module itself. A final example is IS*1999*, which when inserted upstream in novel antibiotic resistance genes mediating very-large spectrum beta-lactam resistance promotes its mobilization (59). In principle, most modules involved in adaptive functions, including antibiotic resistance of every kind (from detoxifying enzymes to porin genes) might be recruited and translocated by IS modules. Other elements involved in module mobilization are DNA transposons and retrotransposons (that move by means of an RNA intermediate).

Modularization might act at the genome level as mutation acts at gene sequence level. Just as in the case of mutations, we should admit stochasticity as the major source of different modular combinations. We can expect that probably most of the combinations do not provide any fitness benefit, or might even reduce fitness of some module-associate functions. Nevertheless, some models suggest that even in the absence of any selective advantage, genotypic modularity might increase through the formation of new sub-functions

under near-neutral process (60). Certainly it might be well conceived that some of these combinations could provide some direct adaptive benefits to the host cell, such as antibiotic resistance. Probably, successful combinations tend to perpetuate the connection among particular series of modules that act more and more now as a single complex module. For this reason there is a synthetic dimension of modularity, which during evolution tends a number of genetic and biological orders, in a "doll-inside-doll" model. Note that modularity implies that bacterial entities are not formed or maintained as strict hierarchies, either from the top down (from ecosystem, communities, species, phylogenetic sub-specific groups, clones, genomes, long or short genetic sequences), or bottom-up (from short genetic sequences to ecosystem).

Indeed we know that not every bacterial phylogenetic group within a given bacterial species is represented in different ecosystems; not a single clone is equally distributed among different hosts; not every plasmid is present at equal frequency among different bacterial species or sub-specific groups. We also know that not every type of mobile element is equally distributed in any bacterial clone within a species, or transposon is inserted with similar frequency in each type of plasmid, or any kind of integron in any transposon, or any antibiotic-resistance gene in any integron. These disequilibria are probably the result of cumulated selective events, exerted simultaneously at different hierarchical levels.

2.7 Horizontal Genetic Transfer and Bacterial Variation

Evolution based on gene recombination and modularization is greatly facilitated by horizontal (or lateral) genetic transfer. In particular, many drug resistance determinants spread between bacterial cells and species using plasmids, conjugative transposons and probably phages. The evolution of resistance on these elements occurs in a modular fashion by sequential assemblage of resistance genes in specific sequences which are frequently mediated by specialised genetic elements such as integrons and transposable elements.

2.7.1 Plasmids and Drug Resistance Evolution

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

Facing an increasingly selective antibiotic environment, in the 1950s, historical (pre-antibiotic) plasmids immediately incorporated antibiotic resistance determinants. The study of pre-antibiotic collections of plasmids strongly suggests that the appearance of resistance genes in plasmids has only occurred during the last five decades. Indeed the diversity of the main plasmid families remains relatively limited, illustrating their success in continuous adaptation and spread of old plasmids thanks to antibiotic-mediated selection. An example is the recent dissemination of old plasmids due to the incorporation to their genetic sequence of genes encoding for ESBLs. For instance, spread of CTX-M-1-like enzymes in Spain is associated with classic IncN, IncL/M, IncA/C_2, or IncFII plasmids (61). Inside these plasmids, evolution might continue diversifying the sequence of ESBLs genes: the existence of identical genetic surroundings of *bla*$_{\text{CTX-M-32}}$ and *bla*$_{\text{CTX-M-1}}$ genes in the same IncN plasmids indicates in vivo evolution of this type of beta-lactamase. All these observations indicate that the total plasmid frequency in bacterial populations might be increasing as a result not only of the more and more extensive anthropogenic release of selective agents, as antimicrobial agents, but also to other organic chemicals or heavy metals (62). This absolute increase of plasmids might have consequences on the full evolutionary machinery of bacterial populations, enlarging the number and variety of genetic interactions. In self-transmissible plasmids, there is always a possibility of entering (particularly under stress) into a new host resistant to the new drug, which may harbour another plasmid determining resistance to this drug. Plasmids from natural populations of *E. coli* frequently show a mosaic modular structure. No wonder that a multiple antibiotic environment has led the plasmid evolution towards the acquisition of multiple antibiotic determinants in a single replicon unit, and even in the same gene cluster.

The possibility of a progressive increase in plasmid frequency and diversity (within classic plasmid backbones) in relation to an escalation of stressful and selective forces in nature, including antibiotic exposure, could be theoretically minored by plasmid incompatibility (inability of two related plasmids with common replication controls to be stably propagated in the same cell line), and progressive capture of plasmid genes by chromosomal sequences which make the cost of plasmid maintenance unnecessary. Recent advancements in the possibilities of determining plasmid relatedness, by restriction fragment pattern analysis, or more significantly, by classification into incompatibility groups (Inc) by

PCR-based replicon (rep) typing (PBRT) (63) have permitted the analysis of large series of resistance plasmids. These studies suggest that the limitation of plasmid incompatibility might be eventually surpassed by the evolution of multi-replicon plasmids or by plasmid co-integration.

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

2.7.2 Transposable Elements

It is mainly transposable elements that have produced genetic transference of resistance in *Staphylococcus aureus* and other Gram-positive organisms. Class I transposons are able to mobilise themselves among different DNA sequences due to the presence of IS flanking their structure (64). Different examples of Class I integrons are those involved in the transference of aminoglycosides resistance genes such as streptomycin, kanamycin or bleomycin (Tn*5*), chloranphenicol (Tn*9*) and tetracycline (Tn*10*). Tn*4001*, which is associated with IS*256*, is one of the most successfully disseminated transposon among Gram-positive organisms. This element harboured the *aac6′-aph2″* gene which encodes a bifunctional enzyme able to inactivate most of the aminoglycoside antibiotics (65).

Class II transposons are widely disseminated among both Gram-negative and Gram-positive bacteria. They have a complex structure, which allows their mobilization from the bacterial chromosome to plasmids present in the bacteria. They have a genetic structure flanked by inverted repeated sequences which also include sequences with functional activity (transposase and resolvases) that facilitate their recombination and integration within the chromosome or a plasmid sequence. Some of these class II transposons may contain resistance genes such as Tn*3* which harbour the $bla_{TEM\text{-}1}$ gene or Tn*21* and their derivatives containing mercury or cadmium resistance genes, which may act as cofactors in the selection process (66, 67). Another example of these class II transposons are Tn*916*-Tn*1545* harbouring tetracycline genes in Enterobacteriaceae or Tn*1456* encoding glycopeptide resistance in enterococci. Moreover, some transposons are able to be transferred with a circular structure similar to that of plasmids (conjugative transposons). Some examples include tetracycline resistance (*tetM*) in *S. pneumoniae* or enterococci.

Transposons are important in the dissemination and maintenance of resistance genes and resistance bacteria. A transposon can be inserted inside another transposon and may contain more than one resistance determinant or even an integron structure (65). These latter elements are able to capture resistance genes (cassettes) due to the recognition of homologous sequences (integrase) and facilitate their expression (67, 68). In general, bacteria harbouring integrons are more resistant to antimicrobials than those lacking these structures as an integron may present more than one resistance cassette. It is important to note that integrons can be mobilized by transposable elements which are also located in plasmids. This structure can be considered as an example of the "doll-inside-doll" model which undoubtedly gives advantage for the selection of resistant bacteria.

Most of the integrons have been described in organisms with high sanitary importance such as *Salmonella* Typhimurium, ESBL-producing *Klebsiella pneumoniae* or *E. coli*. Within the integrons, class I integrons (according to the type of the integrase) have been successfully disseminated probably due to their integration in transposable elements and plasmids. The best example is that of integrons associated with the ISCR1 structure (or ORF513) that are commonly associated with certain ESBL genes ($bla_{CTX\text{-}M}$), carbapenemases genes, the *qnrA* gene, which produces quinolone resistance, or ammonium quaternary compound resistance (55, 69).

2.7.3 Phages

The association of antibiotic resistance with bacterial phages has been overlooked for decades. We should remember that bacteriophages are probably the most abundant type of organism on Earth. Their ability to insert in bacterial genomes, to excise from them eventually carrying host DNA sequences, and to transfer to other bacterial cells, makes them potential vectors for disseminating antibiotic resistance. A number of examples of antibiotic-resistant genes spreading by generalized or specialized phage transduction are available for *E. coli*, *P. aeruginosa, Staphylococcus epidermidis*, *S. aureus*, and *Actinobacillus. B. cepacia* transduce the resistance determinants to cotrimoxazol, trimethoprim, and erythromycin to

Shigella flexneri. A multiresistance gene cluster (*tetG, floR, bla_{PSE1}*) has been transduced from *Salmonella enterica* serovar Typhimurium DT104 to other serovars of *S. enterica*. A high variety of β-lactamases (*bla_{OXA-2}, bla_{PSE-1}, bla_{PSE-4}*, or *bla*P) from *Proteus* have been found associated with bacteriophages isolated from sewage samples. The study of the genetic environment surrounding the plasmid *$bla_{CTX-M-10}$* β-lactamase gene has revealed the presence of upstream sequences with homology to conserved phage tail proteins (70). It is not known whether these genes are part of a functional phage carrying $bla_{CTX-M-10}$ gene or only a reminiscent of an ancestral transduction event.

Abundant phage particles have been found in the supernatant of *Streptococcus pyogenes* harbouring the proton-dependent macrolide efflux system encoded by *mef*(A) gene, and these phage preparations have conferred macrolide resistance to a macrolide-susceptible strain (71). High throughput sequencing has revealed phylogenetically diverse macrolide-resistant *S. pyogenes* strains carrying *mef*(A) inserted in different prophage or prophage-like elements, as Tn*1207.3,* alone or in combination with *tet(*O) gene. *Bacillus anthracis* carries a very diverse array of phages; among them are γ phages which contain a gene conferring resistance to fosfomycin.

2.8 Genetic Variation: Clonalization

Bacterial populations inside species are frequently subdivided in clones, particular lineages or units of descent that probably reflect different evolutionary histories. Multilocus sequence typing has pointed out that most isolates in a clonal population belong to one of a limited number of genotypic clusters (clonal complexes) that are thought to emerge from the rise in frequency and subsequent radial diversification of clonal founders (72, 73). Rise in frequency is in most cases the consequence of selective events favouring the outburst of particular clones and clonal complexes in particular environmental circumstances. Each clone will correspond to a fitness peak, to an "ecotype" (74). This means that the clonal structure of a bacterial population might reflect the changing variety of environments (including environmental gradients) to which the *ensemble* of the species is regularly exposed, and small changes among clones favours microevolution (72). Therefore, we can conceive a bacterial species as a macro-structure composed of a number of clones and clonal complexes that might or might not be present or not in a particular location. In this sense, clones might behave as adaptive modules of a hierarchical superior entity, a "regional community structure", able to provide alternative stable states (75). Mobile elements containing antibiotic-resistance genes, as plasmids, might circulate more effectively in such a genetically highly homogeneous multi-clonal structure, leading to typical complex endemic antibiotic-resistance situations (76) also termed resistance "allodemics" (see Sect. 4.3.1), and Fig. 3 (77, 78).

2.9 Generation of Variation in Response to Antibiotic Stress

We have shown the influence of antibiotics in the mutation rate in Sect. 2.4.3. Indeed that is a particular case of adaptive response to stress. Mutational events (base substitutions, frameshifts, excisions, insertions, transpositions) are increased by orders of magnitude under stress (79–81). Probably, bacterial cells under extreme antibiotic-provoked stress (with membrane or cell wall damage, or compromised protein synthesis, or altered DNA supercoiling) may increase the rate of mutation, which may result in this type of adaptive response. Mutation rates can transiently increase depending on conditions of bacterial growth like starvation and environmental situations that cause bacterial stress, including induction of the SOS response. The SOS cascade can be induced by numerous antibiotics, presumably because these antibiotics cause the production of ssDNA (82). DNA topoisomerase subunit A inhibitors, such as ciprofloxacin and other quinolones have a strong inducer SOS response (50, 83), however the subunit B inhibitors as novobiocin are not inducers (84). On the other hand, antibiotics are also enhancing gene spread among bacterial populations: macrolides, tetracyclines, and beta-lactam agents facilitate intracellular and intercellular gene transfer. Most prophages are SOS-inducible, so that SOS-inducing agents will dramatically increase the spread of prophages. This might significantly influence the spread of antibiotic-resistant genes (85), as it does for virulence factors. Indeed antibiotics might contribute to the spread of resistance genes modifying virulence and host-to-host frequency of transfer. For instance the prophage-encoded shigatoxin gene is SOS-induced and treatment of the haemolytic-uraemic syndrome SOS-inducers, as fluoroquinolones, worsens the syndrome, amplifying the population of phages encoding shiga toxin (86). Goerke et al. have demonstrated the increase of the expression of virulence factors and titres of particle phages in *S. aureus* strains carrying ϕ13 lysogen, after being exposed to concentrations of ciprofloxacin near the threshold of growth inhibition (87, 88). Other antibiotics, such as trimethoprim, have also been reported to cause phage induction (88). In summary, antibiotic pressure in the environment may well contribute simultaneously to the increase in mutant resistant phenotypes, to the selection of the fittest among them, and to the dispersal of resistance genes, which is expected to result in an acceleration in the rate of microbial evolution.

2.10 Phenotypic Variation and Genetic Variation: the Baldwin Effect

As stated in Sect. 2.1 there is a certain degree of plasticity in the bacterial cells and populations that are able to tolerate a determined concentration of antibiotics without requiring any inheritable genetic change. Regulatory factors influencing DNA supercoiling, catabolic repression or growth-phase specific regulators, translational modifications, and/or induction or stress responses might provide this flexibility. In a certain sense, the mechanisms of resistance that are induced by the presence of antibiotic agents also provide adaptive phenotypic variation, as is the case of AmpC related chromosomal beta-lactamases in *Enterobacter* or *P. aeruginosa* (89). A classic important and still unanswered question in evolution is: if survival provided by phenotypic variation influences or does not influence the emergence of specific inheritable genetic changes (90). Apparently, phenotypic variation should limit the selective power of antibiotics for heritable changes, slowing evolution. Nevertheless, plasticity might help crossing adaptive valleys in a fitness landscape. For instance, antibiotic selection will favour the cells in the plastic population that are the most effective in resisting antibiotic action. Low-effective antibiotic-resistance mutations arising in this population will be probably more effective than in the cells with lower expression of plasticity, and might be hooked by selection. Cells that are super-inducible for resistance might be prone to evolve to constitutive production of the mechanism. Indeed, stress-inducible phenotype could be selectively enriched to the extent where it is stably (constitutively) expressed in the absence of stress (91).

3 Selection: The Mechanism of Evolution of Drug Resistance

The common wisdom supports that the emergence of drug resistance is a direct consequence of the selective events imposed by the use of antibiotics in clinical infections. That is probably true in terms of clinically relevant antibiotic resistance, involving a relatively high number of strains with high levels of resistance. In reality, the mere discovery of an antibiotic effect frequently reveals the presence of resistance to this antibiotic, and in many occasions the description of relevant mechanisms of resistance precedes the launching of the drug for clinical use (Table 3). Resistance is always there.

3.1 Selection by Low Antibiotic Concentrations

Antibiotic resistance is frequently recognized by clinicians as a therapeutic problem only after an extremely prolonged period of "subclinical resistance". During this cryptic period, a huge number of selective and evolutionary events take place among the originally susceptible bacterial populations challenged by continuous, intermittent, or fluctuating antibiotic pressure, in the same or in different hosts. Bacterial spontaneous variability, perhaps increased after antibiotic-mediated mass extinction events, offers the selective process an important number of mutants, some of them exhibiting very low levels of antibiotic resistance. In most cases, these mutants remain indistinguishable from the fully "susceptible" strains applying the current standard susceptibility testing procedures that

Table 3 Chronological introduction of different antimicrobial agents in therapeutics and emergence of resistance mechanisms

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

(implicitly) assume their selectability, considering that the peak antibiotic concentration in serum by far exceeds the concentration needed to inhibit the variant. Nevertheless, retrospective genetic and populational analysis of recently emerging resistant bacterial organisms, as beta-lactam resistant *S. pneumoniae* or *Enterobacteriaceae* harbouring ESBLs, strongly suggests that low-level resistant variants have indeed been selected during treatments, and that they have evolved, after new cycles of mutation and selection, to high-level resistant organisms.

The discussions on the evolution of antibiotic resistance in microorganisms have been greatly dominated by some *a priori* beliefs. The first of them probably originated from human chemotherapy: to be considered "resistant" to an antibiotic, a given microorganism should express a relevant increase in the minimal inhibitory concentration (MIC) to this drug. In this view, "minor" increases are meaningless, since the patient can still be successfully treated with antibiotic concentrations exceeding this MIC value. A derivative belief is that: "only significant antibiotic concentrations apply in the selection of resistance". Therefore, as antibiotics are mostly excreted in very small amounts by natural microorganisms in the environment, the origin of resistance as a result of these small selective forces (outside of the producing organism) tends to be disregarded. A third belief, closely related to the first, is that "resistance genes" are only those related to "significant" high-level resistance. Under natural circumstances, the preservation of susceptible bacteria may depend on the fact that the selective effect could be preferentially exerted in a given spatial compartment, in a "small niche" according to Smith and Hoekstra (92). We propose that this compartment, responsible for this type of "confined selection", could be considered as the space or niche in which a precise concentration of antibiotic provides a punctuate selection of a particular resistant bacterial variant. The antibiotic concentration exerting such an effect is here designated as the "selective antibiotic concentration".

3.2 Concentration-Specific Selection: the Selective Window

Any antibiotic concentration can potentially select a resistant variant if it is able to inhibit growth of the susceptible population but not that of the variant harbouring the resistance mechanism. In other words, a selective antibiotic concentration is that which exceeds the minimal inhibitory concentration (under the local conditions) of the most susceptible population, but not that of the variant population (even if it is very close). If MICs of both susceptible and variant populations are surpassed, then no selection of the variant is expected to occur, and the same applies when the antibiotic concentration is below the local MICs of both populations. Therefore, the selection of a particular variant may happen *only* in a very narrow range of drug concentrations (93).

Among the more efficient new TEM-beta-lactamase variants that have evolved to hydrolyze cefotaxime are those which differ from the earlier molecules by several amino acids. Assuming that mutation rates in *E. coli* are in the order of 10^{-10} per base pair per generation, it is unlikely that two or more point mutations would appear simultaneously in a beta-lactamase gene. Therefore, if the TEM-1 beta-lactamase is the ancestor of these multiple multiplied variants, it is most likely that the variants arose by a process of sequential point mutation and selection of singly mutated intermediates. For such a scenario to be plausible, each mutation would need to confer a selective advantage over the ancestral strain. In many cases, strains with monomutated TEM-1 enzymes (such as TEM-12, resulting from a single substitution of arginine for serine at position 164) exhibit only a very small increase in resistance to cefotaxime. Typically, TEM-1-producing *E. coli* is inhibited by 0.008 μg/mL, and TEM-12-producing *E. coli* is inhibited by 0.015 μg/mL. Both in-vitro and in-vivo experiments have demonstrated that despite such a small phenotypic difference, TEM-12-containing strains are efficiently selected by cefotaxime exposure, thereby providing the genetic background for double-mutated, more efficient enzymes; for example, TEM-10 (94). Such selection only occurs in particular antibiotic concentrations that define a "selective window for selection".

3.3 Antibiotic Gradients in Antibiotic Selection

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

Theoretically, each particular variant population showing a definite MIC will have the possibility of being selectively enriched by a particular antibiotic concentration. This conclusion appears obvious. Surprisingly, the theoretical and practical consequences of such a conclusion remain to be

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

A more practical conclusion has been developed in this field when Drlica and collaborators proposed to use antibiotics at dosages that should surpass the "mutant prevention concentration" to avoid the selection of resistance mutants (95).

3.4 Fluctuating Antibiotic Environments

Fluctuating antibiotic environments may facilitate the possibility of evolution of a resistant organism towards higher adaptive peaks than fixed environments. Despite the large number of in vitro mutations that increase resistance to extended-spectrum cephalosporins in TEM-type beta-lactamases, only a small number occur in naturally occurring enzymes. In nature, and particularly in the hospital setting, bacteria that contain beta-lactamases encounter simultaneous or consecutive selective pressure with different beta-lactam molecules. All variants obtained by submitting an *E. coli* strain that contains a bla_{TEM-1} gene to fluctuating in vitro challenge with both ceftazidime and amoxicillin contain only mutations previously detected in naturally occurring beta-lactamases. Nevertheless, some variants obtained by ceftazidime challenge alone contained mutations never detected in naturally occurring TEM beta-lactamases. A number of modulating mutations might arise that are neutral by themselves but in addition to others might equilibrate the antibiotic substrate preference in fluctuating antibiotic environments (96). Indeed it can be suggested that extended-spectrum TEM variants in hospital isolates result from fluctuating selective pressure with several beta-lactams rather than selection with a single antibiotic (94).

3.5 Selection Towards Multi-Resistance: Genetic Capitalism

The concept of genetic capitalism has been recently applied to multi-drug resistance pathogens (97). It refers to further adaptive possibilities of organisms to accumulate resistance mechanisms, either via mutational or gene acquisition events. This reflects a kind of genetic capitalism – the rich tend to become richer. In the last years different examples illustrate this concept such as methicillin resistant *S. aureus*, vancomycin-resistant enterococci or ESBL-producing Enterobacteriaceae. Genetic capitalism has determined not only the increase in the prevalence of multi-drug resistance pathogens but also the spread and maintenance of resistance genes among clinical isolates, those belonging to the microbiota and in the environment (98). Obviously, in environments where exposure to different selective agents (antimicrobial drugs) is frequent, the organism harbouring more resistant traits should have higher possibilities of being selected (multi-lateral selection), and a single antibiotic might select multi-resistant strains. This process is illustrated in Fig. 2. Moreover, the acquisition of resistance genes, or even virulence traits, may increase clonal fitness and may facilitate the uptake of more and more adaptive advantages. Examples of dispersion of specific genes among bacterial isolates from different compartments are those conferring resistance to tetracyclines (*tet*), macrolides (*erm*), beta-lactamases (*bla*), aminoglycosides (*aac, aad, aph*), sulphonamides (*sul*), and trimethoprim (*dfr*). In certain cases, the persistence of resistance genes such as those affecting sulphonamides and streptomycin cannot be explained by the current antibiotic selection pressure, as these antibiotics are scarcely used. However, the concomitant presence of other resistance genes may drive this selection process and explains this paradox. Moreover, the genetic support of resistance genes, including integrons, transposons, or plasmids, also facilitates their persistence without selective force (99).

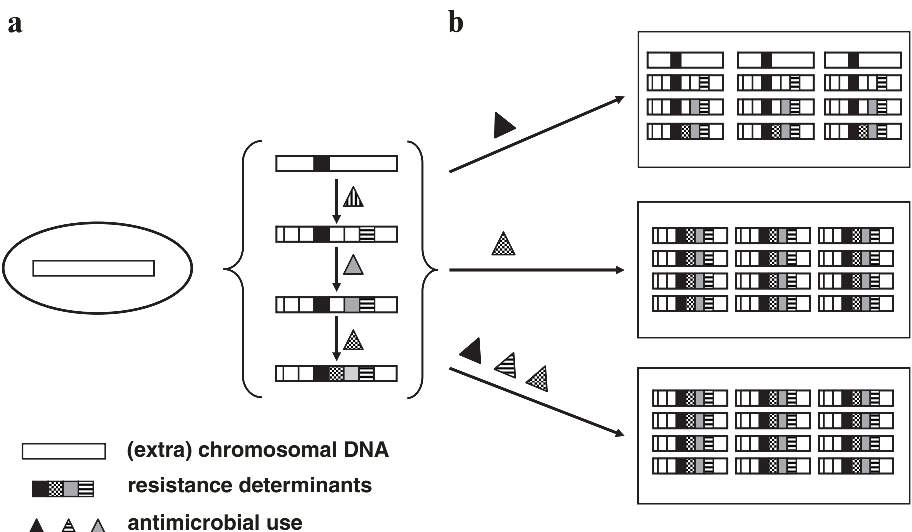

Fig. 2 Emergence of multi-resistance by sequential acquisition of antimicrobial resistance determinants (mutation or gene transfer) and selection of resistant bacteria under different antimicrobial selective pressures. (**a**) The sequential exposure to different antimicrobials may accumulate resistance determinants in bacteria. (**b**) The use of different antimicrobials may select resistant bacteria with different patterns of resistance determinants; note that eventually exposure to a single antibiotic produces the same selective effect for multi-resistance that exposure to different drugs

4 Evolution of Drug Resistance: Future Prospects

4.1 Units of Variation and Units of Selection

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

Suborganismal evolution may involve molecules such as peptides and proteins. Thus, relatively simple forces, such as chemical stability in a certain environment or modular structures within a particular protein conformation, may exert selective pressures within the "protein universe." Suborganismal evolution may also involve genes; operons; stable chromosomal fragments; mobile genetic elements such as plasmids, transposons, integrons, and insertion sequences; and "nuons." This term, coined in 1992 by Brosius and Gould (100), encompasses any nucleic acids that could act as an elementary unit of selection. Thus, nuons might include genes, gene fusions, gene modules encoding protein catalytic domains, intergenic regions, introns, exons, promoters, enhancers, slippage regions, terminators, pseudogenes, microsatellites and long or short interspersed elements. Organismal evolution is exerted on units of selection that are typically microbial clones or cell lineages with particular genomic contents, including also demes or local populations. Supra-organismal evolution is exerted on microbial species, with species considered here as a biological individual with a birth, a transformation and possible death; on clades which are monophyletic groups of species; on communities of microbial species, which include microbiomes, possessing metagenomes; and also on stable associations of microbiomes with particular hosts or host communities (metabiota). We frequently use the term "system" to describe the structure of individuals of higher complexity.

Antibiotics might exert selective activities, or, in other words, disequilibrium at any of these hierarchical levels. Indeed, both between and at each level, the elements composing the system behave as evolutionary pieces, whose relations are governed both deterministically (by affinity or repulsion), and stochastically (by chance or opportunity). The result of these interactions is a constant buildup of complex patterns, in most cases offering nothing advantageous, and in a few cases something deleterious. Occasionally, a coincidence of one of these patterns with a particular environmental challenge determines its selection, and the pattern

(for instance a particular combination of resistance gene, a plasmid and a set of related bacterial clones) is selected. This view enlarges the classic knowledge about selection of just a number of resistant bacterial organisms, and helps to shape the selective landscape of antimicrobial agents.

4.2 The Limits of Drug-Resistance Evolution

4.2.1 Saturation Constraints, Short-Sighted Evolution

There are potential bottlenecks for the evolution of antimicrobial resistance. For instance, genetic variation inside the modified target, determining more and more effective antibiotic resistance levels, may arrive to exhaustion. As the efficiency of the mechanism of resistance improves incrementally, the selective advantage of each increment will diminish, until a saturation point is reached at which increments in functional efficiency result in negligible improvements in fitness (101). Typically this may occur in enzyme kinetics (for instance, hydrolyzing ability of a beta-lactamase for a given beta-lactam antibiotic). When this stage is reached, random changes in the amino-acid sequence are more often expected to impair enzyme performance than improve it. In the case that the modified antibiotic target retains some vital functions in the bacterial cell, the mutational modifications required to reach very high-level antibiotic resistance may reach a lethal situation. This can be considered as a case of "short-sighted evolution".

4.2.2 Minimizing the Costs of Evolvability

In a well-adapted organism, any change including acquisition of drug resistance, has a biological risk. Hence bacterial organisms have developed mechanisms to reduce variation to the lower possible level compatible with evolvability, evolutionary innovation, and ability to adapt. The most obvious way to reduce the necessary costs associated with variation is by reducing genetic variation itself, even at the expense of decreasing variability. The most basic mechanism reducing genetic variation is the degeneracy of the genetic code as a number of nucleotide changes are not reflected in the changes in the amino acid sequence (synonymous nucleotide substitutions). Variation is also reduced by assuring a high-fidelity transcriptional process during DNA replication, or by using highly effective mechanisms of repair of transcriptional mistakes, including increased homologous recombination or daughter strand gap repair. Interestingly, a number of bacteria might have evolved effective mechanisms to reduce the mutation frequency below the average (hypomutation). Mechanisms for stress reduction should also reduce evolvability; indeed the full adaptation of an organism to a very specific niche reduces stress, but stress is maximized when this well-adapted strain is obliged to leave its normal environment. A number of antibiotic resistance mechanisms involved in detoxification of the drug or by its expulsion decrease antibiotic-mediated stress and probably reduce variation and evolvability (102).

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

4.2.3 Cost of Antibiotic Resistance

As said before, gene mutants that have been selected for novel resistance phenotypes may have maladaptive pleiotropic effects (103). This means that acquisition of resistance may de-adapt the resistant organism to its environment thus reducing its competitiveness. Under antibiotic pressure, the competitor organisms may be incapable of taking advantage of this, and therefore the resistant bacteria genotypes have a chance to compensate maladaptation by selection of modifiers (103, 104). This process of adaptation to its own resistance determinants may completely eliminate the biological cost of resistance. The costs associated with the acquisition of non-advantageous changes might be compensated by the acquisition of new changes. Intragenic or extragenic changes (including for instance restorative mutations, gene silencing, or excision) might compensate the cost in a particular environment, but this compensation might even increase the cost in other circumstances. Gene duplication might compensate for decreases in the functioning of a mutated gene and this compensatory effect alone might have important evolutionary consequences. Interestingly, compensatory changes in the bacterial genome may be fixed by reasons other than antibiotic resistance, thus perpetuating the resistance characters in particular genotypes, even in the absence of antibiotic selection. Indeed chromosomal compensatory mutations may eventually increase the bacterial fitness, even if the antibiotic resistant determinant is lost. At the same time, these organisms may be in the optimal situation of being able "without cost" to lose the mechanism if necessary. Frequently, resistant genes are located in large plasmids, but plasmid carriage usually reduces the competitive fitness of bacteria in the absence of selection for plasmid-encoded functions. It could be expected that plasmid-mediated antibiotic resistance

may not be able to persist in bacterial populations in the case of discontinuation of antibiotic use. Interestingly, the cost of plasmid carriage may be compensated in some cases by the mechanisms of resistance encoded, even in the absence of selection. For instance, a tetracycline-efflux pump (determining resistance to this antibiotic) may be used for exporting toxic metabolites from the cell (105). The in-practice non-functional bleomycin-resistance gene in plasmids harbouring the transposon Tn*5* may confer improved survival and growth advantage (106).

4.3 *Epidemiology and Evolution of Antibiotic Resistance*

Bacterial selection may result from the acquisition of resistance to environmental changes that are deleterious for competing populations as happens after exposure to antibiotics. Apparently, resistance does not add new capabilities to the survivor: it just compensates (equilibrates) the reduction in reproductive output imposed by the antibiotic. Consequently, immediate intuition associates selection of antibiotic-resistant microbes with the classic expression "*survival* of the fittest". Note that resistant organisms are only "the fittest" in the presence of antibiotics. Certainly natural selection also acts on positive differences when the acquisition of a novel trait is able to increase the ability of the bacterial organism to exploit a given environment thus provoking a selective difference with the competitors. It is frequently unrecognized that antibiotic resistance provides this type of selective advantage, which is not only a compensation for a loss but *at the same time* is also the gain of a new possibility of habitat exploitation. Frequently, antibiotic-producing microorganisms simultaneously produce antibiotic-resistance mechanisms (18, 19). It may be that the objective (benefit) of antibiotic production is to obtain an *exclusive* environment where only the producer is able to survive, because of resistance. As a consequence, all the resources of the environment can be exploited exclusively by the producing strain. In other words, in the presence of the antibiotic, antibiotic resistance is a colonization factor to gain *exclusivity* for resources. Etymologically, exclusive means "closed for the others". It may be well conceived that in a world in which antibiotics have become frequent components from the microbial environments (in particular in humans and animals), the acquisition of antibiotic resistance is evolving not only a protective mechanism but also a factor assuring *exclusivity* for the resistant populations in antibiotic-containing areas. The increase in the absolute number of antibiotic-resistant organisms is the proof of the benefits of this strategy.

4.3.1 Resistance, Epidemics, Endemics, and Allodemics

Antibiotic resistance is expected to have a minor biological or clinical effect in the absence of effective spread of resistant organisms. As stated in the last paragraph antibiotic resistance might help a given organism to spread, particularly in environments assuring frequent exposure to these drugs. Eventually hyper-mutable organisms might be better suited for host colonization, host-to-host transmission, survival in inert environments and also for developing antibiotic resistance, either by mutation or homeologous recombination with exogenous genes. On the other hand, pathogenic and epidemigenic organisms are probably more frequently exposed to antibiotic therapy. Therefore, a certain convergence between virulence, epidemigenicity, and resistance could be expected to occur (44). Interestingly, antibiotic resistant clones frequently coincide with "successful clones" well adapted for colonization or spread *before* acquiring antibiotic resistance. This convergent process of selection, leading to the dissemination of antibiotic resistance determinants in different bacterial populations is illustrated in Fig. 3. Examples of this can be found in beta-lactam-resistant *S. pneumoniae, E. faecalis,* and *S. aureus* or in glycopeptide-resistant *E. faecium* (107–111).

However, and consistently with the concept of the multiplicity of units of selection stated before (Sect. 4.1), a particular epidemigenic "resistant clone" does not constitute the only selectable unit of antibiotic resistance. The wide application of molecular techniques, such as restriction pulsed field gel electrophoresis (PFGE) to the definition of bacterial clones is offering a totally new view of several "epidemic" phenomena. A surprising diversity of clones was found when the clones responsible for the progressive and steep increase of enterobacterial strains harbouring ESBLs in a single hospital were studied. For instance, *K. pneumoniae* strains harbouring $bla_{CTX\text{-}M\text{-}10}$ belonged to 13 different clones! Therefore, the case was an "epidemic of $bla_{CTX\text{-}M\text{-}10}$ resistance" but not a classic "epidemic" in the classic acception. The term "*allodemics*" (from Greek *allos*, other, different; and *demos*, people), in the sense of "something is being produced in the community by different causal agents" has been proposed to describe this pattern (Fig. 3) (77). Note that the infection (or in our case the frequency of antibiotic resistance) may cluster but not necessarily be its causative organism. In other words, the phenotype may cluster, but not the genotype. Indeed the concept of allodemics emphasises the importance of the asymmetry between phenotype and genotype in natural selection. Its practical consequences are quite obvious. In documented allodemic situations, interventions should be focused more to the environmental causes of the problem than to the classical approaches including

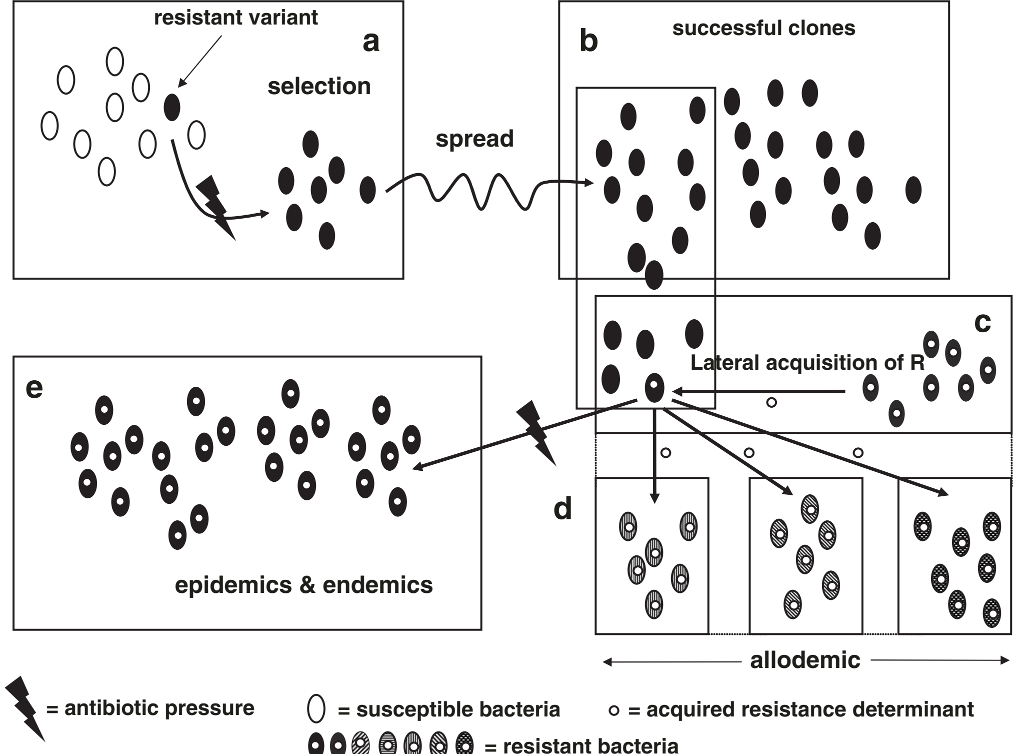

Fig. 3 Epidemiological scenarios for the selection and spread of antimicrobial-resistant bacteria: (**a**) The use of an antimicrobial agent may select resistant bacterial variants within a susceptible population; (**b**) Selection might contribute to the dominance (success) of the resistant clones, favouring spread in different compartments; (**c**) Because of the dominance, successful spreading clones are prone to contact with resistant organisms and to acquire resistance genes by lateral transfer processes; (**d**) At their turn, these resistant clones might act as donors of resistance to other clones depicting an allodemic (or polyclonal) resistance situation); (**e**) Resistant clones with acquired resistance genes may become dominant in particular environments depicting epidemic or endemic situations

clone-directed measures to limit host-to-host spread, or search-and-destroy strategies. For instance in our particular case, a reduction in the intensity of the use of antibiotics potentially able to *select for* ESBLs could be an appropriate environmental intervention for controlling our allodemic situation.

4.3.2 Resistance as a Colonization Factor

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

Certainly, natural selection also acts on positive differences when the acquisition of a novel trait is able to increase the ability of the bacterial organism to exploit a given environment thus provoking a selective difference with the competitors. It is often unrecognized that antibiotic resistance provides this type of selective advantage of being not only a compensation for a loss, but *at the same time* the gain of a new possibility of habitat exploitation. Antibiotic-producing microorganisms produce antibiotic-resistance mechanisms simultaneously (18, 19). When this occurs it may be that the biological benefit of antibiotic production is to obtain an exclusive environment, in which only the producer is able to survive because of resistance. The same might be true if a bacterial organism resistant to antibiotic A were able to induce production of antibiotic A in another antibiotic-producing organism such as another bacteria, fungus, plant, or animal. Antibiotic release will eliminate competitors. In a certain sense, antibiotic-resistant bacteria have taken ecological advantage of human production and release of a number of antibiotics. The increase in the absolute number of antibiotic-resistant organisms is the proof of the benefits of such an evolutionary trend.

4.3.3 Biogeography and Local Biology of Antibiotic Resistance

Biogeography of resistance is the study of the distribution of diversity of resistance over space and time (112). In the words of Brendan Bohannan, "space is the next frontier in biology". The world is a spatially structured place, with localized dispersal, localized interactions, and localized selective events. In environments under high intensity of selective forces (for instance, in the hospital, because of pathogenesis, host-to-host spread, and local usage of antiseptics and antimicrobial agents), the local tool-kit of evolutionary active elements should be very large. Locally successful sub-specific groups, clones, plasmids, transposons, integrons, or antibiotic-resistance genes (see Sect. 4.1 about individuals and units of selection) will be cumulatively selected, and possibilities of interaction (accessibility-connectivity) will necessarily increase. Consequently in these environments we can expect acceleration in the evolution (construction-selection) of complex structures eventually involved in antibiotic resistance. Organisms that are ecologically and/or phylogenetically distant, present in a low density or submitted to environmental isolation might have reduced possibilities for genetic exchange and evolvability. The term "exchange community" has been proposed to identify the biological systems able to exchange genes (113). It is possible that genetic exchange might occasionally occur among organisms sharing similar lifestyles across a wide phylogenetic range; as such "ecologically-close" ensembles of organisms tending to conserve equivalent regulatory networks (114). Note that "genetic exchange communities" are necessarily local ones. Different environments with different cumulative histories of antibiotic use and local epidemics/endemics may harbour different ensembles of evolutionary pieces. Therefore the emergence and development of new antibiotic-resistance patterns is probably of biogeographical dimension. Of course "global spreading clones" disseminate a number of the genetic elements involved in antibiotic resistance but once in touch with local biological ensembles, a local phylogeographic diversification tends to take place.

4.3.4 Antibiotics as Ecosystem-Damaging Agents: the Role of Resistance

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

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

4.3.5 Might Evolution of Antibiotic Resistance Be Predicted?

The ultimate reason for any human scientific knowledge is the optimization or improvement of our current and future interactions with our environment. The reason for research in

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

Antibiotic resistance is a relevant model process in biology. In this respect, predicting the emergence and dissemination of antibiotic resistance is just an exercise of predictive evolution. This exercise is frequently based on qualitative genetics, on the molecular analysis of the genetic elements and functions involved in antibiotic resistance. However prediction of both the emergence and dissemination of resistance needs the aid of quantitative studies of genetics based on molecular phylogeny and epidemiology of all genetic pieces whose interactions result in antibiotic resistance (97). In particular, prediction of evolutionary trajectories in antibiotic resistance need better measurements for selection, consideration of environmental variance and the associated evolutionary constraints.

The evolvability of a known antibiotic-resistance gene towards resistance to new antibiotics should also be explored by for instance using a combination of DNA shuffling and error-prone-PCR. However, the "potential" to evolve towards novel antimicrobial resistance phenotypes is not limited to known antimicrobial resistance enzymes. The chemical structure of new antibiotics should be thoroughly analyzed for detecting potential "enzyme-inactivation points", and bacterial enzymes capable of doing this or a similar function identified. Determination of the three-dimensional structure of such enzymes, including the ones with known antibiotic resistance, docked to potential substrates and followed by site-specific mutagenesis, evolvability challenges and selection experiments might be helpful for predicting these novel enzymatic activities. The possibility of selection of very small phenotypic differences is critical in this process (see Sect. 3.2). In the case of modular structures associated with resistance, the predictive process should be based on research about the "grammar of affinities" between modular elements. Techniques of comparative genomics have been used to infer functional associations between proteins based on common phylogenetic distributions, conserved gene neighbourhood, or gene fusions. The use of scoring-schemes in the building up of networks describing possible associations between modules facilitates the prediction of novel functions (115, 116). Similar types of methods could be developed to predict functional associations between modules involved in the emergence, expression, mobilization, or evolution of antibiotic resistance. A concern of these studies is their unaffordable complexity. Nevertheless, as in the case of mutation, genetic architectures based on modules might have an affordable complexity as they show reuse of alignments or circuit patterns which allow construction of complex adaptive systems by using common series of modules (117, 118). From the perspective of a modular "genome system architecture" (119) it is possible to find in different organisms, plasmids, transposons, integrons or protein sequences such as recombinases, identical modules combined in different ways. The study of the corresponding linkage patterns has become critical for understand the evolution of evolvability (120). Indeed multi-resistance is the result of combinatorial genetic evolution (121, 122). If it were possible to make comprehensive catalogues of modular functional units, combination of these modules in local alignments could be predicted that might fulfil the expected bacterial adaptation (123). The building up of comprehensive interconnected databases where modules could be stored in function of their combinations has been proposed (124). Bioinformatics (network genomics and proteomics) using approaches like combinatorics, fuzzy logic models and principles learned from linguistics and semiotics may be able in the future to accomplish the task of finding a grammar of modular affinities (97, 119, 125) to approach one of the major objectives of all biological sciences: to be able to predict evolutionary trajectories of living beings. To define such a "topology of the possible" (126), a huge amount of work will have to be developed to efficiently identify the most significant modules in particular environments and their mutual linkages: this is the task for a new sub-branch of science, predictive molecular epidemiology based on synthetic biology, that is arising in this new century (127, 128).

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Chapter 3
Pharmacology of Drug Resistance

G.L. Drusano

1 Introduction

Resistance of pathogens to antimicrobial agents is a major problem for all fields of anti-infective therapy. Certainly, we are in a crisis of resistance with respect to antibacterial agents (1). Therapy of human immunodeficiency virus (HIV) is often restricted by multidrug resistance (2). Multidrug-resistant TB is exploding in several areas of the world. Most of the discussion below will focus on bacteria, but the ideas are applicable to any pathogen.

In order to start to address the problem, it is important to start with a definition of antimicrobial resistance. Resistance to antimicrobial agents may be defined in a number of ways. Here, the focus will be on two differing definitions. Each definition has important differences in the outcomes that result. The first definition of resistance, the one with which most clinicians are familiar, is the idea that a specific pathogen, when infecting a patient, will have a low probability of responding to a "normal" drug regimen. This idea is intrinsically bound up with the idea of breakpoints. Another definition of resistance is one often used by microbiologists who perform large surveillance studies. Here, "resistance" is defined by a strain's acquisition of DNA allowing a changed minimum inhibitory concentration (MIC) through drug destruction or other mechanisms (e.g., β-lactamase-containing plasmids), an altered target site, pump overexpression, or any other mechanism that increases its MIC over that of a wild-type isolate, even if it is still treatable by a standard therapeutic dosing regimen. This definition has been used for epidemiological purposes. However, it is also critical to the idea of suppression of emergence of resistance through dose, schedule, and duration choice. We shall examine each of these definitions in turn.

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2 Resistance Defined by Breakpoint: Good Clinical Response for "Sensitive"

Breakpoints dividing measured MICs into different categories (sensitive, intermediate, resistant, nonsusceptible) can be determined in a logical manner. The first question that must be asked and answered is what result is desired by labeling a pathogen as being sensitive (or resistant) to a specific drug (and, by implication, drug dose and schedule)? Normally, when an organism is labeled as "sensitive" to a drug, we mean that a clinician treating a patient infected with this pathogen will have an "acceptably high" probability of the patient responding to therapy with a specific drug dose and schedule.

In order to rationally choose such a breakpoint for determination of susceptibility/resistance, there are a number of pieces of information required:

1. What goal of therapy is desired
2. What is the protein binding of the drug in man and, if the goal of therapy is set pre-clinically, what is the protein binding of the drug in the animal species used for target-setting
3. What is the distribution of drug exposure in a population of patients after being given a specific dose
4. What is the distribution of MIC values (or EC_{50} values, if dealing with viruses) for the pathogen(s) for which the drug is to be used

2.1 Goal of Therapy

Generally, exposure–response relationships in clinical patients are not available a priori. Consequently, such relationships must be developed in preclinical models, such as animal model systems or in vitro systems, such as a hollow fiber infection model. Such models allow linkage of differing amounts of drug exposure to differing amounts of microbiological activity.

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Craig and colleagues have popularized the mouse-thigh and mouse-lung infection models (3, 4), in which the pathogen of interest is injected into the mouse thigh or inhaled into the lungs, a time period elapses (usually 2 h) to allow the infection to progress, and this is followed by treatments that differ by dose and schedule. After a period of time (usually 24 h), the mouse is sacrificed, the posterior thigh is dissected off or the lungs removed, homogenized, and plated after serial dilution to identify the bacterial burden at the time of sacrifice. Since different doses and schedules are administered, this allows linkage of the true dynamically linked variable (peak/MIC ratio, area under the concentration–time curve/MIC ratio (AUC/MIC ratio) or time > MIC) which serves as the independent variable, to the effect achieved, which serves as the dependent variable.

This can be done with microbiological effect, as in Fig. 1a or with a dichotomous end point, such as survivorship, as shown in Fig. 1b. Resistance suppression can also serve as a dependent variable, but will be discussed separately.

In Fig. 1a, the dashed horizontal line is the organism concentration present at the time of therapy initiation. Having the number of organisms at that concentration 24 h later is a definition of stasis for a drug regimen (i.e., no net growth), although it should be recognized that the number of organisms in between the 0 h and 24 h time points can take many trajectories to wind up at the same place. One can also see that it is straightforward to estimate 1, 2, or 3 $\log_{10}$ (CFU/g) decline from stasis and see at what level the measure of drug exposure needs to be to mediate that particular degree of effect. Finally, it is important to recognize that, in this instance, where a cephalosporin-type β-lactam antibiotic is being tested, the time that drug concentrations exceed the MIC (time > MIC) is the measure of drug exposure most closely linked to microbiological effect.

In Fig. 1b, neutropenic rats are challenged intraperitoneally with 10^9 CFU of *Pseudomonas aeruginosa* (6). Previous work had demonstrated that the AUC/MIC ratio or peak/MIC ratio was most closely linked to outcome (6, 7). Here, three isogenic mutants were created that had three differing MIC values to the fluoroquinolone antibiotic lomefloxacin being evaluated (1, 4, and 8 mg/L). Control cohorts are not shown for clarity. Three cohorts with differing MIC values are treated with 80 mg/kg/day and survivorship was observed. As can be seen, the cohort infected with the MIC = 1 mg/L had a 65% survivorship, while the cohort infected with the isogenic isolate with an MIC of 4 mg/L had a 15% survivorship and the cohort with the MIC of 8 mg/L had a survivorship of zero. Clearly, MIC does make a difference. However, there was a fourth cohort, where the infecting organism with an MIC of 1 mg/L was treated with a dose of 20 mg/kg/day. Dose also matters, as the survivorship is 10% here versus 65% when the dose was 80 mg/kg/day. It is also important to recognize that the 4 mg/L cohort treated with 80 mg/kg/day and the cohort infected with the strain having an MIC of 1 mg/L had the same peak/MIC ratio as well as AUC/MIC ratio. Clearly, the survivorship curves are nearly identical. So, even though the doses were different and the MICs were different, the same outcome results when the peak/MIC and AUC/MIC ratios are the same.

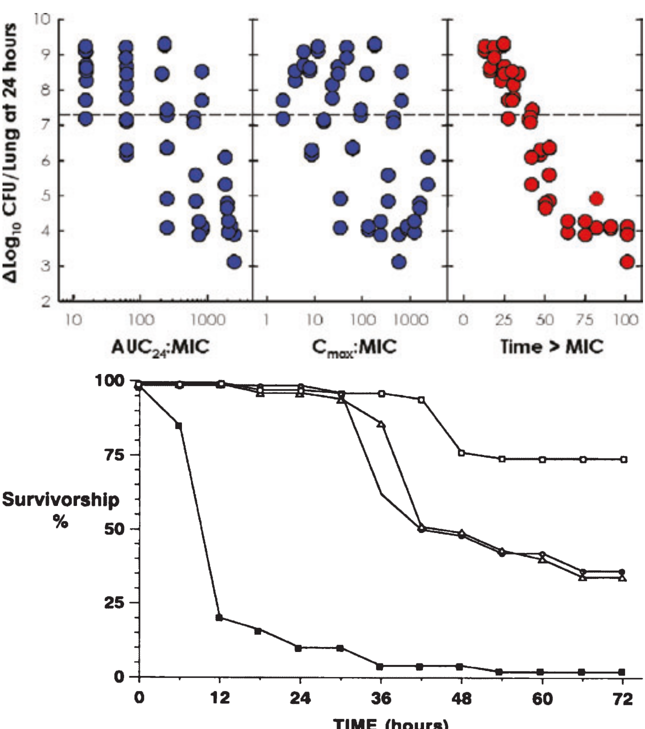
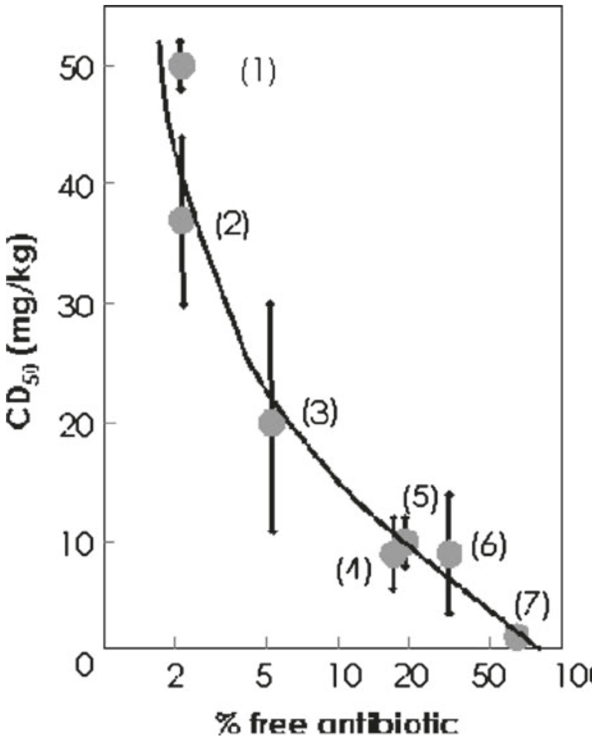


Fig. 1 Demonstration of the pharmacodynamically linked variable for a microbiological endpoint (top panel), as demonstrated by Craig (after Reference 4). Demonstration of the pharmacodynamically linked variable for a survivorship endpoint. Three isogenic strains with 3 different MIC values were studied: parent strain, fluoroquinolone MIC = 1 mg/L; Daughter Mutant #1, fluoroquinolone MIC = 4 mg/L; Daughter Mutant #2, fluoroquinolone MIC = 8 mg/L. Regimen/Strain pairs examined were: (□) Parent Strain: Rx: 80 mg/kg/day; (∇) 4X Daughter Mutant, Rx: 80 mg/kg/day; (Δ) 8X Daughter Mutant, Rx: 80 mg/kg/day; (⊗) Parent strain, Rx 20 mg/kg/day, producing the same Peak/MIC ratio and AUC/MIC ratio as the 4X Daughter Mutant treated with 80 mg/kg/day. After Ref. (6)

From Fig. 1a, if one is contemplating the administration of ceftazidime for pneumonia, it will require that drug concentrations remain above the MIC for about 30–35% of the dosing interval to achieve a static effect. For one, two and three log kills of microorganisms, it is clear that about 40%, 50%, and 60–70% of the dosing interval needs to be covered to achieve the desired microbiological endpoint. Consequently, the target will differ, depending on the drug class as well as the ultimate effect one wishes to achieve.

2.2 Protein Binding

While this topic is sometimes controversial, it should be stated that, in the vast majority of instances, protein binding matters, in that free drug is microbiologically active. There are



Drug	MIC (mg/L)	% bound	Peak (mg/L)	T 1/2 (hr)
1	0.25-0.5	98	3.1	0.3
2	0.25-0.5	98	4.3	0.2
3	0.25-0.5	95	5.0	0.3
4	0.25-0.5	81	2.9	0.3
5	0.25-0.5	79	4.5	0.2
6	0.25-0.5	71	3.7	0.2
7	0.25-0.5	36	5.1	0.1

Fig. 2 Relationship between non-protein bound drug and survivorship in an IV challenge model using the Smith strain of *Staphylococcus aureus* After Ref. (8)

circumstances in which the degree of activity decrement is not mathematical with respect to free drug (e.g., daptomycin). However, there is almost always a decrement in microbiological activity with small, free drug fractions. Where it is not straightforwardly mathematical in nature, it is almost always due to having avidity for the effect target that is near to the avidity of the molecule for the protein binding site (e.g., albumin, α-1 acid glycoprotein).

Merriken et al. (8) demonstrated that protein binding does matter. They took seven isoxazolyl penicillins from the Beecham Pharmaceuticals collection and tested them against *Staphylococcus aureus* strain Smith in a sepsis model. As can be seen, all had the same MIC, and the half-lives were virtually identical, but the protein binding ranged from 2% free drug (drug 1) to 74% free drug (drug 7). Survivorship was clearly related to the degree of protein binding (Fig. 2).

2.3 Distribution of Drug Exposure

When a fixed dose of drug is administered to a large patient population, it is critically important to understand that there will be true between-patient variability. Patients differ by height, weight, sex clearance, organ function, and in ways that we do not measure. The consequences of these differences are that there will be a distribution of drug exposures in the population taking a fixed dose.

Levofloxacin is a very well-behaved fluoroquinolone antibiotic in a pharmacokinetic sense. It is, in the main, renally cleared. We studied 272 patients with community-acquired infections. Patients were required to have serum creatinine values of <2.0 mg/dL. A fixed dose of levofloxacin was administered (500 mg). The resultant distribution of AUC values from administration of the dose is shown in Fig. 3 (9).

2.4 Distribution of MIC Values

In general, organisms have a relatively broad range of MIC values for a specific drug. Some of the range is due to acquisition of resistance mechanisms (e.g., stable derepression of ampC β-lactamase or loss of an outer membrane porin protein) or overexpression of a protein such as an efflux pump. This would create a multimodal distribution. Some is due to the intrinsic ±1 tube dilution variability of the test. However, even without a readily apparent mechanism of resistance and

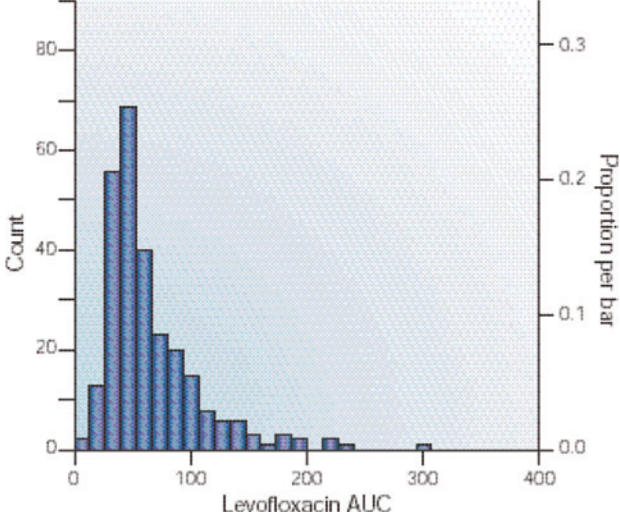


Fig. 3 Distribution of Levofloxacin Area Under the concentration time Curve (AUC) values after a 500 mg daily dose, as derived from the population kinetic parameter values measured in a large clinical trail After Ref. (8).

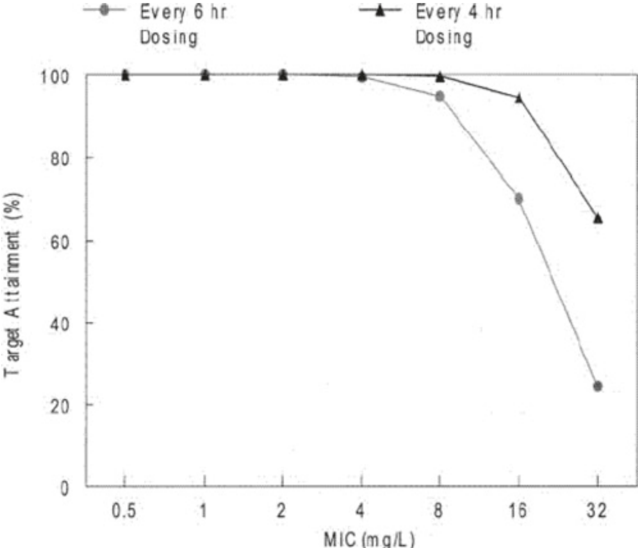


Fig. 4 Comparison of target attainment profiles of 3.375 g of piperacillin/tazobactam administered every 6 h or 4 h as a 0.5 h infusion for hospitalized patients

accounting for the intrinsic test variability, one can still encounter a considerable range of MIC values. As seen in Fig. 1b, this information is important relative to outcome, in that, for a fixed drug dose, a higher MIC provides a lower probability of a good outcome being achieved.

2.5 Integration of Factors for Dose Choice and Sensitivity Breakpoint

In order to choose a drug dose to attain a specific target, it is important to recognize, as stated above, that the dose needs to attain the target at a high probability, recognizing that true between-patient variability exists for pharmacokinetic parameter values. For a specific dose, we can then make a rational judgment about the MIC values that represent "sensitive" and "resistant". We can approach this judgment through the use of Monte Carlo simulation. This allows the full range of exposures attendant to the administration of a fixed drug dose to a large population to be observed. These exposures can then be corrected for protein binding and the resultant free-drug values normalized to different MIC values, to obtain the frequency that the target exposure will be obtained "if the MIC is X mg/L". This can be done irrespective of the exposure target or the pharmacodynamically linked variable (peak/MIC ratio, AUC/MIC ratio, time > MIC). This technique was first described at an Food and Drug Administration (FDA) Anti-Infective Drug Product Advisory Panel (October 15, 1998) and subsequently published (10). An example of this technique is shown above in Fig. 4. Here, 3.375 g of piperacillin/tazobactam is administered intravenously (IV) every 6 or 4 h to steady state in a 10,000-subject Monte Carlo simulation. For each simulated subject, the times that free drug concentrations remain in excess of the MIC are calculated for MIC values between 0.5 and 32 mg/L, for an exposure target of 50% of the dosing interval, at which point near-maximal bacterial kill is achieved (11). As can be seen, the percentage of the simulated subjects attaining the target approximates 100% out to an MIC of 4 mg/L, whether the dose is administered every 6 h or every 4 h. Beyond this, the two dosing regimens diverge. So, what MIC value represents "susceptible" and for what values can we think of the organism as being "non-susceptible"? There is no absolute answer and this technique provides the clinician not with a decision, but rather with a decision support. How high a target attainment percentage is high enough? Obviously, the answer is "It depends". For the wag, it might be said that the percentage should be well in excess of 90% if the person being treated was their mother, but that 75–80% would be just fine for their mother-in-law. Others might take the view that the consequences of the infection being treated needs to be taken into account. For the patient with bacterial meningitis, a more conservative breakpoint might be reasonable relative to a patient with an uncomplicated skin and skin structure infection. The Clinical Laboratory Standards Institute's (CLSI) Antimicrobial Subcommittee has decided that a target attainment percentage of 90% is a reasonable compromise to allow determination that MIC values of that degree of target attainment or higher shall be deemed "susceptible". For Fig. 4, the six-hourly administration

schedule attains 90% target attainment out to an MIC of 8 mg/L, while the four-hourly administration still exceeds 90% target attainment out to an MIC of 16 mg/L.

If one has an MIC value distribution, one can take the product of the probability of an MIC value in the total MIC population and the probability of target attainment at that MIC value, add them together for all the MIC values (take a weighted average) to attain an estimate of how well that specific dose and schedule will perform (target attainment), accounting for the variability in the population for pharmacokinetics and the variability in the MIC values for the pathogen with which the patient is likely to be infected.

3 Resistance Defined by Mechanism

Change in drug susceptibility on the part of a pathogen can occur in many ways. In all the examples below, the resistance mechanism has some, if only a small, cost to the organism. Consequently, it will be maintained in the population only so long as it also provides the organism with some survivorship advantage. One resistance mechanism is related to the acquisition of foreign DNA. An example of this is found in *Streptococcus pneumoniae*, where acquisition of DNA from oral streptococci results in the creation of mosaic chromosomes with altered β-lactam binding proteins and, consequently, altered MIC values for drugs of the β-lactam class. This mechanism is responsible for the 15–30% rate of high-level penicillin resistance seen across the United States. A more familiar example can be found in the acquisition of resistance plasmids or DNA on other mobilizable elements. Here, multiple resistance determinants can be found on a single piece of DNA. More importantly, all resistance determinants can be maintained within the organism by pressure against only one of the resistance elements. For example, theory would set forth the idea that withdrawal of a drug from use would remove that drug's pressure and, over time, the organism would lose the resistance mechanism as being not worth the price of its maintenance. Chloramphenicol is an agent that has had little to no use in the United States for over two decades. Yet chloramphenicol resistance commonly persists through the mechanism of plasmids carrying chloramphenicol acetyl transferase. This occurs because these plasmids may also carry aminoglycoside-modifying enzymes, a β-lactamase, or TMP/SMX-resistance determinants. Pressure against any of the resistances in the cassette causes maintenance of the plasmid with all the determinants present.

In both instances, creation of the initial resistant variant becomes amplified through horizontal transmission. For pneumococci and other respiratory pathogens, day care centers serve as an efficient horizontal transmission mechanism. Resistance-plasmid-bearing organisms can be spread horizontally within hospitals, often on the hands of healthcare workers. Once a sufficient degree of horizontal spread has occurred, it is difficult to impossible to get the resistant pathogen out of the population, as has been seen with *Staphylococcus aureus* and vancomycin-resistant enterococci. Consequently, identification of a new resistance mechanism, perhaps on a mobile DNA element, calls for strict infection control policies to prevent horizontal spread.

In many instances, the acquisition of a resistance mechanism, such as a target site mutation in a topoisomerase enzyme for resistance to quinolone antimicrobials, will cause only a moderate change in the MIC value (e.g., fourfold or less). This is a critical issue, as it is possible in this circumstance to suppress amplification of such a resistant subpopulation by dosing. Before entering into this discussion, it is important to recognize the factors that can lead to the generation of such a population.

The first is the presence of bacteria that have resistance determinants that are present a priori, but are under the control of a promoter or are being repressed in the baseline state. An example of the former is the presence of efflux pumps. For fluoroquinolone antibiotics, *Streptococcus pneumoniae* possesses several efflux pumps for which these agents are good substrates. At baseline, some pumps are expressed. With quinolone pressure, the expression of pumps can be quickly upregulated. In some instances (12), the pumps can be stably overexpressed. This can produce unstable (induction) or stable (usually mutation in a control sequence) relatively low-level change in the MIC. In the case of the baseline suppression of a resistance determinant, perhaps no better example can be set forth than induction of ampD β-lactamase in organisms such as Enterobacter species, Serratia species, Indole-positive Proteeae, Citrobacter species and *Pseudomonas aeruginosa*. It is important to differentiate induction from its cousin, stable derepression. In the former, the presence of a β-lactam causes the induction of increased enzyme production that is unstable and decrements toward baseline after drug withdrawal. In the latter, mutations most often found in ampD result in a sub-population of organisms that produce enzyme at a very high rate all the time, irrespective of the presence of drug. This production is stable and not lost upon drug withdrawal.

In the case of both inducible pump overexpression and β-lactamase induction, these subpopulations, while labile, can be selected and amplified during therapy, as will be discussed below.

More familiar to many clinicians is the idea of an organism harboring a target site mutation, which provides it with a survivorship advantage in the presence of a drug. The

mutation also comes with a fitness cost in many instances. It is the balance of pressure and impact of fitness cost that will ultimately determine the size of the population in a mixed-population environment.

The baseline size of the resistant population is generally determined by the mutational frequency to resistance and the total bacterial population burden. The size of the mutant subpopulation is then approximated by the product of the bacterial burden with the mutational resistance frequency. This has been well understood for many decades in the realm of *Mycobacterium tuberculosis*, where combination chemotherapy has been de rigueur. Here, frequencies to resistance approximating $1/10^5$–$1/10^8$ have been documented for many front-line drugs. In a cavity, total burdens exceeding 10^{10} are not uncommon. Consequently, it is to be expected that organisms with mutations to a primary drug are present at baseline.

Less well known, but highly important is the idea of creation of target site mutations during the course of therapy by error-prone replication. In *Escherichia coli*, the recAB gene sequence is involved and produces a number of error-prone polymerases. Other organisms have homologs that perform a similar function. We have observed this in *Bacillus anthracis* with the addAB gene sequence. Further, certain drugs have a special role to play in the induction of error-prone replication. Fluoroquinolones, as they act at the heart of DNA replication, certainly activate error-prone replication readily. This error-prone replication essentially throws out mutations randomly. Most of the mutations are deleterious and result in the death of the organism. However, some will provide a survivorship advantage under drug selective pressure and it is these clones that will preferentially amplify. We will discuss more about this below.

4 Suppression of Resistance by Dosing

However the resistance mechanism got there, a central question is whether we can use something that we control (like dose selection) to limit the amplification of the resistant subpopulation. The answer is that we can.

Our laboratory examined a strain of *Pseudomonas aeruginosa* in our in vitro hollow fiber infection model and tested the exposure to a quinolone necessary for both early kill and suppression of resistance (13). The organism concentration at baseline exceeded 10^8 CFU/mL, with a system volume of 15 mL. Consequently, the total population burden exceeded 1.5×10^9 CFU/mL. This value exceeds the inverse of the mutational frequency to resistance, resulting in a subpopulation of resistant organisms present at baseline resistant to three times the value of the baseline MIC for the drug. This can be seen in Fig. 5 at time zero. As can be seen in panels E and F, there is little difference between the early (24 h) kill achieved with AUC/MIC ratios of 108 and 201. What does differ is the emergence of resistant mutants after hour 24. This clear differentiation of end point (organism kill versus suppression of resistance) is critical. In circumstances of high bacterial burden, where there is a high likelihood of resistant subpopulations being present a priori, the suppression of resistance will almost always require more drug exposure than will the attainment of an early maximal rate of kill.

We also fit a large mathematical model to the data, using the subsequent results to identify exposures that would suppress resistance and also would generate maximal cell kill, followed by resistance. The results of the prospective validation are shown in Fig. 6. Again, at hour 24, the decline in organisms is virtually identical between the AUC/MIC ratios of 137 and 200. After this time point, however, there is considerable divergence, with the lower AUC/MIC ratio exposure allowing complete replacement of the population with resistant mutants, while the slightly higher exposure holds the resistant subpopulation in check.

Consequently, there is an intervention available that can help suppress the amplification of a pre-existent population of organisms that are less susceptible to the drug in question. This has been demonstrated in an in vitro model with a prospective validation. It raises the question of whether this can be shown in an in vivo setting.

We examined this question in a mouse thigh infection model employing the same strain of *Pseudomonas aeruginosa* as above, but studying a different fluoroquinolone antimicrobial (14). Preliminary studies were performed with differing drug doses. Again, a large mathematical model was fit to the data. This allowed identification of drug exposures predicted to suppress the population in vivo and to also optimally amplify it. We identified a ratio of AUC_{24}/MIC of 52 as optimally amplifying the subpopulation, while a ratio of 157 would suppress it. These results are slightly different from our in vitro studies and the differences are likely due to the presence of an intact immune system, including granulocytes, in the mouse. As seen in Fig. 7, the prospective predictions worked quite well, with the lower exposure allowing subpopulation amplification, while the larger exposure suppressed the population. Indeed, the lines shown are not the best-fit lines, but rather prospective prediction lines, around which the experimental data have been scattered. Consequently, we have demonstrated both in an in vitro model of infection and in a mouse model of infection that choice of dose is important in being able to suppress the amplification of pre-existent populations of

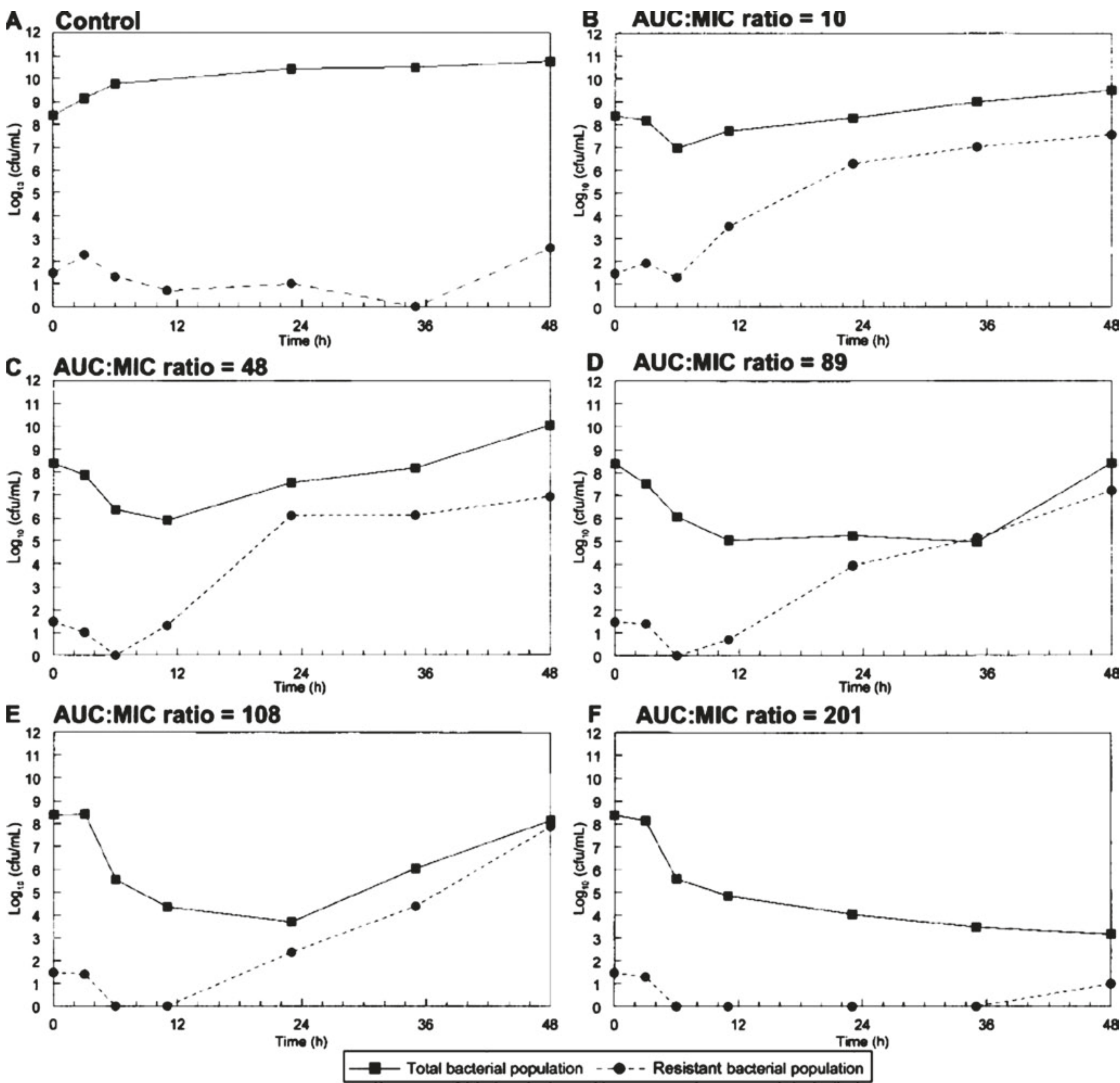


Fig. 5 Response of *Pseudomonas aeruginosa* at various quinolone exposures (AUC_{24}/MIC). After Ref. (13)

organisms that are less susceptible to the drug than their wild-type brethren.

The existence of a less susceptible population of organisms at the outset of therapy depends upon the population burden being high, the mutational frequency to resistance being frequent, or both. There are instances when neither of these conditions is met, yet target site mutations occur. This can be explained in some instances by the induction of error-prone replication (see above). Our group examined the Δ-Sterne strain of *Bacillus anthracis* in our hollow fiber infection model (15). For new agents to gain an indication of this pathogen, the "two animal rule" must be followed. Consequently, in addition to examining the fluoroquinolone levofloxacin against this pathogen simulating human pharmacokinetics, we also employed Rhesus monkey pharmacokinetics. The short half-life seen in the Rhesus resulted in surprisingly poor activity, when given once daily. This was resolved by

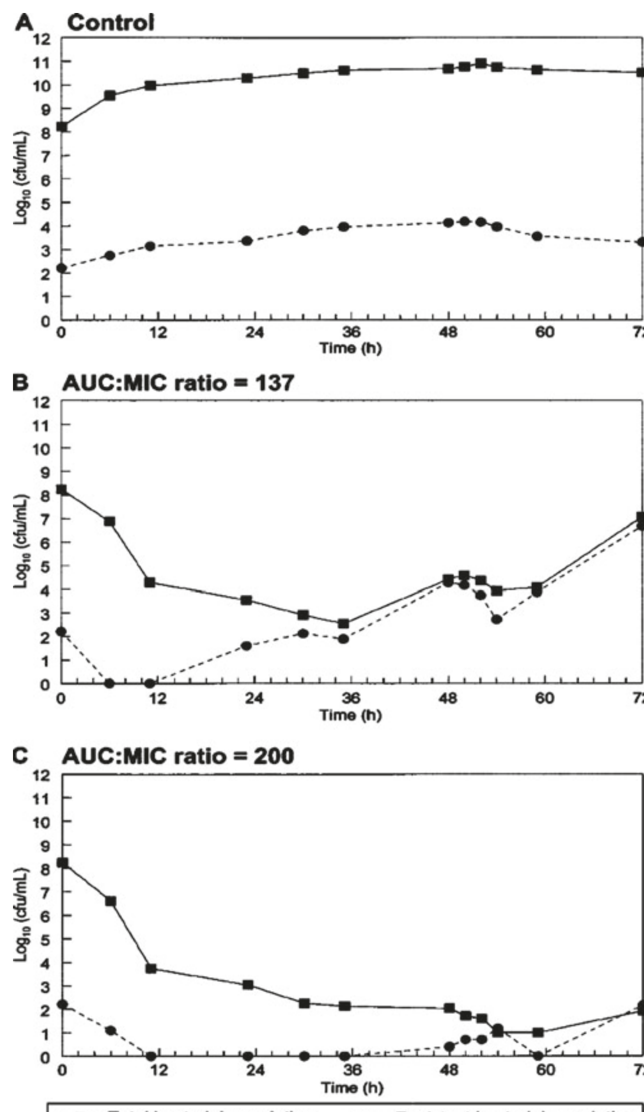


Fig. 6 Prospective validation experiment for regimen success and failure. After Ref. (13)

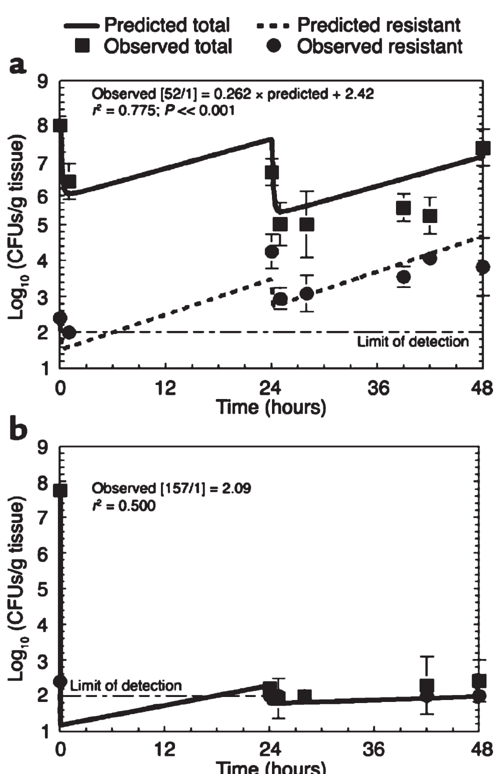


Fig. 7 Prospective validation of resistance suppression in the mouse: (**a**) suboptimal exposure of an AUC/MIC of 57 allowing resistant subpopulation amplification; (**b**) amplification suppressed by the predicted exposure of 157. After Ref. (14)

"humanizing" the dose. However, as part of the evaluation, we noted that emergence of resistance occurred at several of the exposures during the 10-day experimental evaluation (Fig. 8).

The regimens with AUC/MIC ratios of 150, 200 and 300 administered with Rhesus pharmacokinetics allowed emergence of resistance observed first between days 3 and 6. The mutational frequency to resistance for this pathogen is $<1/(5 \times 10^8)$ at three times the baseline levofloxacin MIC. The starting inoculum was approximately 3×10^6 CFU/mL, with a 10 mL volume, giving an approximate burden of 3×10^7 organisms. While the process is fully stochastic and one cannot know for certain without sampling the entire population, there is a low probability that a resistant mutant was present in the population at baseline. It is highly likely, therefore, that error-prone replication was responsible for the emergence of resistance during therapy. The differing lag times until the resistant population became manifest also supports this hypothesis.

It is also important to note that emergence of resistance was NOT seen in the higher AUC/MIC ratio groups over the period of time of the experiment. One can make the inference, then, that even with error-prone replication, the generation of resistant mutants can be suppressed by dosing.

5 Choosing a Dose for Suppression of Resistant Subpopulation Amplification

In both the in vitro as well as in vivo settings, a target exposure could be developed to suppress emergence of resistance. As shown above (Fig. 4), we can apply Monte Carlo simulation techniques to identify the likelihood that the resistance-suppression exposure identified in the mouse (AUC/MIC ratio of 157) would be obtained in man by a standard 750 mg dose of levofloxacin.

Figure 9 demonstrates that a 750 mg dose cannot counterselect resistant subpopulation amplification reliably if the

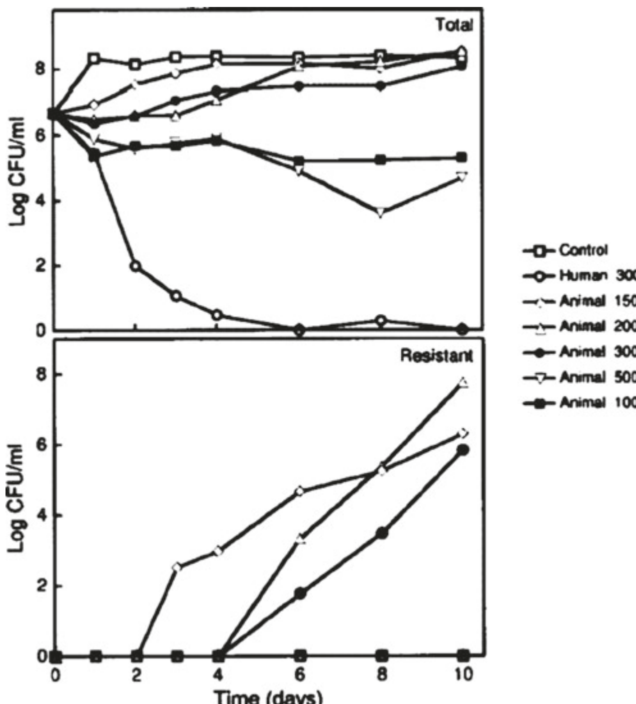


Fig. 8 Activity of levofloxacin (daily dosing) against *B. anthracis* in the hollow fiber infection model (human and rhesus monkey PK). Upper panel shows effect on the total population. The legend shows the exposure for each regimen as AUC/MIC ratio with human or Rhesus PK. The lower panel shows whether resistant mutants emerged by plating on plates containing three times the baseline MIC to levofloxacin. After Ref. (15)

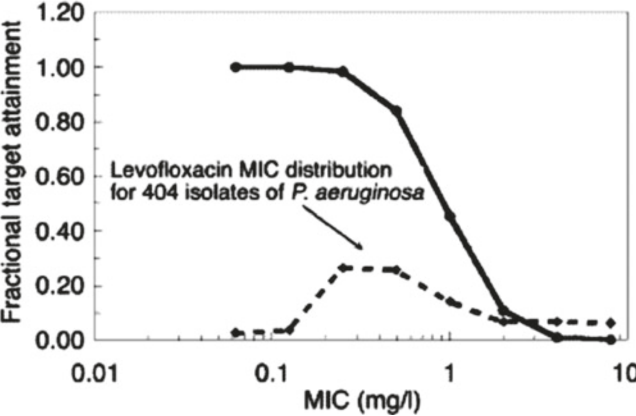


Fig. 9 Target attainment analysis: the fraction of 10,000 simulated subjects that attained an AUC/MIC ratio of 157 (target for suppression of resistance) is displayed as a function of the MIC for a distribution of 404 isolates of *Pseudomonas aeruginosa*. After Ref. (14)

MIC value for levofloxacin exceeds 0.5 mg/L. For the organism population as a whole, one can calculate that this levofloxacin dose suppresses the amplification of the resistant subpopulation with a probability of approximately 61%. This prediction can be shown to be accurate clinically. For another fluoroquinolone (ciprofloxacin), there are two studies of nosocomial pneumonia for which the *Pseudomonas aeruginosa* emergence of resistance rate has been published (16, 17). These studies used very different doses and schedules of ciprofloxacin (200 mg i.v. every 12 h for one and 400 mg i.v. every 8 h for the other). If one performs a Monte Carlo simulation for the two different regimens from ciprofloxacin data drawn from patients with nosocomial pneumonia (16, 17) and uses the resistance-suppression target of 157, one obtains two predictions for suppression of resistance. For the 200 mg every 12 h regimen, there is a prediction of 25% suppression of resistance (75% rate of emergence of resistance). For the 400 mg every 8 h regimen, the prediction is 62% resistance suppression (38% emergence of resistance). For the first trial (16), there were ten patients with *Pseudomonas pneumonia* and three patients with other Pseudomonas respiratory infections (two patients with empyema and one with bronchiectasis). For the subset of patients with pneumonia, there was a 70% rate of emergence of resistance. If one takes all 13 patients, 10/13 (77%) had emergence of resistance, which tallies quite well with the prediction of 75% emergence of resistance. For the other trial (17), there were 36 patients with *Pseudomonas aeruginosa* pneumonia. There were 12/36 (33%) of patients whose organism emerged resistant during therapy. Again, this is quite concordant with the 38% emergence of resistance prediction.

6 Summary

Pathogen resistance to our antimicrobial compounds has reached crisis proportions. The definition of resistance is important in understanding the problem. It is straightforward to generate a pathway to identify a target drug exposure that will suppress emergence of resistance in certain circumstances. This is important because it allows us to intervene in the clinical circumstance through the modality of something that we control (drug dose) to suppress emergence of resistance. Once the target is identified, Monte Carlo simulation techniques can be applied to allow a dose choice to attain the target with a high probability, accounting for patient and pathogen MIC variability. Hopefully, application of such approaches will prolong the useful lifespan of currently available agents as well as new agents entering the physician's therapeutic armamentarium.

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Chapter 4
Antimicrobial Resistance Versus the Discovery and Development of New Antimicrobials

David M. Shlaes and Steven J. Projan

1 Introduction

In many areas of infectious diseases, there is a disparity between the intensity of medical need and the perceived commercial potential for the appropriate products. Well-known examples include malaria and tuberculosis. For a variety of reasons, the same disparity is becoming reality for new antibacterial compounds which could address existing and emerging pathogens that are resistant to current antibiotics. In this chapter, we will review the history of antibiotic discovery and development to put everything else in an appropriate context. We will then explore the scientific challenges that have resulted in the paucity of novel antibacterials in today's pipeline. Then we will examine the various factors that have coalesced to make antibacterials seem less commercially attractive for large companies. We will compare the situation in biotechnology and small pharmaceutical companies with that in large pharmaceutical companies. Finally, we will speculate on the future of antibacterial discovery and development given the emerging trends in science, in the marketplace, within the regulatory environment, and in the context of the pharmaceutical business.

2 Antibiotics and the Pharmaceutical Industry

Penicillin was discovered by an observant and imaginative Fleming in 1928. It was not produced in any sort of large scale until Florey, Chain, and Heatley succeeded during the early days of World War II (1). They used a homemade countercurrent apparatus running at 12 L of culture broth per hour followed by lyophilization. After some initial tests of toxicity in animals, they were ready to produce the drug on a larger scale. But this was not possible with their junkyard Rube Goldberg apparatus at Oxford University. Shortly after Florey's group published results of their work on penicillin in the *Lancet*, an American group under Martin Henry Dawson at Columbia University was able to replicate their experiments using *Penicillium notatum* stock cultures from Fleming's original mold. At the same time, Merck, Squibb, and Pfizer began preliminary work on the manufacture of penicillin. The first successful human therapeutic use of penicillin came in 1941 and 1942 on both sides of the Atlantic. Florey's group, desperately seeking help in further scaling up production, approached the Lister Institute and Wellcome Research Laboratories in England without success. They then decided to come to the US where they attempted to scale up production of penicillin at the Bureau of Agriculture facility in Peoria, Illinois. At the same time, they began discussions with a number of US pharmaceutical companies, of which Merck, Lederle, Squibb, and Pfizer expressed serious interest. Abbott and Winthrop soon joined the effort as well. To facilitate the work, the US government exempted the companies from anti-trust provisions of the law for the purpose of pursuing the manufacture of penicillin for the war effort. During 1943, enough penicillin was available only for the military, although occasional civilian cases were treated on a compassionate basis. By D-day, the production had increased hundreds-fold, but was still not enough for general civilian use. Manufacture to the scale required for general civilian use did not occur until 1945.

The experience gained by the pharmaceutical companies in the manufacture of a natural product from a culture of mold would serve them well as the great antibiotic era would begin with streptomycin, the tetracyclines, the macrolides, and others.

3 The Rise in Resistance to Penicillin

Before penicillin was manufactured on any significant scale, Abraham and Chain reported the existence of bacterial β-lactamases capable of hydrolyzing the antibiotic molecule,

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rendering it ineffective (2). By 1952, 75% of hospital isolates of *Staphylococcus aureus* in some areas of the US were resistant to penicillin by virtue of β-lactamase expression (3). This was a fascinating epidemic that probably started in Australia, where, in 1957, this strain accounted for 61% of outbreaks in that country (4). At the National Institutes of Health (NIH) in Bethesda, phage type 80/81 penicillin-resistant strains peaked at 8.7% of all strains tested in 1960. Indeed, there were reports from some US centers that up to 30% of hospital staff were nasal carriers of 80/81 complex strains, with over 5% developing overt signs of infection (5–8). Some hospital nurseries had nearly all newborns colonized (6–8). The 80/81 complex strains were involved with 1–2% of all nosocomial mortality (9). By 1964, phage type 80/81 was disappearing or had disappeared entirely for reasons that remain obscure, although by then penicillin resistance had already invaded the community (4). By the 1970s, 85–90% of strains from both the hospital and the community were resistant to penicillin (10), in large part because of the transmission of virulent phage 80/81 strains from the hospital to the community via colonized or infected healthcare workers or patients. The prevalence of resistant strains in hospitals and the community was part of the motivation for the era of antibiotic discovery to follow.

3.1 The Golden Era

Perhaps the full flowering of antimicrobial drug discovery was the generation that spanned 1955–1985. Well over 100 antibacterial agents were clinically tested and at least 60 were brought to the market. Not only were newer, more potent, better-tolerated, and easier-to-administer versions of older antibiotics discovered, but novel classes of agents, including those with new mechanisms, were also put into clinical practice. Below are listed some of the highlights of that era. An important lesson is that, over time, clinical practice was able to sort out which of these agents provided the best therapeutic utility (and even discriminate between resistance "issues" and those which were less problematic). It was only the large number of options to choose from that allowed for the cream of the antibiotic crop to rise to the top. Contrary to the publicly expressed views of pharmaceutical industry critics, the fact is that even "slight" differences in properties such as tolerability, bioavailability, spectrum of activity, and potency can translate into a significantly superior antibiotic. The simple fact that the "me too" antibiotic methicillin (11) was not hydrolyzed by the *Staphylococcus aureus* β-lactamase was the key to restoring the clinical utility of the β-lactam antibiotics until the emergence and dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

What made this the Golden Era was, first and foremost, the enhanced ability to solve the molecular structures of antibacterial agents. Indeed, unraveling the structure of tetracycline in the early 1950s led directly to both doxycycline (12) and minocycline (13), semisynthetic tetracyclines which are both clearly superior to the original natural product.

Discovered during this era were antibiotics in new classes including the glycopeptides (vancomycin (14)), the rifamycins (15), the fluoroquinolones (16), the cephamycins (17), the lincosamides (18), and the β-lactamase inhibitors (19). Also, this period witnessed an amazing expansion in previously discovered classes such as the aminoglycosides (e.g., tobramycin, gentamicin, kanamycin (20)), the macrolides (clarithromycin, azithromycin (21)), the penicillins (piperacillin (22)), and especially the cephalosporins (23). Perhaps the three most widely used antibacterial products were all discovered and developed during this era: ciprofloxacin (24), amoxicillin/clavulanate (25), and ceftriaxone (26) (primarily a hospital IV antibiotic).

This activity was fueled by large numbers of medicinal chemists and aided and abetted by a phalanx of microbiologists at scores of relatively small to mid-sized pharmaceutical companies. Interestingly, the large number of therapeutic options meant that price competition was a real factor in this field. Indeed, even today antibiotics still represent one of the most inexpensive types of therapy, and, if there were ever such a measure as a cure per cost index, antibacterial agents would easily rank at the top of all treatments (one of the reasons for their "overuse").

4 Microbial Genomics: The Failure to Deliver and the Hope for the Future

The last novel class of antibiotics to be launched into the market place was trimethoprim in the late 1960s (IDSA report). With the rise of antibiotic resistance, especially the pandemic of MRSA, a medical need for new antimicrobials was being created. To answer this, discovery scientists began to turn to the new technologies available to them. In the early 1990s, Human Genome Sciences established a collaboration with Smith Kline Beecham Pharmaceuticals to provide the complete genome sequence of *Staphylococcus aureus* for use in antimicrobial drug discovery. This was, perhaps, the first of the genomic alliances in anti-infective drug discovery. During the rest of that decade a number of biotechnology companies, on the basis of their ability to provide genomic sequences or carry out functional genomic studies, emerged mainly from academia. They quickly established collaborations with other biotechnology companies or with large pharmaceutical companies desperate for new antibacterial targets.

The parallel evolution of X-ray crystallography, combinatorial chemistry, and high-throughput screening held the promise of new antibiotics with novel modes of action. To the present time, none of these efforts has resulted in a marketed antibiotic or even one in advanced-stage clinical development. On the other hand, many new and novel essential genes have been identified and some have had their 3D structure elucidated by either NMR or X-ray methods, or both. A few have even been described with small molecule ligands. Yet none of these efforts has yet been ultimately successful in terms of delivering a new antibacterial to the marketplace. Why?

There are many potential explanations for the failure of the genomics revolution in antimicrobial discovery. The most likely one revolves around the nature of the novel targets that were discovered. Some targets were unsuitable because they were not sufficiently conserved across bacterial genera, thereby restricting potent antibacterial activity to a small number of organisms. The best example of this might be the FabI inhibitors from Glaxo Smith Kline (27), which have potent activity against staphylococci but have no activity against streptococci in which FabI is replaced by FabK (28). For other targets, the active site seemed unsuitable for binding pharmaceutically favorable ligands found in the compound libraries of large pharmaceutical companies. This was either because the active site was too open and available to solvent or too hydrophobic such that favorable molecules could not enter. Frequently there was a dichotomy between activity of compounds against the target in vitro and those most likely to penetrate the bacterial membrane (or evade efflux), thus making optimization more than challenging. Sometimes, there was simply an inability to assay for the relevance of compound binding to a novel target in vitro, since the activity of the target in the cell was not known.

Genomics did, on the other hand, also provide for a wealth of available assay formats that allowed researchers to obtain key information on potential inhibitors of any target. Perhaps the most immediately important aspect of this was the development of sensitive assays that allowed one to determine whether the inhibitor, which also had antibacterial activity, was acting through its target in situ. If one could make that demonstration, then optimization against the target at least provided hope that antibacterial activity might improve in a parallel manner.

Today, genomics of mammalian cells is contributing to our understanding of toxic effects of compounds, and, in some circumstances, in vitro assays based on genomics predict animal (and human) toxicity.

Some bacterial targets, such as peptidyl deformylase (29), FabF (30), and others, are still being explored, with late preclinical and early clinical candidates in the pipeline. Thus, it is still possible, even likely, that bacterial genomics will deliver novel antibacterial compounds in the foreseeable future.

5 Medical Need, Antimicrobial Resistance, and the Anti-Infective Marketplace

As noted earlier in the chapter, there was a time when the pipeline of new antibiotics was so robust that practitioners were less concerned about emerging resistance, being assured that new products active against resistant strains were around the corner.

Another dynamic in this arena is the need to provide physicians with the means to treat empirically. Although bedside diagnosis of some infections is coming, the absence of such capability and the expense of the diagnostic tests already available (at least in the community setting) have forced physicians to treat empirically in most cases. This means that antibiotics targeting specific bacterial species, such as those active primarily against staphylococci or against *Pseudomonas* will be difficult for physicians to use on a practical, real-world basis. Another factor contributing to this desire for broad-spectrum agents is the fear that pathogens not diagnosed may still be partly responsible for the patient's infection, and the resulting desire to continue broad-spectrum therapy "in case."

In general, antibacterials constitute one of the few areas in pharmaceuticals where medical need and marketplace can diverge. There are a number of reasons for this. First, there is the probable link between antibiotic use and emergence of resistance. Thus, as an antibiotic is used, the risk of emerging resistance grows, and once resistance appears, there is a certain time period, which varies considerably with the antibiotic in question, until the resistance becomes prevalent enough to alter the perceptions of medical practitioners. The level of prevalence of resistance required to trigger physician awareness also seems quite variable.

Resistance to penicillin was discovered before penicillin was marketed. Yet it was not until the outbreaks of penicillin-resistant *Staphylococcus aureus* in US hospitals in the mid-1950s through the 1960s, especially those caused by virulent phage 80/81 strains, that there was sufficient medical need to give rise to markets for the first cephalosporins, methicillin, and even for vancomycin with its reputation for toxicity. This market expanded with the spread of penicillin-resistant staphylococci and *E. coli* to the community in the 1960s and 1970s.

Aminoglycosides share a similar history. With the discovery of streptomycin, resistance among *M. tuberculosis* followed almost immediately, leading ultimately to the development of isoniazid. Gentamicin, which targeted Gram-negative bacteria and which was almost always used as part of combination therapy, gave rise to resistance peaking in the late 1970s and early 1980s, which led to the development and introduction of amikacin and other aminoglycosides to the marketplace.

In the case of the carbapenems and vancomycin, however, we are seeing a different picture. These are the so-called last-line options for physicians and patients in situations where, at least until recently, there were no other options for treating serious infections caused by multiresistant pathogens. The beginnings of the compromise of vancomycin against methicillin-resistant staphylococci and of carbapenems against multiresistant Gram-negative bacilli have led to serious concerns among physicians and opened the marketplace to new products. For Gram-positive pathogens, linezolid (31) offers oral as well as IV therapy, albeit at a high price. But the prevalence of frankly vancomycin-resistant strains remains, thankfully, low. For carbapenems, there is a growing number of resistant bacterial strains in hospitals, especially among *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (32). The only new broad-spectrum antibiotic to be introduced recently has been tigecycline, which has activity against *Acinetobacter* but not against *Pseudomonas*.

With even our “end of the line” antibiotics being threatened with emerging resistance, there is a tendency to keep new antibiotics that target key resistant pathogens in reserve to preserve their utility as long as possible. As noted previously, from the market viewpoint, this is the great paradox of the anti-infective arena. In almost no other therapeutic area will physicians take a new and effective product that answers a key medical need and “keep it on the shelf” as long as possible (33).

Antibiotic sales, although representing one of the largest sales volumes (around $26 billion in 2004) (33) among the therapeutic areas, have been and are projected to remain relatively flat. In terms of prescription volume, generics account for the vast majority. In terms of value, though, branded products continue to drive the market. One of the most fascinating launches of an antibiotic was that of linezolid. Linezolid is positioned as a hospital product with a fully bioavailable oral version as well as a parenteral one. The oral is priced approximately the same as the IV form (expensive), essentially restricting it to hospital use. Even with its Gram-positive-only spectrum and its bacteriostatic mechanism of action, linezolid sales will achieve blockbuster status if they have not already done so. This demonstrates that with a niche market strategy, given the right niche where there is a lack of competition (oral therapy for MRSA and vancomycin-resistant enterococcal infections), and given the right pricing and sales strategy, commercial success is still possible in anti-infectives.

The other important observation is that linezolid targets the hospital market. This marketplace is manageable for a small pharmaceutical company, since the sales force that is required, especially considering the US alone, is relatively small. Thus, in the recent past, a number of biotechnology companies, including Vicuron (now Pfizer), Cubist, Theravance, and others, have specifically targeted the hospital market. In some cases, they have taken their clinical candidates through Phase III trials all the way to the marketplace without a partner. Thus, a small biotechnology company by itself could launch a US product, and perhaps even a US-European product, given access to appropriate public capital markets to fund the development costs.

In this overall dynamic, we must consider the role of community-acquired infections by virulent strains of methicillin-resistant *Staphylococcus aureus*. These infections strike otherwise healthy young adults and children and cause considerable morbidity and mortality (34). It is fascinating that these strains appear to resemble the old bacteriophage 80/81 strains genetically, except that they have now acquired SCCmec and, therefore, methicillin resistance (35). Certainly, the strains are following a similar pattern to the phage 80/81 pandemic of the 1950s and 1960s. This will put further pressure on clinicians to use linezolid outside the hospital for empiric therapy. At the same time, this medical need, given the success of linezolid, might provide enough of a market opportunity to motivate large pharmaceutical companies to continue working in the antibacterial area and to further encourage biotechnology companies and their investors.

6 The Regulatory Environment for Antibacterials

A good description of the regulatory environment for antibacterial compounds at the time of writing would be “confused.” This state of affairs is an improvement over the previous condition of “hostile.” The confusion comes mainly from the Food and Drug Administration (FDA) in the United States, which is, of course, the most sought-after marketplace for pharmaceuticals in general. It arises from three main sources. The FDA has traditionally provided specific guidance for the development of antibacterial compounds, reaching to partners such as the Infectious Diseases Society of America for help in the development of these guidelines. The guidelines now are seriously out of date and badly in need of revision. Further, the FDA has initiated an indication for activity against resistant strains, which is much sought after by companies for competitive purposes but for which there is no guidance. Although this does not, and in our opinion should not, stop companies from pursuing such an indication, the lack of guidance from the FDA is certainly new. Large pharmaceutical companies, being essentially risk averse, prefer a clear path forward and a level playing field for everyone – a role filled previously by the guidance documents. It is of interest that no such guidance documents exist

in Europe and yet companies are able to register antibacterials in the European marketplace.

The FDA has now mandated placebo-controlled trials for indications where they are concerned that there could be a large placebo effect. In 2007, based partly on concerns regarding serious hepatotoxicity, they withdrew the indications for treatment of acute bacterial sinusitis and acute bacterial eacerations of chronic bronchitis from the label of telithromycin stating that the absence of placebo-controlled trials made it impossible to establish a positive risk-benefit ratio. They conveniently ignored the fact that most other antibiotics approved for these indications were not approved based on placebo-controlled trials either and some of them have safety profiles no better than telithromycin. It is highly questionable whether such trials can be completed. Replidyne was unable to complete such a trial for faropenem and has since gone out of business. The NIH has several ongoing placebo-controlled trials and the world is watching for their progress and ultimately their data.

The commercial implications of the insistence of the FDA on placebo-controlled trials for the indications of Acute Bacterial Sinusitis and Acute Bacterial Exacerbations of Chronic Bronchitis is that the marketplace for new oral antibacterials in the community is decimated and no large pharmaceutical company will venture into this area until there is less risk. This will push the industry towards hospital-based infections which has been traditionally less lucrative although that may now be changing.

7 Large Pharmaceutical Companies Consolidate and Abandon Antibacterial Discovery while Biotechnology Enters the Field

The costs of development for pharmaceuticals have been increasing for many years to the point where, accounting for all failures, each successful drug launched requires $0.8–1.7 billion of investment (36). In addition, post-launch costs in terms of additional surveillance for resistance and for safety have also risen. These costs, in combination with a general failure of large companies to replace blockbuster products which have lost or are about to lose patent protection, have led to consolidation within the industry. A recent analysis of the years 1980–2003 revealed that 70 pharmaceutical companies existing in 1980 had collapsed to just six companies in 23 years (Table 1). Thus, it requires >10 older large pharmaceutical companies to make one current large pharmaceutical company. This represents over a 90% collapse in the number of large pharmaceutical companies during the years of study.

The high costs of development have also forced companies to prioritize their project portfolios and to focus on those projects most likely to yield a return on the development investment. In this process, in many large pharmaceutical companies antibacterial projects have been de-prioritized. Prioritization coupled with difficult science, a poor perception by much of the antibacterial marketplace, and what some considered to be a "hostile" regulatory environment have led many companies to abandon the area of antibacterial research entirely. Only a minority of large pharmaceutical companies today continue their antibacterial discovery efforts (Table 2).

These developments, coupled with potential opportunities in the hospital marketplace, have resulted in an incursion of

Table 1 Consolidation within the pharmaceutical industry 1980–2003

2003 Pharmaceutical Company	Number of original companies since 1980
Aventis[a]	17
Bristol-Meyers Squibb	8
Glaxo Smith Kline	12
Novartis	7
Pfizer	12
Wyeth	14

[a]Now Sanofi-Aventis

Table 2 Evolution of antibacterial research in large pharmaceutical companies since 1990[a,b]

Large pharmaceutical companies active in antibacterial research in 1990	Companies active today	Companies not pursuing antibacterial research today
Abbott	Merck	Abbott
Bayer	Pfizer	Bayer
Bristol-Meyers	J&J	Bristol-Meyers Squibb
Ciba	Astra-Zeneca	Lilly
Glaxo	Glaxo SmithKline	Roche
Hoechst	Novartis	Wyeth
J&J		
Lederle		
Marion Merrell Dow		
Merck		
Parke-Davis		
Pfizer		
Roche		
Rhone-Poulenc		
SmithKline Beecham		
Squibb		
Upjohn		
Zeneca		

[a]Authors' recollections, press releases, company presentations
[b]Only US and E.U. companies listed

biotechnology companies into the antibacterial space. Of interest, it seems that many of these companies are focused on the development of one or two clinical candidates and that few have significant discovery capabilities or pipelines of candidates spanning preclinical through clinical stages of development. The explanation for this finding is straightforward. Only relatively near-term products, that is those at least in early clinical development or at worst very late preclinical development (where something is known about both safety and efficacy), can attract the private funding required by small biotechnology companies. Further, there is a reticence on the part of investors to continue funding discovery activities, since they are frequently perceived as playing no important role in recovering their investments. The biotechnology companies can be squeezed from both ends in the sense that it can be difficult for them to raise the capital required to take their products through Phase III development given the very high costs associated with these large and complex trials. Some companies (e.g., Vicuron and Cubist) have been able to go to the public marketplace during the Phase II development stage to garner the funds required for them to complete their development plans. Whether other companies will be able to follow their example is as yet unclear, although many are counting on it.

In looking at the biotechnology pipeline of antibacterials in clinical development, and eliminating topical products from consideration, the vast majority of products seem to have originated in large pharmaceutical companies. In a recent review by the Infectious Diseases Society (37), eight antibiotics in advanced clinical development were listed from biotechnology companies. Seven originated with large pharmaceutical companies (Table 3). The tendency of large pharmaceutical companies to "spin off" small biotechnology companies (e.g., Novexel from Aventis, Basilea from Roche, Nabriva from Sandoz) will contribute to this state of affairs. Of course, from the point of view of medical need, this is a good thing. But we cannot yet say that biotechnology has taken up the cause of novel antibacterial discovery. In fact, a view of the current state of affairs in antibacterials within biotechnology leads one to ask, "Where is the innovation?"

Table 3 Antimicrobial compounds in Phase II or later stage development from biotechnology companies[a]

Compound	Manufacturer	Company of origin
Dalbavancin	Pfizer from Vicuron	Merrell Marion Dow
Iclaprim	Arpida	Roche
Oritavancin	Targanta	Lilly
Telavancin	Theravance	Theravance
Ceftibiprole	Basilea-Johnson&Johnson (J&J)	Roche
Cethromycin	Advanced Life Sciences	Abbott
Doripenem	J&J from Peninsula	Shionogi

[a]Modified from (37)

Table 4 Antibacterial discovery and development: the future

The medical need for new products is not going away
The shrinking landscape of companies means less competition
Niche market strategies for resistance can succeed
E.g., linezolid (Zyvox)
Small companies can market products in hospitals
Expand into novel indications
E.g., Cubist

8 The Future of Antibacterial Research

We are cautiously optimistic regarding the future of antibacterial discovery and development. The factors driving our optimism are summarized in Table 4. Antimicrobial resistance continues, and, as a medical need, will continue to drive the marketplace. The experience with the niche strategy of Pharmacia, now Pfizer, for linezolid may have set an example for a niche market approach in the antibacterial arena. From the competitive point of view, consolidation within the industry could mean less competition. The departure of many large companies from antibacterial development further decreases competition, but also opens opportunities for small companies. Biotechnology companies can either pursue antibacterial compounds with the possibility of partnering them with larger companies or can even take them all the way to the marketplace themselves, provided the market is a manageable one like that in hospitals. A caveat for biotechnology is that discovery research in biotechnology remains difficult to fund and, partially as a result of this funding problem, biotechnology seems, so far at least, unable to innovate in terms of identifying and developing novel antibacterial compounds.

9 Conclusions

As most of us know, bacterial resistance will be a continuing need as long as we use antibacterial products. The question for which we do not have an answer is whether the perceived market for antibacterial products will be sufficient to continue to drive investment and, hence, discovery and development of new products, or whether antibacterials will go the way of antimalarials or antituberculosis drugs. We are more optimistic today than we were even a few years ago (33). The market success of Pfizer with linezolid (Zyvox), the more open approach of regulatory agencies, the increased participation of biotech in the antibacterial space, and the continuing medical need driven by resistance are the bases for our guarded optimism. But all of these factors remain tentative. It is not clear whether others will be able to repeat the approach of linezolid, given the encroachment of MRSA into the com-

munity and the concomitant price sensitivity for community products. Biotech seems unable to innovate itself, and we are running out of candidates discontinued by larger pharmaceutical companies. The good will of regulatory agencies can be evanescent, as we have seen over the last decade.

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Section B
General Mechanisms of Drug Resistance

Chapter 5
Genetic Mechanisms of Transfer of Drug Resistance

Paul H. Roy

1 Introduction

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

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2 Conjugative Plasmids

Most antibiotic resistance genes, in both Gram-negative and Gram-positive bacteria, reside on conjugative plasmids. Conjugative plasmids have four groups of genes: DNA replication, partition and control of copy number, conjugative transfer, and auxiliary genes including antibiotic and heavy metal resistance, and degradation of complex organic chemicals (2, 3). The gene names and functions vary from plasmid to plasmid; the well-studied IncP-1 plasmid R100 is used here as an example.

2.1 DNA Replication

Plasmids are divided into several incompatibility groups based on their DNA replication machinery. This determines the host range of the plasmid. For example, IncF plasmids are limited to Enterobacteriaceae while IncP-1 plasmids can be maintained in a wide variety of Enterobacteriaceae, Pseudomonadaceae, and many other Gram-negative bacteria. The term "incompatibility group" arises from the fact that two plasmids of the same group are incompatible, i.e. are not stably maintained together since the replication and partition machinery cannot tell them apart, and daughter cells will, after a few generations, end up with all of one or all of the other plasmid. Plasmids have an *oriV* site, containing sites for binding of DnaA and TrfA, as the origin of replication (4). Replication of plasmids of Gram-negative bacteria is usually by a theta structure intermediate and may be unidirectional or bidirectional; while Gram-positive plasmids may use either a theta-intermediate or rolling-circle method of replication. A plasmid-carried replication initiation protein, such as TrfA of IncP-1 plasmids, is often involved in replication, although most replication functions (DnaA, DnaB, DnaC, DNA polymerase III and DNA gyrase) are furnished by the host cell (5).

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2.2 Partition and Control of Copy Number

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

2.3 Conjugative Transfer

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

2.4 Antibiotic Resistance and Other Auxiliary Genes

Antibiotic resistance genes on conjugative plasmids are often on other mobile elements: composite transposons, simple transposons, and integrons. In addition to favoring rapid evolution of conjugative plasmids, there is an additional advantage for the resistance genes: if the plasmid is unable to replicate in the recipient cell due to limitations of the host range, the resistance gene can "hop" to another plasmid or to the chromosome, either as part of a transposon or as an integron cassette. Again, R100 (8) provides a good example (Fig. 1). Tetracycline resistance is carried on the composite transposon Tn*10*. Chloramphenicol resistance is carried on another composite transposon similar to Tn*9*. Within the latter element is a simple transposon, Tn*21*, coding for mercury resistance, and the whole element is called Tn*2670*. Within Tn*21* is a defective site-specific simple transposon similar to Tn*402*, and this element contains an integron containing antiseptic and sulfonamide resistance and additionally a streptomycin resistance gene cassette, *aadA1*. The defective transposon also carries an insertion sequence, IS*1353* and within the latter, another insertion sequence, IS*1326*.

3 Composite Transposons

Many of the earliest described mobile elements encoding antibiotic resistance are composite transposons (Fig. 2). Among these are Tn*9*, encoding chloramphenicol resistance, Tn*10*, encoding tetracycline resistance (9), and Tn*5*, encoding kanamycin, bleomycin, and streptomycin resistance (10).

3.1 Structure and Mechanism

Composite transposons consist of a region of DNA flanked by insertion sequences, either as direct or inverted repeats. Insertion sequences (ISs) are mobile DNA elements that are cryptic, i.e. coding only for their own mobility. They typically encode a transposase that acts at short inverted repeats at the ends of the element. The insertion sequence can move either conservatively or replicatively. In conservative transposition, the transposase cuts at each end of the IS, holding the ends together while it finds a target site. The target site is then cut and the ends of the IS are ligated to the site. In replicative transposition, the transposase binds to the target site, cuts it and attaches the ends to the ends of the IS so as to create a structure that resembles a nearly completed round of replication, with two replication forks approaching each other. Replication of the IS then occurs, creating a cointegrate structure in which the donor and recipient molecules are joined by two copies of the IS, each of the latter consisting of one parental and one newly synthesized strand. Site-specific recombination at a *res* site within the IS then separates the donor and recipient molecules, leaving each with a copy of the IS. The mechanism of replicative transposition was worked out for bacteriophage Mu (11) and also applies to simple transposons of the Tn*3* family (see Sect. 4.1).

When insertion sequences insert on either side of a short region of DNA such as an antibiotic resistance gene, a composite transposon is created. The transposase can then act at

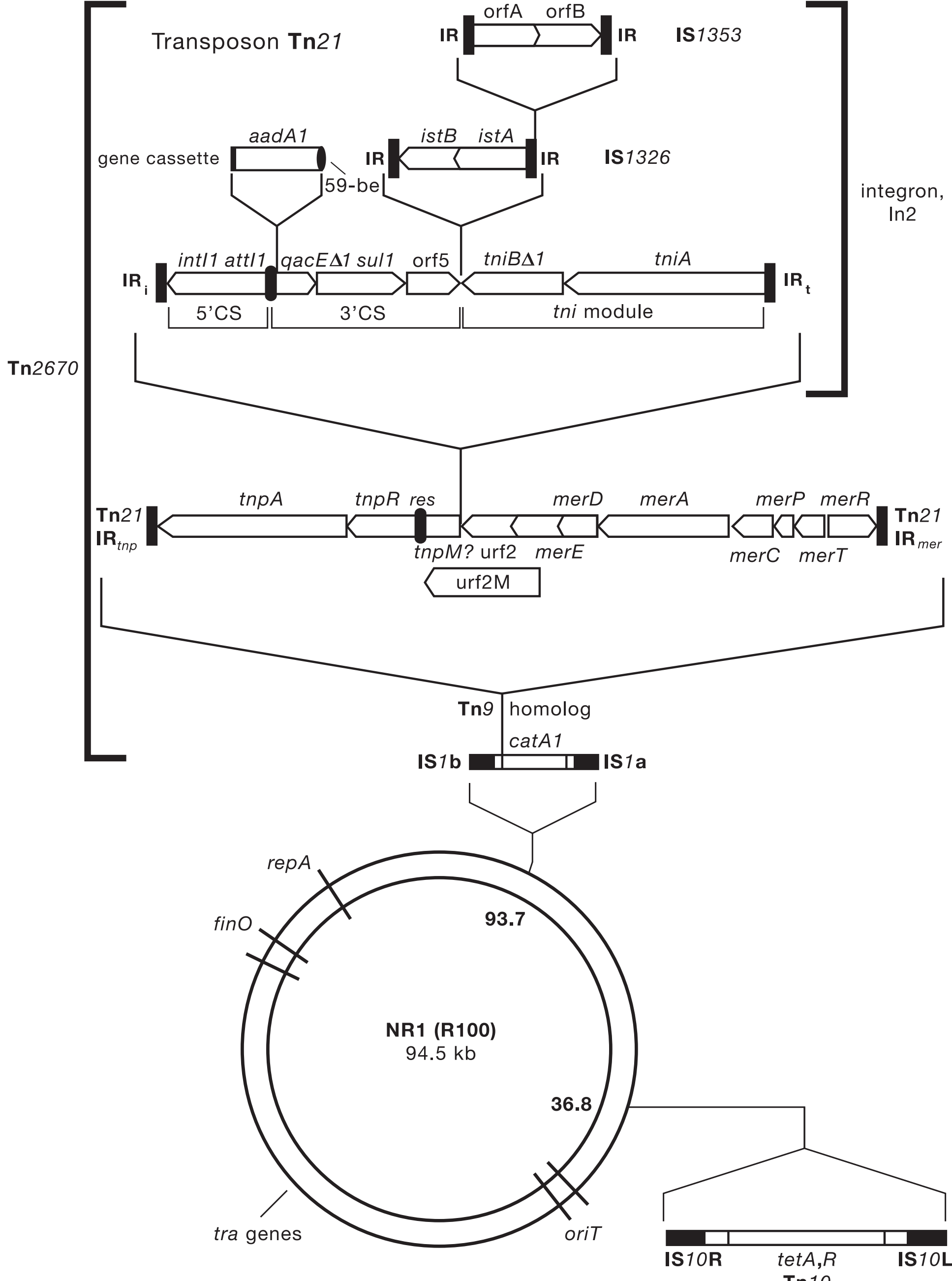
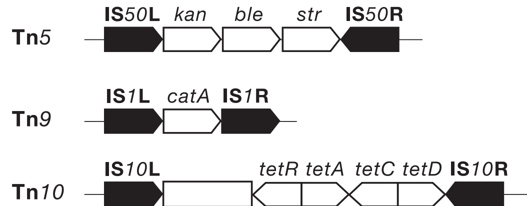


Fig. 1 The conjugative plasmid R100. This plasmid contains several embedded mobile elements including Tn*10* and Tn*2670*, which is made up of Tn*21* within a Tn*9*-like transposon. Tn*21* contains an integron within a defective Tn*402*-like element. The integron contains one mobile gene cassette, and the Tn*402*-like element contains two insertion sequences, one within the other. Adapted from (8)

the extremities of the entire element, rather than the extremities of a single IS, thus moving the two IS copies and the central region together. For example, Tn*9* consists of two directly repeated copies of the insertion sequence IS*9*, flanking a *catA* gene coding for a chloramphenicol acetyltransferase. Tn*10* consists of two copies of IS*10* in inverted orientation flanking a tetracycline resistance determinant. Since the short inverted repeat sequences of the two IS ends are identical, the transposase can recognize the entire composite transposon as if it were a single copy of the IS. Tn*10* undergoes conservative transposition (12) and possesses an interesting mechanism of regulation. The DNA ends contain GATC sequences that are target sites for the Dam methylase. Newly replicated DNA is methylated on only the parental strand (since the Dam methylase lags behind the DNA polymerase) and this hemimethylated state favors transposition. Tn*5* also undergoes conservative transposition (10, 13) and has a central region with three resistance genes (*kan* encoding an aminoglycoside phosphotransferase, *ble,* and *str* encoding a streptomycin phosphotransferase flanked by two copies of IS*50* in inverted orientation. These copies are nearly identical; one copy furnishes the active transposase while in the other copy a point mutation creates a premature stop codon for the transposase but at the same time provides a



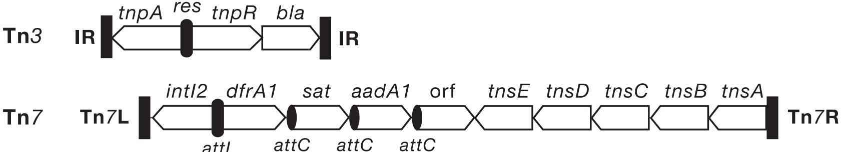


Fig. 2 Composite and simple transposons. Composite transposons have a central region with one or more genes flanked by insertion sequences in direct (Tn*9*) or inverted (Tn*5*, Tn*10*) orientation. Simple transposons are flanked by short (38 bp for Tn*3*) inverted repeats. Tn*3* has two transposition genes and carries the TEM-1 Beta-lactamase gene; Tn*7* has five transposition genes and carries an integron with three mobile cassettes

promoter for the transcription of the antibiotic resistance gene cluster.

3.2 Newer Elements and Their Antibiotic Resistance Genes

The flanking ISs are often involved in expression of the resistance gene in the central region. The *aacC3* aminoglycoside-(3)-acetyltransferase was found both in its "original" plasmid context and in a composite transposon formed by flanking insertions of IS*140*. In the latter, the −35 element of the promoter is replaced by a stronger −35 element in the IS end. Moreover, the correct spacing of the −35 and −10 elements is maintained, resulting in increased expression of *aacC3* in its transposon context (14).

Composite transposons are also common in Gram-positive bacteria. An example is Tn*4001*, where a bifunctional *aac-aph* gene encoding gentamicin resistance is flanked by two copies of IS*256* (15). Another is Tn*1547*, in which a chromosomal *vanB* operon is known to have undergone first one and then another insertion of IS*256*, forming an element in which the *vanB* operon, including the *vanRS* two-component regulator, became a transposon that then "hopped" onto a conjugative plasmid (16).

Single insertions of ISs are known to alter expression of nearby antibiotic resistance genes. The TEM-6 extended-spectrum Beta-lactamase is overexpressed by the insertion of an IS*1*-like element upstream, forming a stronger promoter than in its original context (17). Similarly, several extended-spectrum Beta-lactamases of the class A CTX family (e.g. CTX-M-15) are overexpressed by the insertion of IS*ECp1* upstream (18).

4 Simple Transposons

4.1 Tn3 and TEM Beta-Lactamase Genes

Perhaps the best-known simple transposon is Tn*3*, which encodes the TEM-1 Beta-lactamase. Unlike composite transposons, simple transposons (Fig. 2) have no long repeats, only short inverted repeats at their extremities (38 bp in the case of Tn*3*). Simple transposons of the Tn*3* family carry a *tnpA* gene encoding a transposase and a *tnpR* gene encoding a resolvase. These genes may be either divergently transcribed as in Tn*3* or co-transcribed as in Tn*21* (8). They also contain a *res* site. They undergo replicative transposition (11, 19), in which the transposase carries out the first step (nicking of the transposon ends and of the target site) and the resolvase carries out the final step (site-specific recombination at the *res* site to resolve the cointegrate structure) (20). The Beta-lactamase gene of Tn*3* has undergone significant evolution and selection of point mutants to yield extended-spectrum varieties resistant to cefotaxime and ceftriaxone (e.g. TEM-3), ceftazidime (e.g. TEM-5) and/or Beta-lactamase inhibitors (21).

The appearance of penicillin-resistant *Haemophilus* and *Neisseria* beginning in 1974 (22) appears to have been due to transfer of Tn*3*, either by transformation or conjugation, on a plasmid unable to replicate in these species. Tn*3* appears to have transposed onto resident cryptic plasmids before the original plasmid was degraded. Versions of the recipient plasmid with an intact Tn*3* have been found in *Haemophilus ducreyi*, while in *Haemophilus parainfluenzae* and *Neisseria gonorrhoeae* truncated versions lacking *tnpA* and *tnpR* are found (23). Remarkably, after 30 years and despite widespread use of ceftriaxone for treatment of gonorrhea, there is still no report of mutant, extended-spectrum Beta-lactamases

from *N. gonorrhoeae*. Could this be related to the fidelity of its DNA polymerase?

4.2 Tn1546 and Vancomycin Resistance

Tn*1546* was first found in vancomycin-resistant enterococci (VRE) and contains *tnpR* and *tnpA* genes at its left end, followed by a complete gene cluster encoding vancomycin resistance. The *vanRS* two-component system encodes a sensor and regulator that respond to the presence of vancomycin in the medium (24, 25) and induce expression of the *vanHAX* operon encoding a D-Ala-D-Lac depsipeptide in the place of D-Ala-D-Ala at the termini of the pentapeptide of the peptidoglycan layer, thereby altering the target of vancomycin (26, 27). Auxiliary genes recycle the components of the wild-type termini. Tn*1546* has now been found in *Staphylococcus aureus*, producing a fully vancomycin-resistant VRSA (28).

4.3 Site-Specific Transposons: Tn7 and the Tn5053 Family

Tn*7* is a site-specific transposon that was found to insert into unique sites in the chromosome of *E. coli* and in bacteriophage lambda. It carries short inverted repeats at its ends, five transposition genes (*tnsA-E*) in its right half and a class 2 integron (see below) and *dfrA1, sat*, and *aadA1* genes encoding trimethoprim, streptothricin and streptomycin/spectinomycin resistance. Tn*7* usually undergoes conservative transposition, but also has a replicative transposition mode (29).

A related family of site-specific transposons is the Tn*5053* family, including Tn*402*. They have four transposition genes, *tniA, tniB, tniQ*, and *tniR*. The *tniA* gene encodes a D,D(35)E protein and is homologous to *tnsB* of Tn*7*. The *tniB* gene has an ATP-binding motif and is homologous to *tnsC* of Tn*7*. The *tniR* gene encodes a serine recombinase of the invertase-resolvase family. Tn*402* carries a class 1 integron (see below) with *dfrB3* and *qacE* genes encoding trimethoprim and quaternary-ammonium-compound resistance, respectively. Tn*402* and the mercury resistance transposon Tn*5053* are closely related, and the products of their transposition genes can complement each other in *trans* (30).

5 Conjugative Transposons

Conjugative transposons are elements that can reside either on the bacterial chromosome or on plasmids. They have a broad host range and are important vehicles of antibiotic resistance in Gram-positive bacteria, notably in streptococci, but also occur in *Bacteroides* and Enterobacteriaceae. They are capable of excision from the donor chromosome or plasmid to form a nonreplicative circle, which then undergoes conjugal transfer into the recipient bacteria (31).

5.1 Tn916-Like Elements and Their Antibiotic Resistance Genes

The type element of this group is Tn*916*, discovered in 1981 (31a), that carries the tetracycline resistance gene *tetM*. Tn*916* is 18 kb in size and has 18 ORFs among which are an integrase and an excisase similar to those of lambdoid phages, as well as genes coding for intracellular and extracellular transposition functions. There is also an *oriT* site for an origin of transfer of single-stranded DNA from the excised circle. The frequency of transfer is relatively low and may be related to the DNA sequences flanking the donor element (32). A closely related element, Tn*1545* (33), carries kanamycin and erythromycin resistance genes in addition to *tetM*. A newer conjugative transposon, Tn*5382* (34), also called Tn*1549* (35) was first isolated from *Enterococcus faecium* and carries the *vanB* vancomycin resistance operon in place of *tetM*. Although *E. faecium* carrying *vanB* represents a smaller proportion of vancomycin-resistant enterococci than *E. faecalis* carrying *vanA* mediated by the simple transposon Tn*1546*, the former is clinically significant.

5.2 Mechanism of Transfer

Transfer (Fig. 3) is initiated by excision of the conjugative transposon into a nonreplicative circular intermediate, mediated by the *int* and *xis* genes located at one extremity of the element. These genes are similar to the *int* and *xis* genes of phage lambda, and the process is analogous to phage excision, except that the “sticky ends” produced are not complementary, as there is no equivalent of an *attB* site. The *oriT* site is then nicked, and then DNA replication by strand displacement begins. The details of the conjugal transfer have yet to be worked out, but it probably resembles that of conjugative plasmids and involves the genes *orf13* through *orf23* at the other extremity of the element. The second strand is synthesized in the recipient, and integration involves the Int protein. In contrast to phage lambda, integration, while showing site preferences, is not site-specific (31, 36).

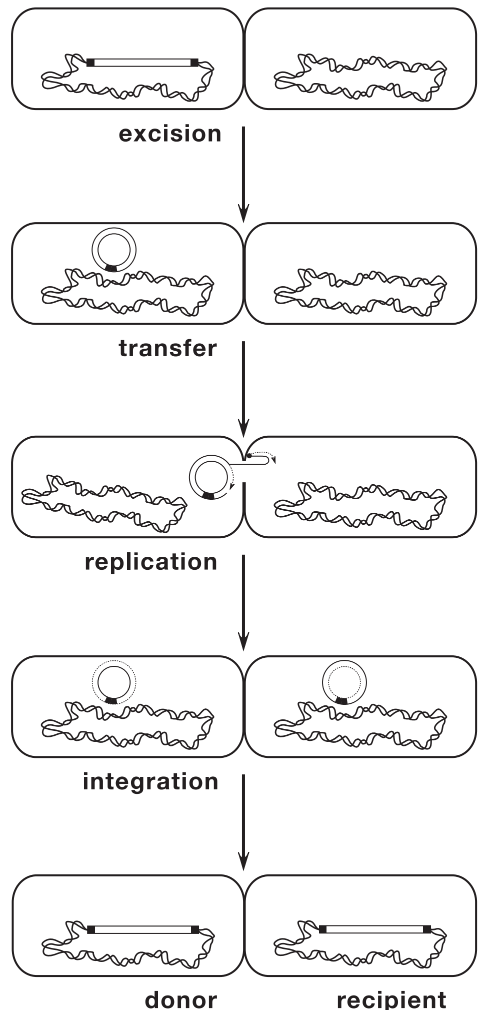


Fig. 3 Transfer of a conjugative transposon. The transposon excises and forms a circle; a single strand is then transferred into the recipient while replacement synthesis takes place in the donor. The second strand is synthesized in the recipient; circular copies are then able to integrate into the chromosome. Adapted from (37)

5.3 Related Elements

CTnDOT is a conjugative transposon from *Bacteroides* that carries the *tetQ* gene coding for a ribosomal protection mechanism and the *ermF* gene for MLS resistance (37). The latter gene, instead of being in the usual central region, is at the extremity, beyond the *int* gene. The *tetQ* and *ermF* genes are also found in *Prevotella* and *Porphyromonas*, providing evidence for horizontal transfer. CTnDOT has a greater degree of site specificity than Tn*916*.

Bacteroides also has mobilizable transposons such as NBU1. This element has its own integrase and *oriT* site, analogously to mobilizable plasmids; and can be transferred by the transfer genes of a conjugative transposon such as CTnDOT. Integration of NBU1 is site-specific, with a 14-bp recognition site (38). Tn*5398* of *Clostridium difficile* is another mobilizable transposon, and carries the *ermB* MLS resistance gene (39).

Another group of site-specific conjugative transposons is the SXT elements of *Vibrio*. The content of resistance genes varies, but they often encode resistance to trimethoprim-sulfa as well as streptomycin and chloramphenicol. These elements are site-specific, and have been referred to as ICEs (integrative conjugative elements) and constins (conjugative, self-transmissible, integrating elements) but are best described as conjugative transposons that happen to be site-specific. Most of the resistance genes are in a cluster but the trimethoprim resistance gene is a cassette, near the *intI9* gene, in a distinct class of integron (40).

6 Integrons and IS*CR* Elements

6.1 Class 1 Integrons

Integrons are elements composed of an integrase of the tyrosine recombinase (phage integrase) family, an *attI* site, and one or more gene cassettes usually composed of a single, promoterless structural gene and a palindromic *attC* site (also called 59-base element) with conserved consensus sequences at its ends and conserved structure (but not sequence) in the center (41). In integrons, the mobile element is the gene cassette. The integrase mediates the site-specific excision and integration of gene cassettes (42). In the early 1980s, restriction enzyme digests and electron microscopy heteroduplex experiments pointed to the existence of gene-sized insertions in otherwise identical plasmids. DNA sequencing resulted in the discovery of gene cassettes flanked by a "5′-conserved sequence" with the integrase gene and *attI* site, and a "3′-conserved sequence" that turns out to contain a truncated *qacE* gene cassette followed by a nonspecifically inserted *sul1* gene encoding a sulfonamide-resistant dihydropteroate synthase (43). The 5′-CS also contains a promoter directed toward the cassettes and is responsible for their constitutive expression (44, 45). Class 1 integrons are often found within transposons related to Tn*21* and often (erroneously) referred to as "Tn*21*-like elements". Many Tn*21*-family transposons in fact differ only by the cassette content of their integrons, e.g. Tn*2603* that differs from Tn*21* only by a single additional cassette encoding the OXA-1 Beta-lactamase gene (46). Most class 1 integrons are in fact on defective simple transposons of the Tn*402* family; some of these have found their way onto competent transposons related to Tn*21* while others occur on plasmids such as R46, R388, pMG7, etc. where they do not "piggyback" on another mobile element.

6.2 Recent Evolution of Class 1 Integrons

Sequence analysis gives us some clues concerning the evolutionary history of class 1 integrons (Fig. 4). This suggests an ancient association of the integrase and *attI* site with a Tn*402*-like transposon containing only the four transposition genes and associated 25-bp inverted repeats (47). The first cassette to be integrated would have contained the *qacE* gene, encoding resistance to quaternary-ammonium compounds by a *smr* family efflux mechanism. This cassette, unlike the rest of the element, is AT-rich and has a very different codon usage pattern from the other five genes, indicating that *qacE* was laterally transferred, probably from a low-GC Gram-positive organism (class Firmicutes). Two subsequent events provided the ancestor of ca. 95% of the class 1 integrons seen today: (1) addition of a sulfonamide resistance gene by a non-site-specific event that also removed the *attC* site of the *qacE* cassette, effectively locking it into place as the 3′-conserved segment, and (2) deletion of the *tniQ* and *tniR* genes as well as part of the *tniB* gene. This resulted in a defective transposon as a vehicle for most class 1 integrons. Subsequent events included insertion of IS*1353* and/or IS*1326* between the *sul1* and *tniB* genes (48), and the acquisition of the *aadA1* cassette encoding an adenylyltransferase conferring streptomycin and spectinomycin resistance. This cassette is very common, but not ubiquitous, in class 1 integrons. It is interesting to speculate that the *qacE* cassette may have arrived in the integron early in the twentieth century with the use of antiseptics. The incorporation of the *sul1* gene may have taken place in the late 1930s, and the *aadA1* cassette in the 1950s.

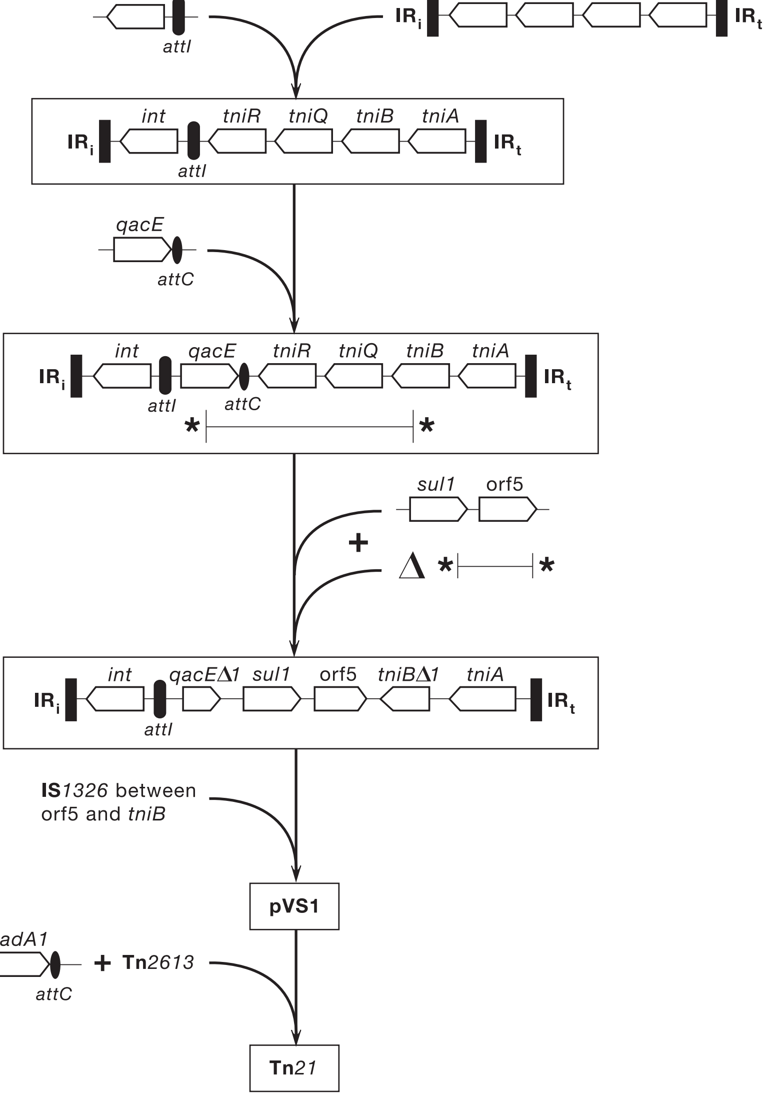


Fig. 4 A schema for integron evolution. There was probably an ancient association of the *intI1* gene and adjacent *attI1* site with a Tn*5053*-family transposon to create an immediate precursor of Tn*402* (without gene cassettes). A first cassette, qacE, encoding antiseptic resistance, differs greatly from the Tn*402*-precursor in G+C content and codon usage, and may have arrived from a Gram-positive organism in the late nineteenth or early twentieth century. Two subsequent events, the truncation of the *qacE* gene by the arrival of *sul1* and orf5 and the deletion of *tniQ* and *tniR*, immobilized *qacE* and resulted in a defective transposon. These events may have occurred in the 1930s or 1940s. Subsequently, insertion sequences and gene cassettes (beginning with the common but not ubiquitous *aadA1*) may have occurred in the 1950s. The defective transposon can "piggyback" on a competent transposon (the mercury resistance transposon Tn*2613* of the Tn*3* family) to reacquire mobility

6.3 Antibiotic Resistance Genes Carried by Integrons

Class 1 integrons contain a great variety of antibiotic resistance gene cassettes, and the order of the first occurrence of these cassettes closely mirrors that of the first clinical use of the corresponding antibiotics. Chloramphenicol resistance is often mediated by *cmlA*, which encodes a specific efflux protein of the major facilitator family (49, 50). A closely related florfenicol resistance protein often occurs in veterinary isolates, and can occur on other mobile elements in addition to integrons. Chloramphenicol resistance can also be mediated by *catB* genes such as *catB2* from Tn*2424*, which encodes a chloramphenicol acetyltransferase that is very different from that encoded by Tn*9* (51). CatB2 is in fact a member of the xenobiotic acetyltransferase family, and is closely related to Vat and Sat proteins that mediate virginiamycin and streptogramin resistance in Gram-positive bacteria (52, 53). The *catB2, catB3*, etc, genes in integron cassettes are closely related to the chromosomal *catB1* and *catB7* genes of *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*, respectively (54).

Aminoglycoside resistance genes include various adenylyltransferases and acetyltransferases whose spectrum depends on the availability of specific target positions on the aminoglycoside molecule. The *aadA* genes encode aminoglycoside-(2″) adenylyltransferases giving resistance to streptomycin and spectinomycin. Gentamicin resistance is commonly mediated by gene cassettes such as *aadB* encoding an aminoglycoside-(2″) adenylyltransferase (55), or *aacC1* encoding an aminoglycoside-(3) acetyltransferase (56). Gentamicin resistance can also be conferred by a version of the *aacA4* gene. This gene differs from a version conferring amikacin resistance by a single point mutation, with a serine codon in the gentamicin-resistant, amikacin-sensitive version and a leucine codon in the amikacin-resistant, gentamicin-sensitive version (57). While the latter was discovered first, the former is the more probable ancestor. As in the case of the ESBLAs mentioned above, a point mutation resulted in resistance to the more recent, semisynthetic antibiotic, amikacin. However, an extremely large variety of *aacA* gene cassettes have now been found in integrons, including *aacA7* that encodes resistance to both gentamicin and amikacin (58).

Integrons carry a wide variety of genes encoding Beta-lactamases. Although less common than the widespread TEM- and SHV- Beta-lactamases (including their extended-spectrum variants), integrons encode OXA-, PSE-, and CARB- Beta-lactamases. A few years ago, it appeared that there were relatively few novel resistance genes appearing in integrons as compared to other mobile elements. Except for a variety of *aacA* amikacin resistance genes, most of the new genes found in integrons were for resistance to older antibiotics such as trimethoprim and streptomycin. This situation changed dramatically with the discovery of a gene encoding a class B metallo-Beta-lactamase conferring resistance to carbapenems. The *bla*IMP-1 gene was first found in a class 3 integron (with a distinct integrase and *attI* site) in *Serratia marcescens* in Japan (59), but then spread to class 1 integrons as well as to other genera (*Klebsiella* and *Pseudomonas*) and geographically also. A gene for another class B Beta-lactamase, *bla*VIM-1, was first found in an isolate from Italy (60) and has also undergone worldwide dissemination. Integrons also carry, particularly in *Acinetobacter baumannii*, a variety of novel class D ("oxacillinase") Beta-lactamases conferring carbapenem resistance (61).

6.4 Gene Expression in Class 1 Integrons

Class 1 integrons are a sort of natural expression vector, analogous to constructed plasmids used in recombinant DNA experiments, for the inserted cassettes. The *attI* site in an integron is analogous to a multiple cloning site in an expression vector plasmid, and both integron and vector plasmid possess an upstream promoter for expression of the inserted genes. Most of the small number of polymorphisms in the 5′-CS is related to the promoters directed toward the cassette array. Three versions of varying strength exist, one of which is stronger than the *tac* promoter used in many expression vectors. In addition, a second promoter, 100 bp downstream of the first, can be created by an insertion of three G residues, changing the spacing of −35 and −10 elements from 14 to 17 bp (44, 45). Again, just as some expression vectors permit the expression of cloned genes as fusion proteins, integrons can do the same. A 19-bp insert permits the AAC (3′)-I protein to be expressed as a fusion protein from a start codon in the 5′-CS, using an efficient ribosome-binding site to maximize its translational expression (56).

6.5 Chromosomal Integrons

Chromosomal integrons were first observed in *Vibrio cholerae* (62) and genome sequencing projects have revealed their presence in several Beta-, Gamma-, and Delta-Proteobacteria, as well as a few taxonomically distant bacteria (spirochetes and planctomycetes). Chromosomal

integrons typically have many more cassettes than class 1 integrons, but few antibiotic resistance genes. Most cassette genes are unidentified, but those that are include nonessential genes such as those encoding virulence factors, restriction-modification systems, and plasmid addiction (toxin-antitoxin) systems (63). Chromosomal integrons tend to have more uniform *attC* sites, and cassette codon usage and G+C content reflects that of the parent organism. In contrast, class 1 integron cassettes have a wide variety of codon usage patterns and G+C contents indicating a wide variety of origins. Some chromosomal integron integrases have been shown to be active in integration and excision of cassettes (64–66).

6.6 Origin of Integron Cassettes

While chromosomal integrons may serve as a reservoir for some antibiotic resistance gene cassettes, a major unanswered question is how genes are recruited into cassettes and become attached to their *attC* sites. Recent evidence suggests a role for an RNA element called a group II intron in this process. Some group II introns (class C) target transcriptional terminators (67). A group II intron was found in a class 1 integron, inserted exactly at the junction of the *aadB* gene and its *attC* site (68). This structural gene-intron-*attC* may represent a "frozen" intermediate in cassette formation, along with structural gene-intron and intron-*attC* intermediates found in chromosomal integrons. Group II introns may thus target separately to the ends of structural genes and to *attC* sites, and subsequent steps of homologous recombination, transcription, RNA splicing, and reverse transcription may lead to the formation of novel cassettes.

6.7 CR Elements

In some class 1 integrons, a region downstream of *sul1* is replaced by a region containing an open reading frame that was first called orf513. Sequence analysis showed similarity of orf513 to some insertion sequence transposases. A short distance downstream, sequences diverged at a specific site and were followed by antibiotic resistance genes such as *catA2* and *dfrA10* (69). These results recalled the discovery of the *attI1* site in the early 1980s. The antibiotic resistance genes are not cassettes, and the region is followed by a partial duplication of the integron's 3′-conserved sequence. The Orf513 protein is a transposase of the IS*91* family, and the point at which the sequences diverge has been identified as an extremity of an insertion sequence, now called IS*CR1*. The other extremity has not been identified; and it is probable that "single-ended" transposition events result in the acquisition of part of the 3′-conserved sequence, allowing subsequent insertion, by homologous recombination, into other integrons (70).

6.8 Antibiotic Resistance Genes Carried by CR Elements

The first two genes found associated with the region now called IS*CR1* were a *catA* gene coding for a chloramphenicol acetyltransferase in In6 and the *dfrA10* gene coding for a trimethoprim-resistant DHFR in In7 (69). CTX-M extended-spectrum type A β-lactamases, beginning with CTX-M-2 (71) have been found in IS*CR1* elements. This gene is virtually identical to the chromosomal β-lactamase of *Kluyvera ascorbata*, and the precise extent of the DNA sequence incorporated into the IS*CR1* element is evident. In contrast, while some resistance gene cassettes in integrons are very similar to chromosomal genes (compare *catB2* etc. with *catB1* of *Agrobacterium tumefaciens* or *catB7* of *Pseudomonas aeruginosa*, or *aac(6′)-Id* of Tn*4000* with *aac(6′)-Ic* of *Serratia marcescens*) no cassette gene in its "original" chromosomal context has yet been found.

A series of class C β-lactamases (referred to as cephamycinases), including DHA-1 (72) and CMY-9 (73) has also been found in IS*CR1* elements. In the former, the β-lactamase gene is accompanied by the divergently transcribed *ampR* regulatory gene. A novel quinolone resistance, *qnrA*, mediated by a gyrase protection mechanism is associated with an IS*CR1* element (74). Recently, a novel aminoglycoside resistance mechanism involving 16S rRNA methylation, coded by *armA*, has been described (75).

7 Outlook

In the years following the introduction of each new antibiotic, resistance has appeared, either by point mutations of chromosomal genes or by recruitment and lateral transfer from antibiotic producers or other bacteria sharing their environment (e.g. CTX-M-2), see Sect. 6.8, or *qnrA* from *Shewanella algae* (76). Nonetheless, many antibiotics have had many years of useful life before the emergence of resistance reduced their utility. Certain species have still not developed resistance to certain antibiotics, e.g. *Haemophilus* and *Neisseria* are still susceptible to third-generation cephalosporins. Many broad-spectrum antibiotics have seen their spectrum narrowed by resistance, but can still be useful

when the use of rapid molecular diagnostics becomes common practice.

Novel classes of antibiotics, acting against new targets, are sorely needed. Resistance against new antibiotics of existing classes will be quicker to emerge, often by point mutation of an existing resistance gene. This is illustrated by the multiplicity of TEM Beta-lactamase genes resistant to third-generation cephalosporins and to Beta-lactamase inhibitors. Existing resistance genes have to be known and taken into account, as in the case of resistance to quinupristin-dalfopristin conferred by the combination of *satA* and *vgb* genes (77). On the other hand, resistance to new classes of antibiotics should take longer to emerge and be disseminated. Linezolid resistance, for example, has been limited to point mutants in rRNA genes, although another mechanism is suspected to exist. However, unpleasant surprises do occur. Fluoroquinolone resistance was for a long time limited to the accumulation of multiple point mutations in DNA gyrase and topoisomerase (although even this chromosomal resistance has been disseminated by lateral transfer). Only later did plasmid-specified gyrase protection mechanisms, mediated by *qnr* genes, emerge (78). Another unexpected event was the adaptation of an aminoglycoside acetyltransferase to fluoroquinolone resistance (79).

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

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Chapter 6
Mutations as a Basis of Antimicrobial Resistance

Maja Babic and Robert A. Bonomo

1 Prefatory Remarks

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

2 Genetic Diversity and Mutator Strains

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

We recognize now that genetic diversity is based on mutations. DNA polymerases, the enzymes that replicate bacterial genomes, are of limited fidelity. If a polymerase introduces the incorrect nucleotide, repair enzymes generally correct the "mistake" (3, 4). If the incorrect nucleotide is introduced without "correction," a point mutation occurs. Nucleotide sequences in a codon are permanently changed as a result of substitutions, deletions, or additions. Point mutations can be "silent" if the new codon encodes the same amino acid. They can be "nonsense mutations" if the new codon is one of the chain-terminating ones, or they can be "missense mutations" that encode a different amino acid in the peptide chain. Missense mutations are point mutations that can sometimes confer resistance to an antibiotic, because point mutations can affect the key amino acid residues that are important in protein function. Deletions or insertions usually cause "frame shifting" mutations that are deleterious to the tertiary structure of a protein, and may also result in premature chain termination. In general, mutations that are harmful or deleterious to a particular bacterial phenotype do not get passed to the next generation (5).

In times of normal growth, a perfectly adapted clonal population has a mutation rate close to zero. The absence of mutations, however, may prevent adaptation to the environment, should something suddenly change. A high mutation

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

3 Mismatch Repair-Deficient Permanent Hypermutators

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

Hypermutators are utilized in evaluating the frequency at which resistant genotypes arise in vitro while assessing a novel antimicrobial agent. The recovered mutants can provide insight into likely mechanisms of resistance. Hypermutators can potentially be utilized for selecting rare, interesting mutations with modified metabolic capabilities of biotechnological relevance (10). For example, taking a culture of fully grown *E. coli* with a density of 10^{10} CFU/mL and resuspending this culture in 1/10 the volume, followed by incorporation of 1-mL aliquots on ten agar plates, will detect mutants that arise at a frequency of about 10^{-12}. If hypermutators of *E. coli* exhibiting a 1,000-fold higher mutation rate are used, mutants that arise at frequencies as low as 10^{-15} can potentially be identified. This approach has been used to detect rare *ampC* promoter mutations in *E. coli* that confer increased ampicillin resistance (10).

4 Transient Hypermutators and the *SOS* System

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

SOS consists of three major polymerases – Pol II, Pol IV, and Pol V – that actively generate mutations in the genome (13, 14). All three polymerases collaborate in generating nucleotide substitutions, the so-called "translesions," by dNTP mis-insertions followed by mis-pair extension (15) (see Fig. 1).

As a result of exposure to DNA-damaging antibiotics, SOS^+ bacteria actively increase the number of mutations. Therefore, transient hypermutators are responsible for postexposure mutations, arising under selective antibiotic pressure

DNA damage→RecA activation→LexA proteolysis→Derepression of SOS genes→Pol II+PolIV+PolV→Translesions

Fig. 1 Derepression of SOS

and offering a better evolutionary tool for diversity, incurring an overall lower cost (9). The SOS system renders itself a suitable target for new antimicrobial agent development, as the inhibition of mutation could serve as a novel strategy in combating the evolution of antibiotic resistance (9).

5 Antimicrobial Resistance Determinants

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

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

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

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

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

In most Gram-negative bacteria, resistance to β-lactam antibiotics generally involves inactivation of β-lactam antibiotics by β-lactamases. The majority of clinically important Gram-positive bacteria, along with a handful of Gram-negative organisms, demonstrate low-affinity penicillin binding proteins (PBPs) that confer resistance to β-lactam agents. PBPs are cell wall synthesizing enzymes. Based on size, PBPs are divided into high molecular weight and low molecular weight enzymes. The high molecular weight group is comprised of transpeptidases and transglycosidases, which are essential for cell wall synthesis. Low molecular weight enzymes are carboxypeptidases, which re-arrange and degrade the three-dimensional murein structure. Low molecular weight PBPs serve some regulatory functions, but are not essential (see Table 1). All cell wall containing organisms described to date have from four to eight PBPs. To illustrate, *Staphylococcus aureus* has five PBPs, whereas *E coli* has eight different PBPs.

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

The bactericidal activity of β-lactams is based on their effective inhibition of high molecular weight essential PBPs. Some bacteria manage to escape this action by the presence of PBPs that do not readily bind to the β-lactam and are thus not inactivated by the drug. The origins of these "low affinity PBPs" are very diverse. Point mutations have been described only in the high molecular weight essential PBPs (18, 19). In transformable species like *Streptococcus pneumoniae*, "mosaic genes," acquired through homologous recombination and natural transformation from neighboring intrinsically resistant organisms, have given rise to highly resistant strains. PBP2b, 2x, and 1a are encoded by mosaic genes that can be transferred between *Streptococcus sanguis, S. oralis, S. mitis,* and *S. pneumoniae* (20). A succession of seven amino acid substitutions in PBP2b is responsible for penicillin resistance (21). An interesting point mutation also causes significant modification of PBP2b affinity codes for the substitution of Thr446 by an Ala. This mutation alone confers significant resistance to penicillin when found in wild-type *S. pneumoniae* strains. PBP2b production is associated with much slower cell wall hydrolysis at high β-lactam concentrations. While all other PBPs are inhibited, PBP2b continues active synthesis of the cell wall and thereby counters the action of cell wall autolytic enzymes, which are activated by a process unleashed by interference with cell wall synthesis. This effect is great enough to slow the hydrolysis down and prevent cell lysis. Resistant PBP2x variants differ from the wild-type by only 8–10 amino acids. Apart from the major

Table 1 Major PBP characteristics[a]

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

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

mutation involving a Thr to Ala substitution immediately following the active-site Ser337, the Thr550 to Ala change is noteworthy for conferring resistance to extended spectrum cephalosporins, and also for producing increased susceptibility to oxacillin.

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

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

7 Quinolone-Resistance Determining Region in Fluoroquinolone-Resistant Bacteria

Fluoroquinolones are inhibitors of DNA replication. Quinolones target prokaryotic topoisomerase enzymes whose major function is unwinding of DNA (25). In binding to the enzyme-DNA complex, they stabilize it. This inhibits the movement of proteins such as DNA and RNA polymerases along the DNA chains, thus arresting the replication fork. In Gram-negative bacteria, resistance to fluoroquinolones arises from alterations in the DNA gyrase (topoisomerase II), an enzyme responsible for the relaxation of supercoiled DNA. The DNA gyrase enzyme has two subunits: A and B. Amino acid substitutions resulting in quinolone resistance usually occur in Gyrase A. In *E. coli*, these mutations are clustered between amino acid positions 67 and 106 at the amino terminus of the polypeptide chain. This domain is called the quinolone-resistance determining region (QRDR). The most common mutations encountered in resistant strains involve Ser83 and Asp87. It appears that the above amino acid changes caused by point mutations in the QRDR region of Gyrase A alter the structure of the quinolone binding area at the interface of the enzyme-DNA complex, thereby reducing its affinity for the drug. Many other Gram-negative bacteria, *Mycobacteria,* and atypical pathogens with amino acid substitutions in positions equivalent to Ser83 and Asp87 display fluoroquinolone resistance. Amino acid substitutions in Gyrase B usually result in low-level resistance. In Gram-positive bacteria like *S. aureus*, resistance to fluoroquinolones usually involves point mutations in Topoisomerase IV, which separates intertwined DNA rings. Topoisomerase IV also has two subunits (ParC and ParE). High-grade resistance to fluoroquinolones is linked to amino acid substitutions in ParC (26).

8 Streptomycin Resistance and *Mycobacteria*

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

9 Rifampin Resistance

In *E. coli*, rifampin resistance arises from point mutations in highly conserved regions of the *rpoB* gene, which encodes the β subunit of RNA polymerase. One amino acid change in the β subunit causes a large change in the binding-affinity of rifampin to the DNA-dependent RNA polymerase, thereby hindering rifampin's inhibition of mRNA transcription. Curiously, resistance to rifampin occurs at high frequency in many genera of bacteria. *M. tuberculosis, Neisseria meningitidis,* and *Mycobacterium leprae* develop rifampin resistance by accumulating point mutations in the same highly conserved regions of the *rpoB* gene (28). Thus, rifampin is never used as monotherapy, primarily because of the high frequency at which resistant mutants arise. Combining rifampin with a second agent significantly reduces the chances of rifampin resistance arising on therapy. This paradigm forms the basis of our therapy against *M. tuberculosis*. Point mutations that confer resistance to two antibiotics are separate events, and the chance of both mutations occurring

in one organism is the product of the frequencies of each of them occurring alone (29).

10 Fluoroquinolone Resistance Caused by Overexpression of Active Efflux Pumps

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

11 Constitutive Tetracycline Resistance due to a Mutated Repressor Gene

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

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

Glycopeptide antibiotics, both vancomycin and teicoplanin, act as inhibitors of cell wall synthesis by binding to the D-Ala-D-Ala terminus of the pentapeptide precursor of the peptidoglycan molecule (35). Two types of gene clusters, designated *vanA* and *vanB*, account for the majority of acquired resistance to glycopeptides (36). The gene clusters include three genes, v*anH, vanA*, and *vanX*, which encode enzymes involved in incorporating D-Ala-D-Lac instead of D-Ala-D-Ala into the peptidoglycan precursors, thereby reducing the binding affinity of glycopeptides by approximately a 1,000-fold. Though the number of genes in the *Van* cluster is variable, there are five "core genes" present, as illustrated in Fig. 2 and Table 2. The expression of the *vanA* and *vanB* gene clusters are regulated on the transcriptional level by a set of two other genes, *vanS* and *vanR*, whose products comprise the VanRS and VanRbSb regulatory system. VanS and VanSb are transmembrane kinases that autophosphorylate a histidine residue in the presence of glycopeptides, and thereupon transfer the phosphoryl group to an aspartate residue on the VanR regulator protein. The phosphorylated regulator protein activates transcription of both the resistance and the regulatory genes. VanS also functions as a phosphatase, switching off the VanR regulator protein in the absence of glycopeptides. Alterations in the functions of VanS and VanSb give rise to a variety of phenotypical expressions of vancomycin (or glycopeptide) resistance. The phenotypes fall into several major categories: (1) constitutive expression; (2) inducible expression by vancomycin and teicoplanin; (3) inducible expression by vancomycin alone; and (4) repressed under all conditions. Mutations in the transmembrane segments of VanSb affect

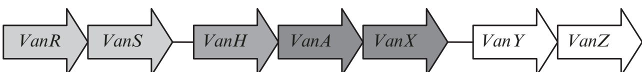

Fig. 2 Vancomycin resistance *VanA* operon

Table 2 *VanA* operon gene function table

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

signal transduction and lead to inducible expression of resistance genes. Mutations in VanSb, causing substitutions at two specific positions located on either side of the His233, give rise to constitutive expression of VanB by conveying resistance to the dephosphorylation of VanR.

13 Unique Regulation of Inducible Macrolide Resistance by Translational Attenuation

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

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

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

Table 3 AmpC regulatory system-specific protein functions

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

The regulatory system responsible for the induction mechanism is rather complex, and under strict control of several other genes: *ampR*, *ampD*, *ampE*, and *ampG*. The most widely accepted explanation of how AmpC production is regulated postulates that the gene product of AmpR has a dual function. It serves as a repressor of *ampC* transcription at baseline, but turns into an activator upon exposure to β-lactams. Current experimental evidence suggests that peptidoglycan breakdown products (i.e., muramyl peptides, and not the β-lactam molecule itself) serve as the activation trigger (41). The product of *ampG* is a transmembrane protein through which peptidoglycan breakdown products enter the cytoplasm. The *ampD* product linked to the transmembrane AmpE protein is a soluble cytosolic *N*-acetylmuramyl-L-alanine amidase that helps to recycle the breakdown products. In the presence of agents whose actions lead to cell wall destruction, like certain β-lactams, the recycling capacity of the amidase is exceeded and AmpR activates the production of AmpC. As a consequence of point mutations in AmpD that render it inactive, the regulatory system breaks down and AmpC production is permanently switched on, conferring resistance to all penicillins and cephalosporins. Strains that hyperproduce AmpC as a consequence of AmpD mutations are designated "derepressed mutants" (42, 43). The functions of individual proteins of the AmpC regulatory system are summarized in Table 3.

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

Ampicillin was the first synthetic aminopenicillin active against *E. coli* and other Gram-negative bacteria. Before long, enzymes capable of hydrolyzing ampicillin and first-generation cephalosporins were discovered. The genes encoding

these β-lactamases were transferred onto plasmids, and propagated with astonishing rapidity among *E. coli* and other *Enterobacteriaceae*. These first "broad-spectrum" β-lactamases were the TEM- and SHV-type. Their mechanism of action is based on catalytically disrupting the amide bond in the β-lactam ring by forming an acyl–enzyme complex. With the help of a strategically positioned water molecule in the active site, the covalent ester link is disrupted, the free enzyme released, and the β-lactam transformed into inactive penicilloyl and cephalosporyl moieties.

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

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

The bases for resistance to extended-spectrum cephalosporins by ESBLs and resistance to β-lactam β-lactamase inhibitor combinations are point mutations in the β-lactamase gene, which cause amino acid substitutions that alter the structure or dynamics of the enzyme. The majority of β-lactamases have more than one amino acid substitution compared to the wild-type enzyme. Interestingly, only a few point mutations at selected loci in the β-lactamase gene give rise to the above-mentioned phenotypes. The corresponding major amino acid positions at which substitutions conferring new resistance occur most frequently are summarized in Table 4.

Table 4 Sites for phenotype-altering amino acid substitutions in TEM and SHV β-lactamases[a]

Phenotype	Position of amino acid (Ambler numbering) substitutions[45] TEM	SHV
ESBL	Gly 104	Gly 238
	Arg 164	Glu 240
	Gly 238	
	Glu 240	
IRT	Met 69	Met69
	Ser 130	Ser 130
	Arg244	
	Arg275	
	Asp276	
CMT	(Gly 238 or Glu 240) + (Met 69 or Ser 130 or Arg 275)	SHV-10

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

16 The G238S ESBL Mutation

This is one of the most frequently encountered, and therefore most studied, mutations that codes for the G238S amino acid substitution. In nature, the substitutions of -Ser, -Ala, or -Asp for Gly at the Ambler position ABL 238 are mutations in SHV β-lactamase that confer resistance to extended-spectrum cephalosporins. There are currently 33 TEM and 25 SHV β-lactamase variants with the substitution Gly238Ser (www.lahey.org). Numerous hypotheses have been advanced to explain why the Gly238Ser substitution results in significant resistance to broad-spectrum cephalosporins. In 2003, the crystallographic structure of SHV-2 was elucidated and compared to the structure of SHV-1 from which it differs in only the one G238S substitution (46).

17 Inhibitor-Resistant TEMs

There are currently 23 inhibitor-resistant TEM and 2 inhibitor-resistant SHV mutants. In general, the inhibitor-resistant mutants are devoid of ESBL activity and are less active against narrow-spectrum cephalosporins than classical TEM (47). The number of Inhibitor-Resistant TEMs (IRTs) in TEM far exceeds the number in the SHV series, although the mutation sites are the same. The reason for this is a subject of ongoing studies.

SHV-10 was the first inhibitor-resistant SHV enzyme discovered in 1997, in a clinical isolate of *E. coli*. It is a derivative of SHV-5, an ESBL enzyme. As a result of a single point mutation in which adenine transitioned to guanine, a glycine is substituted for a serine at Ambler position 130. The enzyme partially retains its ability to hydrolyze penicillins, but loses significant activity against cephalosporins. Only recently, a

second inhibitor-resistant SHV was discovered, SHV-49. This novel β-lactamase was found to be a derivative of chromosomal SHV-1, and differs from the original gene only by the substitution of guanine by adenine at nucleotide position 195, leading to the amino acid substitution M69I. Experiments using site-directed mutagenesis have shown that this change in the SHV-1 conveys inhibitor resistance (48).

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

18 Complex Mutants of TEM

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

19 CTX-M

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

20 Global Suppressors

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

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

21 OXA

The OXA-type enzymes are classified as a group of ESBLs that are different from SHV, TEM, and CTX-M, but share a common substrate spectrum. They are not inhibited by clavulanic acid, and they hydrolyze oxacillin and cloxacillin very efficiently. OXAs are primarily found in *P. aeruginosa* and *Acinetobacter baumannii*. Although the group is genotypically diverse, the most recent additions show some degree of homology to the existing members (59). Most OXAs, including OXA-11, -14, -16, and -17, are derivatives of OXA-10. They differ from the parental enzyme by one to several amino acid substitutions. The two most important substitutions in OXA-10 derivatives are Ser73Asn and Gly157Asp. The latter appears to be necessary for high-level ceftazidime resistance. This substitution is lacking in OXA-17, which, in contrast to the rest of the group, hydrolyzes cefotaxime and ceftriaxone much better than ceftazidime. OXA-31 differs from OXA-1 in only three amino acid substitutions, and was found to confer a rather unusual susceptibility pattern. OXA-31 hydrolyzes cefepime, but not ceftazidime. It is therefore important to avoid reporting ceftazidime resistance solely on the basis of cefepime resistance, as is the routine in most clinical laboratories (60).

22 Concluding Remarks

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

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Chapter 7
Target-Mediated Antibacterial Resistance

Louis B. Rice

1 Introduction

It is axiomatic that all antimicrobial agents have targets – proteins or other structures within the bacterial cell that are essential to the growth and/or function of the microorganism. In order to do their job effectively, antimicrobial agents must interact with these targets in a manner that precludes the normal functioning of the target molecule. Moreover, their interactions with the target must be fairly specific, otherwise the potential for interaction with human molecules, and thereby the potential for toxicity, will be increased. In a circumstance in which the antibiotic-target interaction is specific, changes in the target structure, or in the environment immediately surrounding the target, can have a profound impact on target-antibiotic interaction. This interaction can result in resistance, as long as the changes do not, in a meaningful way, impact the ability of the target molecule to serve its function in cellular structure or metabolism.

Target modifications resulting in antimicrobial resistance are extraordinarily common in bacteria, and quite varied in the forms they take. In some cases, target modifications represent simple point mutations in a protein molecule, usually an enzyme that catalyzes an essential cellular function. Genes encoding the target proteins can also be modified in a variegated manner through homologous recombination with foreign DNA. In some instances, bacteria import entire genes to substitute for the antimicrobial targets. In others, complex and regulated pathways are acquired that modify non-enzymatic cellular structures. Finally, proteins may be made that interact with the target in a manner that "protects" the target from interaction with the antibiotic. Examples of each of these mechanisms are listed in Table 1.

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

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2 Point Mutations that Create Resistance

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

Mutants will emerge more frequently under circumstances in which the rate of mutation increases, such as defects in the mismatch repair mechanisms, and under circumstances where the mutants enjoy a selective advantage over the wild-type phenotype. The most obvious of circumstances in which point mutations confer a selective advantage are those in which the mutations confer resistance to an antimicrobial agent that is present in the environment.

It is, of course, important that the resistance-conferring point mutation not nullify the normal activity of the enzyme. As implied above, point mutations conferring antibiotic resistance may have a moderately deleterious effect on the activity of the target enzyme, resulting in, among other things, prolonged replication times (1). The presence of antibiotics in the environment alters the balance, in that the disadvantage of prolonged replication time will be outweighed by the advantage of continued replication in the presence of

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Table 1 General target-mediated resistance mechanisms and the targets they affect

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

antibiotics. In living systems, further mutations often occur in the absence of antimicrobial selective pressure, which serves to restore some degree of "fitness" while maintaining the mutation that confers resistance (1).

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

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

In general, high-level resistance conferred by point mutations requires the presence of several mutations. In the case of resistance to fluoroquinolones, the most effective mutations occur in the topoisomerase that is the primary target of the specific quinolone (3). Quinolones that target both GyrA and ParC require mutations in both enzymes to confer significant levels of resistance. Very high-level resistance to ampicillin in *E. faecium* also requires the presence of multiple mutations (4). We showed that none of four PBP5 mutations alone conferred high-level resistance to *E. faecium*, but when all were present together (as is frequently the case in highly resistant *E. faecium*), high-level resistance was expressed (4).

There are instances in which a point mutation does confer a high level of resistance, but the effect of this point mutation is diluted out by the fact that there are several copies of the gene present in the microorganism. Such is the case with resistance to linezolid, which inhibits protein synthesis by interacting with the 23S subunit of the bacterial ribosome. *E. faecium* has six copies of the ribosomal RNA genes in its genome, *Staphylococcus aureus* has five, whereas *Enterococcus faecalis* has four. A single point mutation (G2567U) in the ribosomal RNA prevents linezolid binding to the ribosomal RNA. However, when only one of the six copies in *E. faecium* has the mutation, the levels of resistance are very low (7). When four or more of the copies contain the mutation, resistance is very high (128 μg/mL or more) (7). Although it was originally thought that the need for multiple mutations would make the emergence of resistance unlikely, once linezolid was used in clinical settings it was found that resistant isolates (with multiple copies mutated) could be readily identified (8). It has now been shown that the first point mutation is the critical one (9). Once that is in place, the bacterium can increase the percentage of mutants through recombination between resistant and susceptible copies. This recombination to confer resistance has been referred to as "gene conversion." There appear to be some fitness costs to these mutations, however, as continued passage of resistant strains in the absence of antibiotics results in a return to susceptibility, as long as one copy of the wild-type rRNA gene remains (10). If all of the rRNA genes contain the mutation, then the resistance phenotype is much more stable (11), suggesting that gene conversion is responsible for the return to susceptibility as well as the emergence of resistance.

3 Mosaic Genes

In the past decade, the genome sequences of many different species have been determined, annotated, and the results made public for detailed analysis. A consistent theme resulting from these analyses is the remarkable frequency with which gene exchange has contributed to individual variation between members of the same species. It is estimated, for example, that more than 25% of the *E. faecalis* V583 genome has been acquired from other species (12). Differences between uropathogenic and enteropathogenic *Escherichia coli* can be attributed to acquisition of different "pathogenicity islands" (13). Consequently, the exchange of DNA has had a profound impact on the evolution of bacterial species in many areas, including the area of antimicrobial resistance.

Most bacteria acquire exogenous DNA through the action of mobile elements that confer an ability to transfer between bacteria and an ability to integrate into the recipient genome. Examples include conjugative plasmids and conjugative transposons of many sorts (14). A minority of bacterial species have been shown to be naturally transformable. These bacteria have the capacity to absorb naked DNA from the environment, under the proper circumstances. DNA from dead bacteria, which have no genetic material to promote entry into the recipient cell, can be taken up by these bacteria. Once inside the cell, the bacterial homologous recombination functions can integrate this acquired DNA into the genome across regions of sufficient homology. The result is mosaic genes, consisting of parts derived from the parent cell and parts derived from the donor DNA. If this recombination occurs in a manner that maintains the integrity of the open reading frame, a new protein may result. If the gene involved in the recombination encodes a protein that is the target for an antibiotic, and if the acquired DNA contains regions that confer a reduced susceptibility to that antibiotic, then an increase in resistance may result.

Mosaic genes have been found commonly in species that are naturally transformable. Such species include *Streptococcus pneumoniae* and species of viridans streptococci (15), as well as *Neisseria gonorrhoeae*, *Neisseria meningitides*, and several non-pathogenic *Neisseria* (16, 17). Resistance genes that have been shown to be the result of natural transformation and homologous recombination are most commonly penicillin-binding proteins (18) and topoisomerase genes (19). Penicillin resistance in pneumococci is most commonly the result of mosaic genes, with the degree and spectrum of β-lactam resistance varying, depending upon the location of the cross-over and the individual PBPs involved (20). The same can be said of the non-β-lactamase-mediated resistance to penicillin in gonococci. In general, the level of resistance that results from these mosaic genes is modest, probably because the level of resistance that is conferred in the donor bacteria is modest as well. The clinical impact of the low-level resistance can be significant. In areas such as the central nervous system, where it is difficult to achieve bactericidal levels of β-lactam antibiotics even with intravenous administration, low levels of penicillin or ceftriaxone resistance may require use of alternative (and often less effective) agents for successful therapy. In areas such as the middle ear, where inhibitory levels of cephalosporins are difficult to achieve after oral administration, these low levels of resistance can result in clinical failures of this class of antibiotics.

4 Target Overproduction

Occasionally, overexpression of target molecules will be used to overcome the effects of antimicrobial agents. Increased expression of PBP4 in *S. aureus* and PBP5 in *E. faecium* and *Enterococcus hirae* have been implicated in elevated levels of penicillin resistance in these species (21–23). Glycopeptide-resistant staphylococci that emerge after prolonged exposure to vancomycin have been found to have very thick cell walls that are full of unlinked cell wall precursors (24). These precursors are thought to serve as false targets for vancomycin, resulting in sequestration of vancomycin in the outer portions of these thick cell walls, preventing vancomycin arrival at the cell membrane, where the true cell wall precursor vancomycin targets exist. Finally, promoter mutations leading to overproduction of cellular DHFR has been implicated in trimethoprim resistance in *E. coli* (25).

5 Target Substitution

When "home-grown" point mutations confer only a modest level of resistance, high-level resistance can sometimes be achieved by acquiring genes that serve the same function as the target gene, but have a much lower affinity for the antibiotic. Perhaps the most prominent example of acquisition of such a gene is the *mecA* gene of *S. aureus* and coagulase-negative staphylococci. *mecA*, which is incorporated into a mobile element designated SCC*mec*, encodes penicillin-binding protein PBP2a, which binds the anti-staphylococcal β-lactam antibiotics (semi-synthetic penicillins, cephalosporins, carbapenems) with an affinity sufficiently low to result in high-level resistance to these antibiotics (26). PBP2a actually binds ampicillin with a relatively high affinity (27), but the nearly universal production of β-lactamase by methicillin-resistant *S. aureus* (MRSA) negates the efficacy of ampicillin. Whether inhibitor combinations containing ampicillin (such as ampicillin-sulbactam) would be effective therapy for MRSA infections has never been fully explored.

While PBP2a confers a high level of resistance, it appears to be rather specific in its requirements. For example, if the transglycosylase of *S. aureus* PBP2 is inactivated, methicillin resistance is not expressed, suggesting that it requires cooperative interaction with this transglycosylase to function (28). Moreover, several loci have been described – designated either fem (factors essential for methicillin resistance) or aux (auxiliary) – inactivation of which abolishes the expression of methicillin resistance (26). These factors, in most cases, involve the synthesis of the precursors of cell wall structures, suggesting that any alteration of cell wall structures (such as alterations in the peptide bridge that PBP2a cross links) are not tolerated by PBP2a. In this context, it is interesting that recent in vitro data indicate that PBP2a is able to effectively cross-link precursors, in vitro, that are markedly different that the 5-glycine cross bridges present in *S. aureus* (29).

E. faecium resistance to ampicillin results from a combination of point mutations in *pbp5*, and the subsequent substitution of resistant *pbp5* for more susceptible genes. As noted above, high-level resistance to ampicillin in *E. faecium* results from several mutations in its native PBP5 (4). Increasing evidence suggests that the widespread emergence of ampicillin resistance in *E. faecium* results not from the independent mutations of PBP5 in different strains, but more commonly from the spread of highly resistant clonal groups (30). We have shown, in vitro, that resistant *pbp5* is transferable from many *E. faecium* strains (31), suggesting that gene movement contributed to the formation of the clonal groups. In contrast to the *mecA* gene in *S. aureus*, in which PBP2a is expressed along with susceptible PBP2, transfer of *pbp5* between *E. faecium* strains results in replacement of the native *pbp5* (L.B. Rice, data not shown).

Acquisition of individual genes that encode alternative, low-affinity target proteins has been shown to be responsible for resistance to a variety of different antimicrobial classes, including trimethoprim (through alternative dihydrofolate reductases) (25) and sulfamethoaxozole (through alternative dihydropterate synthetases) (25).

Among the more complex and intriguing examples of target substitution resulting in high levels of resistance is the emergence and spread of glycopeptide resistance in enterococci and, on rare occasions, in *S. aureus* (32). Currently available glycopeptides act by binding to the terminal D-alanine-D-alanine of the peptidoglycan pentapeptide precursor. In so doing, the glycopeptides prevent access to this terminus, preventing the PBPs from performing their transpeptidase function (32). It has also been postulated that the large size of these molecules results in steric hindrance of transglycosylation.

The transferable glycopeptide resistance operons, which likely evolved from intrinsic self-defense operons within glycopeptide-producing bacteria, produce a series of enzymes whose activity results in the substitution of normal pentapeptide precursors with those that terminate in D-alanine-D-lactate (32). Glycopeptides bind to these precursor molecules with approximately 1,000-fold lower affinity than they do normal peptidoglycan precursors. Interestingly, the enterococcal PBPs appear to have no trouble utilizing these altered precursors to form a cell wall, and as the terminal amino acid is cleaved from the precursor to form the final cross-linked product, the final product is cross-linked peptidoglycan that is predicted to be identical to that observed in cells lacking the glycopeptide resistance operons.

The two operons of primary importance in conferring glycopeptide resistance in enterococci are designated VanA and VanB (33, 34). VanA operons confer resistance to both vancomycin and teicoplanin, whereas VanB operons confer resistance to vancomycin, but are not induced by the presence of teicoplanin in the media (regulatory mutants resistant to teicoplanin do emerge under teicoplanin selective pressure) (35). Both operons have been identified within transposable elements (36, 37), facilitating their widespread dissemination within *E. faecium*. Why they have not become prevalent in *E. faecalis* and *S. aureus* remains a mystery.

6 Target Modification or Protection

Target molecules can also undergo enzymatic modification that reduces binding of an antibiotic. The most widespread example of this type of modification is in resistance to macrolides antibiotics in Gram-positive bacteria. Macrolides inhibit protein synthesis by binding reversibly to the peptidyl-tRNA binding site of the 60S ribosomal subunit, inhibiting the translocation of a newly synthesized peptidyl-tRNA molecule from the acceptor site on the ribosome to the peptidyl donor site. Resistance to macrolides is commonly achieved by methylating the ribosome, thereby inhibiting macrolides binding (38). Ribosomal methylation results in resistance to all clinically available macrolides (azithromycin, clarithromycin, erythromycin, roxithromycin), lincosamides (clindamycin), and streptograminB (quinupristin).

Several erythromycin ribosomal methylase (*erm*) genes have been characterized. In many instances, these genes are under regulatory control by a translational attenuation mechanism (39). Macrolides induce expression of the resistance operons, whereas clindamycin does not. The presence of even the inducible variety does raise concerns about the use of clindamycin in the clinical setting, as mutations can result in constitutive expression of the *erm* genes, resulting in resistance to clindamycin.

Targets may also be protected by the expression of proteins that bind to the target in a manner that prevents interaction with the antibiotic, but allows normal function of the protein. Among the best studied of these proteins is the tet(M) protein, widely prevalent in Gram-positive bacteria. Tet(M) encodes a protein that exhibits homology to

elongation factors EF-Tu and EF-G, and it exhibits ribosome-dependent GTPase activity (40). It binds to the ribosome, changing its conformation in a manner that precludes tetracycline binding. Tet(M) is most commonly incorporated into broad-host range conjugative transposon Tn*916* and similar elements, explaining its remarkably wide distribution in bacteria (41).

More recently, protection proteins that confer resistance to fluoroquinolone antimicrobial agents have been described. These proteins, referred to as QNR proteins (42), protect DNA from quinolone binding. In general, they confer only a low level of resistance. However, when combined with other mechanisms, such as QRDR mutations or efflux pumps, the level of resistance can be substantial. Finally, acquired resistance to fusidic acid in staphylococci has been attributed to a protein that protects EF-G from fusidic acid binding (43).

7 Conclusion

Target modifications as a route to antimicrobial resistance are extraordinarily common in pathogenic bacteria. These modifications may result from point mutations in the genes encoding the targets, protection of the target, modification of the target, or acquisition of a new molecule that serves the function of the susceptible target, but which is not susceptible to inhibition by the antimicrobial agent. In many cases, these modified targets are incorporated into mobile elements, facilitating their dissemination though many different species. The wide spectrum of mechanistic options available to bacteria for conferring resistance is a sobering aspect of antimicrobial development, as the spectrum of possible resistance mechanisms in nature cannot be known prior to clinical use of a new antimicrobial agent.

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Chapter 8
Biochemical Logic of Antibiotic Inactivation and Modification

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1 Introduction

Bacterial resistance to antibiotics manifests itself in both general and specific protection mechanisms. Consequently, the characteristics of resistance can be paralleled to those of the mammalian immune response. Antibiotic resistance can be differentiated into: (1) nonspecific mechanisms that confer general innate immunity to a class of antibiotics (e.g., broad spectrum efflux mechanisms, target modification), and (2) highly precise responses that include selective enzyme-based mechanisms that mirror the acquired immune response with respect to target specificity and potency. Bacteria deploy both types of mechanisms in response to the presence of cytotoxic antibiotics.

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

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

Walsh described the cellular impact and rationale of biochemical reactions as "molecular logic" (3) and this terminology works very well in dissecting mechanisms of antibiotic resistance. Thus enzyme-catalyzed antibiotic resistance is functionally and structurally linked to the mode of action of these agents. For example, modification of key functional groups on an antibiotic can sterically or electronically block interaction with target (see Sect. 3 below for examples). This review describes mechanisms of antibiotic destruction and modification resulting in resistance in the context of the mode of action of the antibiotic. Our aim is not to provide a comprehensive examination of the details of all known resistance mechanisms but rather to focus on selected examples to decode the molecular basis and biological impact of these inactivation strategies.

2 Destruction of Antibiotics

We classify antibiotic destruction as a mechanism that results in either ablation of a key reactive centre or massive structural rearrangement that is not readily reversed under normal physiological conditions. Hydrolysis of the reactive β-lactam ring of penicillin and cephalosporin antibiotics by β-lactamases is an example of the first class, and linearization of the cyclic depsipeptide of Type B streptogramins by Vgb

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Table 1 Survey of enzymatic mechanisms of antibiotic resistance

Antibiotic destruction		
Antibiotic	Mechanisms	Enzyme(s)
β-Lactams	Hydrolysis	β-Lactamase
Macrolides	Hydrolysis	Macrolide esterase
Type B streptogramins	C–O-bond cleavage	Vgb lyase
Tetracyclines	Mono-oxidation	TetX
Fosfomycin	Hydrolysis	Epoxidase
	Thiol transfer	Thiol transferase
Antibiotic modification		
Antibiotic	Mechanisms	Enzyme
Aminoglycosides	Acylation	Acetyltransferase
	Phosphorylation	Kinase
	Adenylylation	AMP-transferase
Macrolides	Phosphorylation	Kinase
	Glycosylation	UDP-glucosyl transferase
Lincosamides	Adenylylation	AMP-transferase
Rifamycin	Glycosylation	TDP-glucosyl transferase
	Phosphorylation	Kinase
Chloramphenicol	Acylation	Acetyltransferase
Type A streptogramins	Acylation	Acetyltransferase

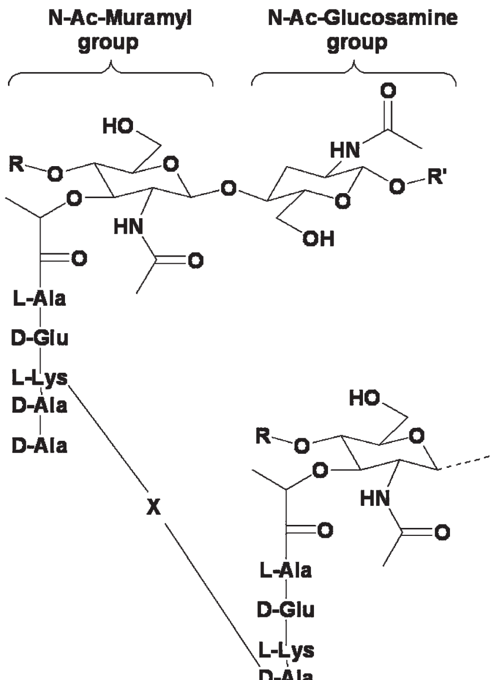

Fig. 1 Structure of the bacterial peptidoglycan unit. Peptidoglycan consists of repeating disaccharyl units (*N*-acetylglucosamine-*N*-acetylmuramic acid), to which a pentapeptide is linked each *N*-acetylmuramic acid. Crosslinking between adjacent pentapeptides provides rigidity to the bacterial cell

lyase is an example of the second. In all classes, the action of resistance enzymes tactically impacts the mode of action of the affected antibiotics to disrupt their biological activity. Examples of each class are discussed below.

2.1 β-Lactam Antibiotics

The β-lactams remain some of the most successful and widely used antibiotics in modern chemotherapy. These natural products and their semi-synthetic derivatives act by covalently modifying so-called penicillin binding proteins (PBPs) (4). PBPs include membrane-associated enzymes important in bacterial peptidoglycan assembly and maintenance. Covalent modification of this subclass of PBPs by β-lactams blocks their enzymatic activity thereby inhibiting cell wall metabolism, which results in impaired wall integrity and cell death. PBPs include the transpeptidases and DD-carboxypeptidases that act on the pentapeptide portion of the peptidoglycan repeating unit that consists of the disaccharyl unit, *N*-acetylglucosamine-*N*-acetylmuramic acid, to which a D-Ala-D-Ala terminating pentapeptide is linked through the lactyl group of *N*-acetylmuramic acid (Fig. 1). Transpeptidases and DD-carboxypeptidases use canonical Ser hydrolase chemistry to either rigidify the cell wall by synthesizing interstrand peptidoglycan crosslinks between the D-Ala-D-Ala termini of adjacent peptidoglycan strands (transpeptidases) or control cell wall strength and flexibility by cleaving the terminal D-Ala-D-Ala peptide bond of the pentapeptide (Fig. 1). Strominger noted 40 years ago that the β-lactam antibiotics sterically and electronically mimic the acyl-D-Ala-D-Ala terminus of the pentapeptide (Fig. 2) (5). This model overlaps the highly strained (and thus chemically reactive) β-lactam ring over the scissile D-Ala-D-Ala peptide bond. Attack of the nucleophilic Ser hydroxyl onto the β-lactam ring carbonyl opens the cyclic structure and generates a covalent intermediate that is resistant to hydrolysis (Fig. 3), thereby chemically titrating PBPs into inactive complexes and shutting down cell wall synthesis.

Penicillin D-Ala-D-Ala

Fig. 2 Comparison of the β-lactam penicillin and the D-Ala-D-Ala peptidoglycan terminus

In order to overcome the action of cytotoxic β-lactams, bacteria have evolved secreted enzymes that hydrolytically cleave the β-lactam ring of penicillins and cephalosporins (6) (Fig. 4). The molecular logic of this resistance mechanism therefore involves the destruction of the reactive "warhead" of the β-lactam antibiotics, thereby eliminating the essential chemical structure necessary for PBP inactivation.

These hydrolytic enzymes, appropriately named β-lactamases, fall into two general structural classes: Ser β-lactamases and metallo-β-lactamases (Fig. 4). The former group share structural homology with the DD-carboxypeptidases and operate by similar Ser hydrolase chemistry. However the hydrolytic step, which is slow in PBPs, is fast in β-lactamases, resulting in highly efficient detoxification of the antibiotics. Metallo-β-lactamases adopt analogous hydrolytic chemistry employed by metallo-proteases to cleave the reactive β-lactam ring.

2.2 Fosfomycin

Destruction of a reactive chemical warhead is also employed by enzymes that inactivate fosfomycin. The key structural element of this antibiotic is a reactive epoxide that is attacked by its intracellular target, the cell wall biosynthetic enzyme MurA (Figs. 5 and 6a). This enzyme is essential for synthesis of *N*-acetylmuramic acid and covalent modification of a key Cys residue by fosfomycin efficiently inactivates the enzyme.

Fig. 3 Mechanism of action of β-lactam antibiotics on bacterial transpeptidases and DD-carboxypeptidases. Nucleophilic attack of the PBP Ser hydroxyl on the β-lactam ring carbonyl results in an opening of the β-lactam ring. The active site machinery of transpeptidases or DD-carboxypeptidases is effectively captured as the subsequent covalent intermediate cannot be hydrolyzed

Fig. 4 Mechanisms of enzymatic inactivation of β-lactam antibiotics. β-lactamases catalyze the hydrolytic cleavage of β-lactam rings. (**a**) Serine-β-lactamases form a transient enzyme-antibiotic intermediate, which is quickly hydrolyzed. (**b**) Metallo-β-lactamases utilize a bound Zn^{2+} to activate water for hydrolytic attack of the β-lactam ring

The bacterial countermeasure to inactivate this antibiotic is an epoxide ring opening reaction using one of two distinct chemical tactics. The first, catalyzed by FosX, is a metal-dependent hydrolytic process that generates the vicinal diol (8) (Fig. 6b). The second is via a thiol-dependent ring opening by enzymes that use abundant intracellular thiols such as glutathione (FosA) (9) (Fig. 6c) and cysteine (FosB) (10) (Fig. 6d). Either strategy results in efficient destruction of the antibiotic's reactive centre, thereby blocking its action on the target MurA.

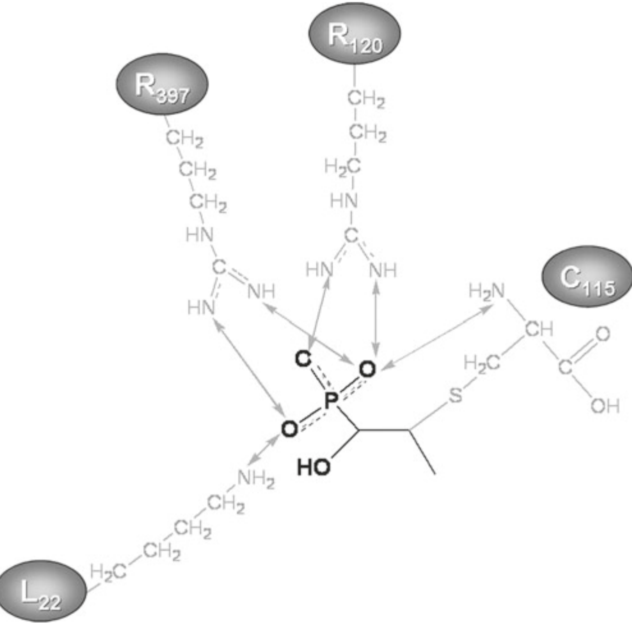

Fig. 5 Interactions of fosfomycin with its bacterial target, MurA. Fosfomycin forms a covalent bond with MurA's active site Cys. Additional interactions with MurA are designated as *arrows*. MurA residues are labeled in *grey*. Adapted from (7)

2.3 Macrolide Antibiotics

The macrolide antibiotics include natural products such as erythromycin and semi-synthetic derivatives (e.g., clarithromycin). These antibiotics are assembled via a polyketide assembly line, cyclized to form a macrolactone ring structure, and subsequently modified by glycosylation to generate a mature antibiotic (11). Macrolides inhibit bacterial translation by binding to the large ribosomal subunit in the vicinity of the peptide exit tunnel (12). This interaction requires an intact cyclic macrolide ring and in most cases the amino sugar desosamine (Fig. 7).

Enzymatic resistance to macrolide antibiotics occurs either by modification of the desosamine sugar (see Sect. 3 below) or by linearization of the macrolactone ring (Fig. 8). The latter mechanism is catalyzed by esterases that hydrolytically cleave the lactone resulting in ring opening and consequently the inability to effectively bind to the peptide exit tunnel. The erythromycin esterases EreA and EreB have been identified in *E. coli* integrons and R-plasmids (14–17).

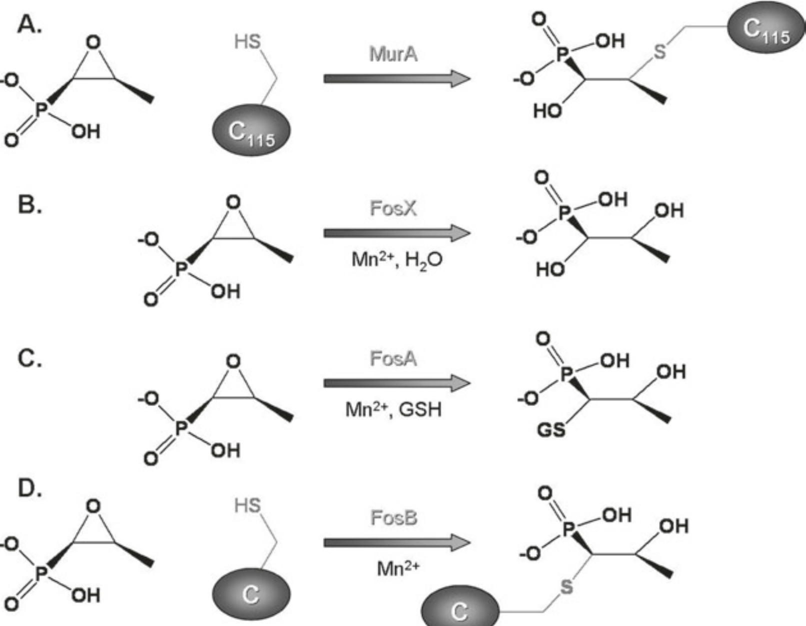

Fig. 6 Mechanism of fosfomycin action and inactivation. (**a**) Fosfomycin targets the active-site Cys residue of MurA, forming a covalent intermediate. (**b**) FosX-mediated inactivation of fosfomycin results in the formation of a diol. (**c**) The product of FosA-mediated fosfomycin inactivation is a glutathione-fosfomycin adduct. (**d**) FosB-mediated resistance to fosfomycin results in a ring-opened inactivated product with free Cys

Fig. 7 Interactions of the macrolide erythromycin with the bacterial ribosomal RNA. Key 23S rRNA residues are shown in grey and the interactions are designated as *arrows*. The hydroxyl group that serves as a site of inactivation interacts with A2058. Adapted from (13)

Fig. 8 Inactivation of the macrolide erythromycin by hydrolysis. Macrolides can be inactivated by hydrolysis of the macrolactone ring. This reaction is mediated by the esterase Ere

2.4 Type B Streptogramins

The streptogramins are natural product inhibitors of bacterial translation that consist of two structurally distinct classes, denoted as Type A and Type B (18). Type B streptogramins are cyclic depsipeptides that, like macrolides, bind to a region of the bacterial ribosome's peptide exit tunnel (19, 20) (Fig. 9). Type A streptogramins are mixed peptide-polyketide antibiotics that bind to the peptidyltransferase centre of the ribosome. Enzymatic resistance to Type A streptogramins occurs via an acetyltransfer mechanism, while enzymatic resistance to Type B streptogramins occurs through a ring-opening reaction catalyzed by the enzyme Vgb. Vgb, originally identified in streptogramin resistant *Staphylococcus aureus*, cleaves the cyclic peptide (21), resulting in depsipeptide linearization. The resulting structure no longer exhibits affinity for the bacterial ribosome, mirroring the biochemical logic of macrolide esterases. However, the mechanism of ring opening is quite distinct. Rather than causing a hydrolytic reaction at the thermodynamically vulnerable ester bond of Type B streptogramins, Vgb catalyzes a lyase reaction that results in a ring opening of the peptide by a C–O cleavage strategy (22) (Fig. 10).

2.5 Tetracycline

The tetracycline antibiotics have found extensive clinical use for almost half a century. This class of antibiotics binds divalent metals and acts by blocking bacterial translation by binding to the small ribosomal subunit (23) (Fig. 11). The principal mechanisms of clinical tetracycline resistance are efflux and ribosomal protection (23, 25). However, an enzymatic mechanism of tetracycline resistance, originally discovered in *Bacteroides* (26), has been identified that inactivates the antibiotic via an oxygen-dependent process. Purification of the enzyme that catalyzes this reaction, TetX, followed by careful analysis of the products of the reaction showed that the enzyme first facilitates mono-hydroxylation of the antibiotic at position 11a, effectively disrupting the essential metal-binding site on the molecule (27) (Fig. 12). Furthermore, this step triggers a nonenzymatic decomposition of the antibiotic to a form of unknown structure that turned the growth media black. This enzyme is also capable of mono-hydroxylation of the latest generation of tetracycline antibiotics, the glycylcyclines, resulting in resistance, but not the subsequent nonenzymatic decomposition of the antibiotic (28).

Fig. 9 Streptogramin B interactions with bacterial ribosome. Quinupristin, a Type B Streptogramin, binds to the bacterial ribosome's polypeptide exit tunnel. Key interactions with the 23S rRNA are designated as *arrows*. Adapted from (19, 20)

3 Antibiotic Modification

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

Fig. 10 Vgb-catalyzed inactivation of the type B streptogramin quinupristin. Quinupristin undergoes a ring-opening elimination reaction, resulting in an inactive derivative

Fig. 11 Interactions of tetracycline with the bacterial 16S rRNA. Interactions are designated by *grey arrows* and key ribosomal RNA residues are indicated in grey. Adapted from (24)

Fig. 12 TetX-mediated inactivation of tetracycline. TetX catalyzes the hydroxylation of the antibiotic, which interferes with the metal-binding site required for activity. Adapted from (27)

decrease in the affinity of the antibiotic derivative for its target in comparison to the unmodified counterpart.

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

3.1 Aminoglycosides

The aminoglycoside class of antibiotics is a diverse group of hydrophilic aminocyclitols modified by amino and neutral sugars that consist of both natural products and their semi-synthetic derivatives. Polycationic aminoglycoside antibiotics, as previously mentioned, act by interacting with the 16S rRNA region of the bacterial ribosome's A-site, impairing its decoding mechanism and consequently resulting in a misreading of the mRNA (29–32). X-Ray crystallographic studies of aminoglycoside antibiotics and the small

ribosomal subunit or fragments of the 16S rRNA reveal that interactions between aminoglycosides and the ribosome span the entire length of the antibiotic (24, 33–36). The primary mode of interaction is through predicted hydrogen bonding and ionic contacts between the antibiotic amino and hydroxyl groups and the 16S rRNA (Fig. 13).

The most prevalent mode of clinically relevant aminoglycoside resistance is via enzymatic modification (38). Three classes of enzymes, whose reactions differ with respect to the functional group transferred and the acceptor site, are responsible for aminoglycoside modification. Aminoglycoside acetyltransferases (AACs) modify amino groups, aminoglycoside phosphotransferases (APHs) target hydroxyl groups, and aminoglycoside nucleotidyltransferases (ANTs) modify hydroxyl groups (Fig. 14). There are numerous examples of each group and the genes encoding aminoglycoside-modifying enzymes are commonly located on mobile genetic elements such as plasmids or transposons, although some have been identified within chromosomal DNA (39–41). The action of all three classes of modifying enzyme changes the electronic properties of the antibiotic, in addition to its size and structure. These alterations result in steric and electronic clashes between the modified antibiotic and the 16S rRNA, impairing efficient binding and resulting in resistance.

3.1.1 Aminoglycoside Acetyltransferases (AAC Family)

Aminoglycoside acetyltransferases (AACs) utilize intracellular acetyl-CoA as a co-substrate, catalyzing the formation of a biologically stable amide with the aminoglycoside. Although AACs primarily modify amino groups (*N*-acetylation), *O*-acetylation has been documented with the acetyltransferase domain of the bifunctional enzyme AAC(6′)-APH(2″) (42) and the mycobacterial enzyme AAC(2′)-Ic (43).

AACs are members of the GCN5 superfamily of proteins (44, 45). Although all enzymes of this class do not exhibit significant primary sequence homology or conserved catalytic residues, analysis of available X-ray crystal structures of four enzymes (AAC(6′Ii), AAC(3)-Ia, AAC(2′)-Ic, and AAC(6′)-Iy) indicates that the aminoglycoside binding pocket commonly contains a highly negatively charged surface to accommodate the polycationic antibiotic (45–48).

AACs are further classified based on the site of acetylation along the aminoglycoside structure. By convention, the position along the amino sugar/aminocyclitol targeted is indicated in brackets, and the amino sugar/aminocyclitol modified is designated in brackets after the position of attack. For example, in Fig. 14, AAC (3) indicates acetylation of the 3-position of the central aminocyclitol moiety, the term (2′)

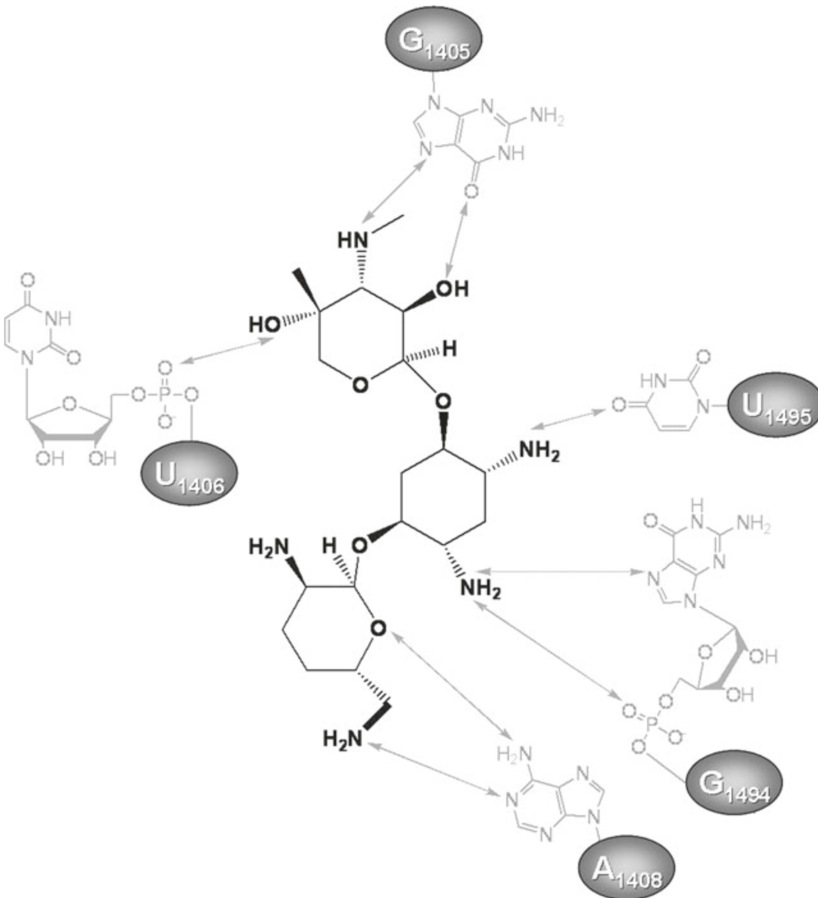

Fig. 13 Interactions of the aminoglycoside gentamicin C1a with the bacterial 16S rRNA. Key 16S rRNA residues are shown in grey and the interactions are designated as *arrows*. Adapted from (37)

Fig. 14 Inactivation of the aminoglycoside gentamicin C1a by aminoglycoside-modifying enzymes. Aminoglycosides can be modified by the addition of acetyl groups, phosphate groups or AMP moieties. These enzymatic reactions are catalyzed by aminoglycoside acetyltransferases (AACs), phosphotransferases (APHs), and nucleotidyltransferases (ANTs) respectively. (**a**) Sites of aminoglycoside inactivation. Groups targeted are labeled by the corresponding resistance enzymes. (**b**) The products of aminoglycoside acetyltransferases, phosphotransferases, and nucleotidyltransferases

suggests modification of the 2-position of the 4-substituent diaminohexose, and (2″) indicates modification of the 2-position of the 6-substituent aminohexose.

Modification of aminoglycosides by AACs results in neutralization of the positive charge on the target amino group, eliminating key ionic interactions and sterically blocking interaction with the 16S rRNA.

3.1.2 Aminoglycoside Phosphotransferases (APH Family)

Aminoglycoside phosphotransferases catalyze the phosphorylation of specific aminoglycoside hydroxyl residues (Fig. 14), using intracellular ATP as a phosphate donor. Classification of phosphotransferases is based on the site of action, analogous to the system described above for acetyltransferases. The APH enzymes are subdivided into seven classes, based on their site of action on the aminoglycoside: APH(2″), APH(3′), APH(3″), APH(4), APH(6), APH(7″), and APH(9). There exists very little primary sequence homology among the subclasses of APHs; however common signature sequences and residues essential for catalysis are evident (49).

The largest subclass of APHs modifies the 3′-hydroxyl of the aminoglycoside and is consequently called APH(3′) (49). Crystal structure analysis of the enzyme APH(3′)-IIIa bound to ADP has established a remarkable similarity to known protein kinases, despite the low primary sequence similarity (50). This may be evidence that APH(3′) and protein kinases evolved from a common ancestor.

Modification of aminoglycosides by APH-catalyzed phosphorylation results in changes in overall charge and size of the antibiotic. This results in electronic and steric clashes with the 16S rRNA and a 10^3-fold impairment of binding to the target 16S rRNA (51).

3.1.3 Aminoglycoside Nucleotidyltransferases (ANT Family)

Aminoglycoside nucleotidyltransferases utilize the co-substrate ATP to transfer an AMP moiety to selected aminoglycoside hydroxyl groups. This class of inactivating enzymes has been identified in some Gram-positive bacterial isolates, as well as a broad range of Gram-negatives (40).

ANTs display very little primary sequence homology, however they exhibit a common core signature region (49). The enzyme ANT(4′)-Ia has been crystallized and its atomic structure determined alone and in complex with the substrates kanamycin and a nonhydrolyzable ATP analogue (52, 53). Although the primary sequence homology is only 10%, the putative active site was determined to be structurally equivalent to that of rat DNA-polymerase β, one of the smallest and simplest of the polymerases (54), and catalyzes a similar chemical reaction.

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

3.2 Macrolides

Macrolide antibiotics are a large class of antibiotics that include both natural products and semi-synthetic derivatives. Most macrolides are derived from bacterial fermentation products, particularly from species of the actinomycete genus *Streptomyces*. Erythromycin was the first member of this class to be identified (1952), a natural product of *Streptomyces*

erythrae (now known as *Saccharopolyspora erythrae*). The name macrolide is derived from the macrolactone ring that characterizes the class, which can consist of 14–16 members and is commonly attached to one or two sugar moieties.

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

Macrolides, as described previously, act by binding with the 23S rRNA of the bacterial 50S ribosomal subunit adjacent to the peptide exit tunnel, blocking polymerization at the peptidyltransferase centre and inducing premature peptide dissociation (13, 20). Interactions with the ribosomal RNA occur primarily through hydrogen bonding, as shown with erythromycin in Fig. 7. Much of the hydrogen bonding ability of macrolides can be attributed to their hydroxyl and amino groups, which interact with the nitrogenous bases or backbone phosphate groups of the rRNA. As shown, the hydroxyl residue of the desoamine sugar plays a key role in the interaction of the macrolide with its target rRNA.

The second mode of enzymatic macrolide inactivation occurs by modification of this essential desosamine sugar (Fig. 15). Modification of the 2′ hydroxyl residue can occur by either phosphorylation or glycosylation. This hydroxyl group, as mentioned, plays an important role in macrolide-target binding, serving as a multiple contact site of hydrogen bonding with the 23S rRNA (Fig. 7). Modification of the antibiotic at this site therefore results in loss of vital structural connections with the target and also results in steric impairment of complex formation.

3.2.1 Macrolide Kinases (Mph Family)

Clinical resistance to macrolides has been documented by means of phosphate transfer from ATP by a family of macrolide-inactivating phosphotransferases, encoded by the *mph* genes (Fig. 15). These enzymes have been identified in both Gram-positive and Gram-negative pathogens (55–57).

Members of the Mph class of resistance enzymes appear to be extremely diverse with respect to the nucleotide sequences that encode the enzymes. The gene *mphA* exhibits a 66% G + C content, uncharacteristically high for the organism it was

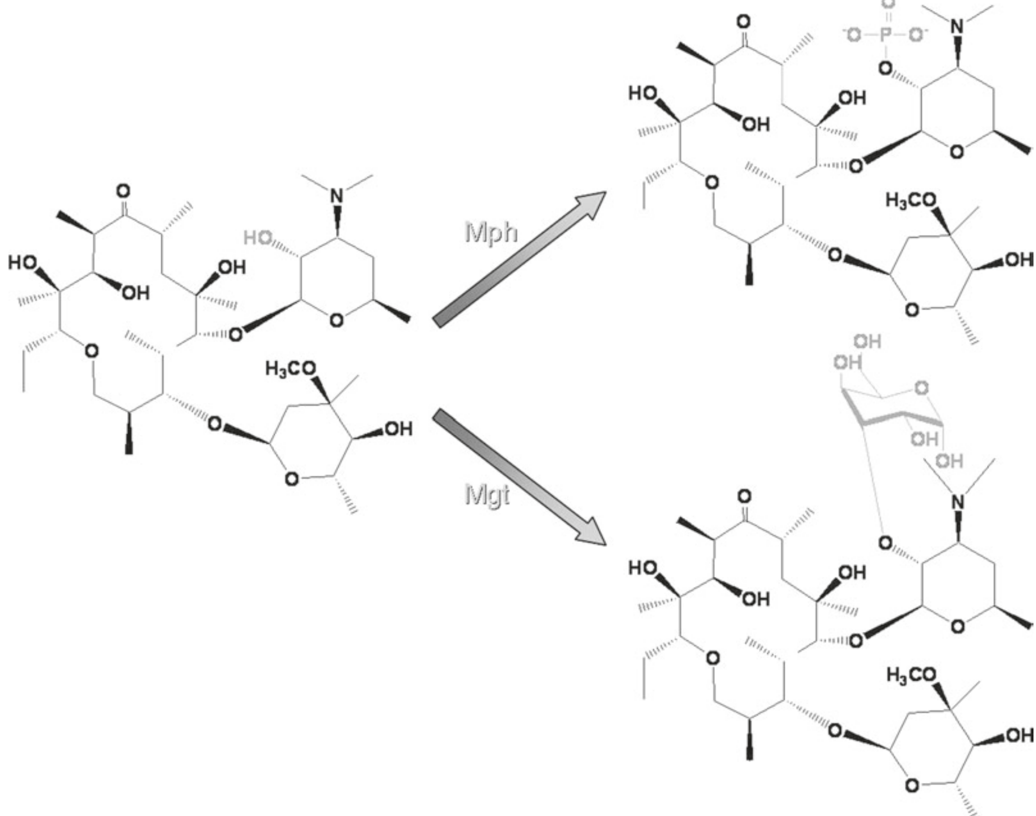

Fig. 15 Inactivation of the macrolide erythromycin by Mph and Mgt. Macrolides can be modified by the addition of phosphate and glucose moieties. The hydroxyl group targeted and the subsequent modifications are labeled in *grey*

originally identified in (*E. coli*, G + C content approximately 50%) (58). The sequences of *mphB* and *mphC*, conversely, display a G + C content of only 38%. The structure of these enzymes has yet to be elucidated, however they share canonical phosphate transfer residues with APHs and probably resemble these aminoglycoside resistance enzymes.

3.2.2 Macrolide Glycosyltransferases (Mgt Family)

Resistance to macrolides in antibiotic-producing strains of bacteria as well as other soil-dwelling organisms is commonly accomplished by intracellular glycosylation of the antibiotic prior to export. This is catalyzed by a class of enzymes called macrolide glycosyltransferases (Fig. 15). Members of this class include Mgt, from the nonmacrolide-producing *Streptomyces lividans* (59, 60), as well as OleD (61) and GimA (62) from the macrolide-producing *S. antibioticus* and *S. ambofaciens* respectively.

Members of the Mgt family are extremely similar with respect to both DNA and primary amino acid sequences, however each enzyme appears to display a unique substrate specificity *in vitro* (63).

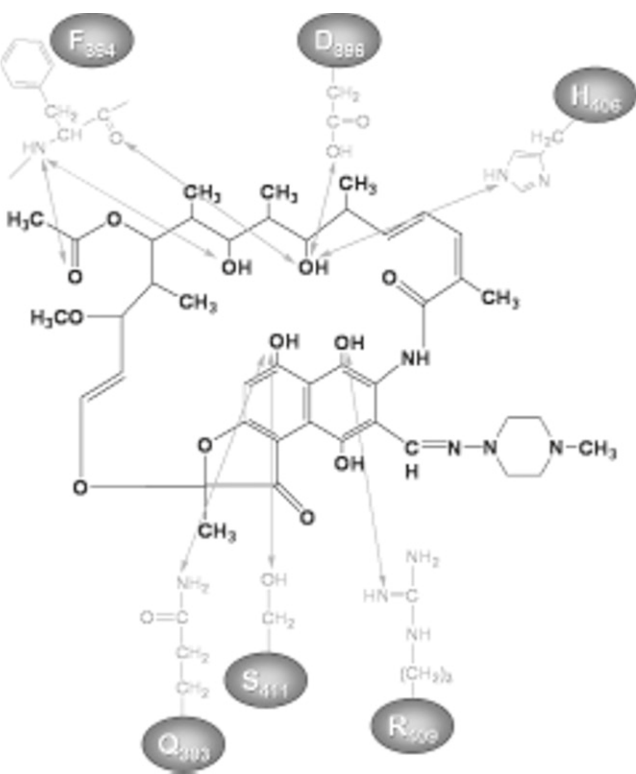

Fig. 16 Interactions of the rifampicin with the bacterial β-subunit of RNA polymerase. Key amino acid residues are shown in grey and the interactions are designated as *arrows*. Adapted from (64)

3.3 Rifamycins

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

Rifamycins target the bacterial β-subunit of RNA polymerase. The crystal structure of rifampicin bound to the RNA polymerase of *Thermus aquaticus* has been determined to 3.3Å (64). Twelve amino acid residues were shown to associate closely with rifampicin, six of which participate in hydrogen bonding, as shown in Fig. 16. The majority of these interactions occur at four crucial hydroxyl residues on the rifampicin molecule including a key interaction between the hydroxyl group at position 23 and the amide of Phe394.

Resistance to rifampicin commonly occurs through amino acid mutations in the RNA polymerase β-subunit. However, inactivating enzymes have also evolved to modify the antibiotic (Fig. 17). Group transfer can result in ADP-ribosylation, phosphate addition, and glycosylation of the rifampicin's 23-hydroxyl. Through the addition of a bulky functional group, rifampicin's tight binding to its target is impaired.

3.3.1 ADP-Ribosyltransferases (ARR Family)

Although both eukaryotic and prokaryotic proteins are commonly modified by means of ADP-ribosyl transfer, this mechanism of antibiotic resistance has so far only been documented for the rifamycin class. Resistance to rifampicin by means of ADP-ribosylation has been documented in numerous nontuberculosis *Mycobacterium* strains, such as *M. smegmatis*. Modification is due to a unique ADP-ribosyltransferase known as ARR (65). Another inactivating ribosyltransferase (ARR-2) with 55% identity to ARR has been identified in a multidrug resistant integron in a Gram-negative *Acinetobacter* strain (66).

The resistance enzymes from the ARR family are characteristically small (approximately 200 amino acids) and do not display sequence similarity to protein ADP-ribosyl transferases. They target the hydroxyl residue at position 23 of rifampicin (Fig. 17), and utilize nicotinamide adenine dinucleotide (NAD^+) as a donor for the ADP-ribosyl moiety. It has also been shown that this ADP-ribosylated antibiotic can undergo subsequent decomposition to release the ADP moiety (65, 67, 68).

Fig. 17 Inactivation of rifampicin. Rifampicin can be enzymatically modified by the addition of ADP-ribose, glucose, and phosphate moieties. The hydroxyl group targeted and the subsequent modifications are labeled in *grey*

3.3.2 Rifampicin Kinases

Inactivation of rifampicin by phosphorylation (Fig. 17) has been documented by species of *Nocardia* (69, 70), *Rhodococcus* (71), as well as *Bacillus* (72). The kinases responsible for this inactivation have yet to be identified or studied. Phosphorylation of rifampicin's hydroxyl at position 23 logically impedes interaction with the RNA polymerase target, although little has been done to elucidate the details of this mechanism.

3.3.3 Rifampicin Glycosyltransferases

Glycosylation of the 23-position of rifampicin has also been reported in *Nocardia* species (69, 73) (Fig. 17). Glycosylation at this position prevents hydrogen bonding with the 23-hydroxyl, hindering effective target binding to RNA polymerase β. The genes encoding the enzymes that catalyze these reactions have yet to be elucidated.

4 Summary and Conclusions

Bacteria use enzymes to strategically incapacitate and neutralize antibiotics. Tactically this includes deployment of mechanisms that either destroy the essential chemical "warhead" or "active site" of the antibiotic (e.g., cleavage of the β-lactam ring by β-lactamases), or mechanisms that modify key structural elements that are essential for binding of the antibiotic to target (e.g., phosphorylation of aminoglycosides). The molecular logic of these approaches is revealed with knowledge of the interaction of the active antibiotic with its cellular target. Study of enzymatic resistance therefore not only can inform on molecular aspects of antibiotic-target interactions, but can serve to guide target identification where this is not yet known.

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

Finally, antibiotic-modifying enzymes also have the opportunity to be exploited as novel reagents in antibiotic semi-synthesis as protecting agents. In some cases, antibiotic-modifying proteins are employed by antibiotic-producing bacteria as a means of self-protection. For example, during streptomycin biosynthesis in *Streptomyces griseus*, the

enzyme StrA modifies mature antibiotic to the inactive 6-phosphoderivative. Export of this “pro-drug” is followed by unmasking of the cytotoxic agent by an extracellular phosphatase (74). These enzymes could serve as reagents to chemically protect and deprotect sensitive structural elements in the synthesis of libraries of semi-synthetic antibiotics.

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

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Chapter 9
Antibiotic Resistance Due to Reduced Uptake

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1 Introduction

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

2 Envelope Structure

2.1 Cytoplasmic Membrane

The cytoplasmic membrane is common to all bacterial species. For Gram-positive bacteria it is the primary barrier to antibiotic penetration, while an outer membrane further protects Gram-negative bacteria (6). In both cases, the cytoplasmic membrane is the site of essential functions such as nutrient transport, energy generation, the enzymatic assembly of lipid-linked monomers of cell envelope macromolecules (e.g. the peptidoglycan or lipopolysaccharide), and protein secretion. The cytoplasmic membrane is a phospholipid bilayer that acts as a hydrophobic barrier controlling the movement of solutes into the cell and enclosing the cytoplasmic contents of bacteria. This bilayer is studded with integral membrane proteins that carry out essential membrane functions. The density of cytoplasmic membrane proteins is high enough such that proteins are separated from each other by only three or four phospholipid molecules (7).

Phospholipids generally contain a glycerol 3-phosphate backbone attached to a hydrophilic head group and hydrophobic fatty acids. The lipids often have a positive charge to balance the negative charge on the phosphate and are termed zwitterionic, or have no charge on the headgroup giving the phospholipid a net negative charge. Although the type and proportion of phospholipids produced will vary under different environmental conditions, a typical membrane composition for *E. coli* is 75% zwitterionic phosphatidylethanolamine (PE), 20% anionic phosphatidylglycerol (PG), and 5% anionic cardiolipin (CL, or diphosphatidyl glycerol) (8). Membrane lipids are amphipathic and given an appropriate balance of headgroups, will spontaneously form bilayers to create a hydrophobic core that contains the fatty acyl chains separating the polar head groups on both sides of the bilayer. The fatty acyl chains are usually either saturated or contain a single double bond and are termed unsaturated, while the acyl chain may comprise 14–22 carbons. For example, the predominant fatty acids in the cytoplasmic membrane lipids of *E. coli* are saturated palmitic acid (16:0), the unsaturated species palmitoleic acid (cis-$\omega^{9,10}$–16:1) and cis-vaccenic acid (cis-$\omega^{11,12}$–18:1) (7).

The fluid mosaic model describes the properties of a membrane whereby both phospholipids and proteins diffuse laterally along the plane of a membrane, although proteins diffuse at a slower rate than lipids (7). Generally speaking, phospholipids do not readily flip from one leaflet in the

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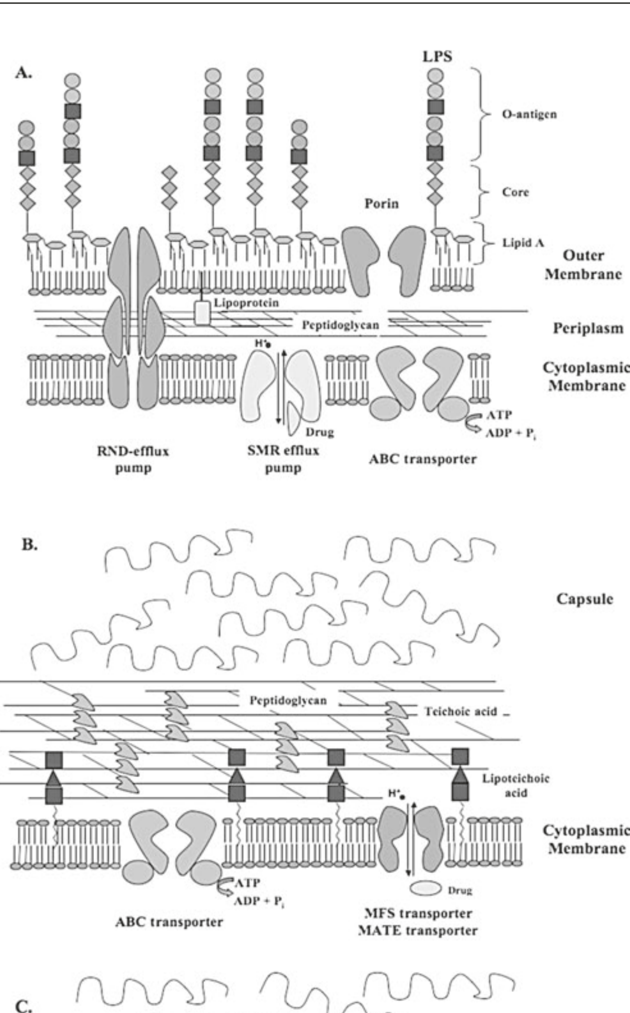
bilayer to the other, since it is thermodynamically unfavourable for the polar head group to pass through the hydrophobic core. When bacterial cells are grown at increasing temperatures, there is generally an increased production of rigid, saturated fatty acids and a decreased production of flexible, unsaturated fatty acids in order to maintain membrane fluidity at a physiologically appropriate level.

2.2 Periplasm/Peptidoglycan

Located between the cytoplasmic membrane and outer membrane of Gram-negative bacteria, is the periplasm (Fig. 1a). Based on thin section transmission electron microscopy, the periplasm is estimated to be between 13 and 25 nm in width (9–11), depending on the sample preparation method used, and this can be compared to the width of membranes that are about 7–10 nm for the inner membrane and 10–30 for the outer membrane (NB, the membrane bilayer of the outer membrane is only slightly larger than that of the cytoplasmic but the long sugar chains of lipolysaccharide, LPS, can thicken the outer membrane adding a capsule-like aspect to the surface of the outer membrane (12). The peptidoglycan layer is located within the periplasmic region.

Given its position, the periplasm plays an important role in buffering the cell from changes in both the intracellular and extracellular environments. To facilitate this function, the periplasm contains anionic sugar polymers termed membrane-derived oligosaccharides as well as many proteins including (a) specific solute or ion binding proteins for the uptake of sugars, amino acids, peptides, vitamins and ions; (b) catabolic enzymes for the degradation of complex molecules into simpler ones that can be transported across the inner membrane; (c) detoxifying enzymes, like β-lactamases and aminoglycoside-modifying enzymes, for the degradation or modification of potential cell inhibitors; (d) hydrolytic enzymes, like nucleases and alkaline phosphatases and (e) proteins which aid in the assembly or translocation of major envelope proteins, peptidoglycan, LPS or capsules (13).

Despite some disparity in measurements of the size of the periplasmic space, the physiological state of the periplasm is thought to be gel-like. Hobot et al. (9) proposed that the periplasm is organized in a gradient of increasing peptidoglycan polymerization from the cytoplasmic membrane to the outer membrane. This peptidoglycan framework is filled with an aqueous solution containing periplasmic proteins, oligosaccharides and other small molecules. More recently, this model has been refined to propose that periplasmic proteins rather than peptidoglycan polymers account for the gel-like state of the periplasm (14). Measurements of periplasmic protein mobility are consistent with this



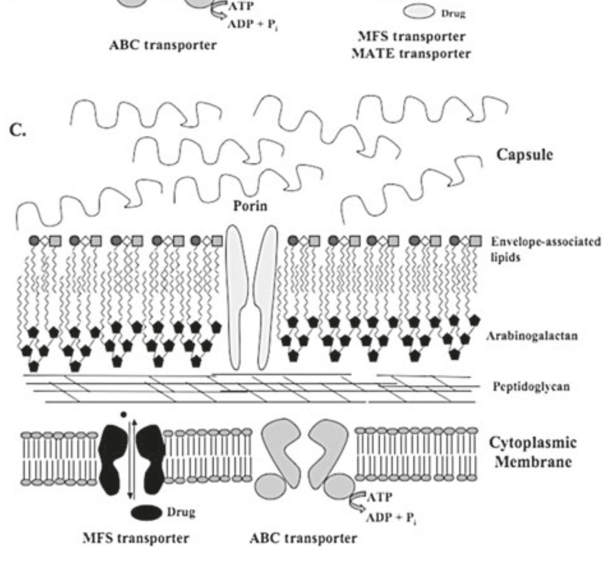


Fig. 1 The structure and arrangement of the cell envelope components of (**a**) Gram-negative bacteria, (**b**) Gram-positive bacteria and (**c**) mycobacteria. Note that although representations of example clinically relevant efflux system are shown, each type of bacterium may contain members of other classes of efflux systems, in addition to those displayed

modification of the model (15). Whatever the physiological state, the periplasm is a dynamic rather than a static environment, and is often underestimated for its significant role in cellular homeostasis.

The term peptidoglycan was first introduced by Weidel and Pelzer (16) to describe a “rigid bag of the volume and shape of the cell.” Peptidoglycan is the polymer that encompasses the bacterial cell providing both strength and structure to the cell and is sometimes called the cell wall or murein sacculus. Due to the high metabolic activity and correspondingly high solute concentration within the cell, bacteria must contain an osmotic pressure that is between five and twenty atmospheres and thus greater than that of the surrounding medium. The peptidoglycan layer is the structure that facilitates maintenance of this pressure difference and is therefore absolutely essential to cell survival. Nevertheless, the peptidoglycan layer has sufficient plasticity to allow for both cell growth and division and specific enzymes that can remodel the peptidoglycan locally to permit these essential functions, with which peptidoglycan is intimately involved.

Although it is conserved in all eubacteria, differences exist in the peptidoglycan layer between Gram-positive and Gram-negative bacteria. In Gram-positive organisms, the peptidoglycan layer is multilayered and relatively thick (5–25 nm) (17, 18). Various acidic and/or neutral polymers like teichoic acid or teichuronic acid are covalently attached to the peptidoglycan layer (Fig. 1b). In Gram-negative organisms, the peptidoglycan layer is located between the cytoplasmic and outer membranes and tends to be only a few layers (19) and 1.5–6 nm thick (20), although recent studies suggest that the peptidoglycan chains may be at least partially oriented perpendicularly to the surface of the cytoplasmic membrane (21). Lipoproteins embedded in the outer membrane and peptidoglycan-associated proteins (covalent and non-covalent) anchor the peptidoglycan layer to the outer membrane.

Peptidoglycan is composed of a polysaccharide backbone made up of β, 1–4-linked alternating residues of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), cross-linked through peptide side chains. A short peptide of four amino acids is attached to the carboxyl group of NAM of mature peptidoglycan. Variability in the peptidoglycan structure is largely due to differences in the short peptide, although differences in the glycan backbone and nature of the crosslink are also observed (22). In *Escherichia coli*, for example, the mature stem peptide is composed of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and D-alanine, whereas in *Staphylococcus aureus meso*-diaminopimelic acid is replaced by L-lysine.

The average glycan strand is about 30 muropeptide units in length (23, 24). Individual strands are cross-linked to each other either directly or indirectly through peptide side chains, and these covalent peptide crosslinks provide the strength required to resist the internal osmotic pressure. In Gram-negative bacteria, for example *E. coli*, direct cross-linking occurs between the carboxyl group of the D-alanine in position 4 of one stem peptide and the free amino group of *meso*-diaminopimelic acid in the adjacent strand. Cross-linking in Gram-positive bacteria is indirect, and occurs through an inter-peptide bridge of five glycines in *S. aureus* for example. The degree of cross-linking and cross-linking position also differs between species of bacteria (25, 26), with Gram-positive organisms having a higher degree of cross-linking than Gram-negative organisms, which have the added protection of the outer membrane.

2.3 Outer Membrane

The outer membrane is an unusual bilayer membrane found only in Gram-negative bacteria (Fig. 1a) (6). What makes this structure unique is the asymmetric nature of the bilayer. The composition of the inner leaflet is similar to that of the cytoplasmic (inner) membrane, with phosphatidylethanolamine being the predominant phospholipid and minor amounts of other phospholipids, e.g. phosphatidylglycerol and cardiolipin (diphosphatidylglycerol). As with the cytoplasmic membrane, the lipid composition of the outer membrane is not static; it varies with the environmental conditions in which the bacteria are found.

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

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

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

The core oligosaccharide of LPS is covalently attached to the Lipid A via the unique sugar molecule 2-keto-3-deoxyoctulosonic acid (KDO). In addition to KDO, this region also includes a variety of other sugar molecules such as L-glycero-D-manno-heptose and its optical isomers, glucose, galactose, rhamnose etc. Some of these sugars may be modified by the addition of phosphate, pyrophosphate, phospholipids (e.g. phophatidylethanolamine, phosphatidylcholine), or amino acids (e.g. alanine). The overall structure of the core oligosaccharide is relatively conserved within a given bacterial genus but may vary somewhat with respect to sugar composition, substitution, and/or connectivity (27).

Between approximately 10 and 25% of the core oligosaccharides are covalently linked to the *O*-polysaccharide (or *O*-antigen), a string of sugar repeat units, that vary substantially even within a species. This diversity is proposed to be driven by selective pressure (e.g. from the immune response or from phage susceptibility) that arises from being exposed to the external environment (28). The basic structure of the *O*-polysaccharide consists of a mono- to octa-saccharide repeat. Over 60 different sugars from different Gram-negative bacteria have been identified as being components of an *O*-polysaccharide. Some examples of these sugars include glucose, mannose, ribose, rhamnose, glucosamine, fucosamine and amino hexuronic acids such as quinovosamine.

The number of *O*-repeats varies from 0 to 50 units and this produces a characteristic ladder pattern when LPS is resolved on an acrylamide gel. Some mucosal pathogens, such as *Bordetella pertussis*, completely lack an *O*-antigen and are thus said to possess LOS (lipooligosaccharide) rather than LPS (29). Other organisms, such as *Pseudomonas aeruginosa*, can have *O*-antigens that extend more than 40 nm from the surface of the cell (12).

In addition to LPS, the outer membrane contains a moderate number of proteins present in high copy number. These proteins are involved in a variety of cellular processes that include selective permeation, cell shape and membrane stabilization, motility, adherence, transport and interaction with the immune system, bacteriophages and other bacteria (6, 30).

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

Outer membrane transporters are involved in both the uptake (porins) and efflux (efflux channels) of compounds into and out of the cell. Both of these protein classes adopt a β-barrel structure in the outer membrane although their architecture is very different with the porins containing one water-filled channel per monomer (or often three per trimer) and the efflux channels containing one channel made from three monomers. Efflux channels have an additional α-helical periplasmic domain, which is discussed in a later chapter and elsewhere (31). Amino acids with non-polar side chains form the outer surface of the barrel and interact with membrane lipids, thus stabilizing the structure. Hydrophilic amino acids line the interior of the channels, providing a polar environment for hydrophilic compounds to travel through.

2.4 Mycobacterial Cell Envelope

Although phylogenetically classified as Gram-positive bacteria, the mycobacteria have a uniquely organized cell envelope (Fig. 1c). As with other bacteria, the cytoplasmic membrane forms an inner barrier between the cytoplasm and the environment, and its lipid composition is similar to that of other bacteria. This is surrounded by a layer of peptidoglycan, with a structure similar to that of Gram-negative bacteria (i.e. relatively thin). External to this is the arabinogalactan layer, consisting of a complex branched network of polysaccharide. Each arabinogalactan residue consists of a polymer of galactofuranose, many of which possess five or six covalently attached arabinose moieties (Fig. 1c). Each of the arabinose groups in these terminal groups are ester-linked via the 1′-hydroxyl moiety to lipidic mycolic acids which extend to the bacterial surface. The mycolates attached to the arabinogalactan are very long (60–90 carbons) and may contain unusual cyclopropane moieties within their acyl chains (32). Due to the length of these fatty chains, they are found in the gel state with phase transition temperatures as high as 60–70°C (33). The composition of the membrane varies due to regulation by temperature and/or environment, analogous to lipid compositional changes in other types of bacteria. There is some evidence for the presence of another glycolipid monolayer consisting of trehalose dimycolates, sulfolipids, phythiocerol dimycocerosate and phenolic glycolipids external to the mycolate residues of the arabinogalactan. The approximate thickness of the mycolate bilayer is ~37–90 nm, substantially larger than that of a Gram-negative outer membrane (34, 35). Like the Gram-negative bacterial outer membrane there are porin-like molecules that traverse the mycolic acid layer but they have a rather unique structure (35, 36). In some senses, the envelope of mycobacteria resembles the outer membrane of Gram-negative bacteria and due to the presence of this thickened highly hydrophobic envelope, mycobacteria are

characterized by their extremely low permeability to most hydrophilic antibiotics.

2.5 Capsule

Many bacteria in their natural habitats produce extracellular polysaccharide capsules. Capsular polysaccharides are either homo- or hetero-polymers of repeating sugar units, connected by glycosidic bonds to form the capsule structure. Because of the broad range of monosaccharide units and glycosidic bond configurations possible, bacterial capsules are extremely diverse. Initially capsules were divided into groups (referenced to *E. coli*) based on the presence of common monosaccharides (37), but more recently capsule classification has been based solely on genetic and biosynthetic criteria to divide *E. coli* capsules into four distinct groups (38). This updated classification scheme (again referenced to *E. coli*) accounts for the observation that not all capsules are composed of polysaccharide K antigens; previous classifications were based on the biochemical division of K antigens, which all form capsules.

Capsule layers are highly hydrated, containing over 95% water (39), and as such may function to protect the organism from desiccation. Consistent with this suggestion, mucoid isolates are more resistant to drying than their non-mucoid isogenic counterparts (40), and changes in extracellular osmolarity are known to induce expression of capsule molecules (41, 42). Polysaccharide capsules also function as adherence factors. Capsules facilitate both biofilm formation and niche colonization (43, 44) by promoting the adherence of bacteria to each other and to surfaces. This ability of bacteria to attach to surfaces and establish a biofilm plays an important role in initiating and maintaining infection (45, 46). For example, *P. aeruginosa* infections of the cystic fibrosis lung are often characterized by overexpression of alginate and biofilm formation (47), which probably helps to protect the bacteria from opsonization and killing by neutrophils and macrophages in the lung.

Infections are further maintained through the ability of the capsule to resist both the non-specific and specific immune responses of the host. Polysaccharide capsules are poor activators of the alternative complement pathway (48–50) and furthermore mask underlying cell surface structures, which do typically activate this pathway (51, 52). This reduced ability to activate opsonic fragments of complement (e.g. C3b), and the net negative charge of the capsule surface works to inhibit phagocytosis (53, 54). Capsular polysaccharides also confer resistance to the host's specific immune response, by mimicking the structure of polysaccharides found in the host, and consequently are usually poor immunogens (55–57).

3 Intrinsic Resistance

3.1 Restricted Permeability

3.1.1 Gram-Negative Bacteria

The outer membrane of Gram-negative bacteria is a semi-permeable barrier to the uptake of most hydrophilic molecules larger than a certain size exclusion limit. An analogy is often drawn to this membrane constituting a molecular sieve although this is only really true for negatively charged or neutral polar molecules, as both positively charged and hydrophobic molecules can pass across the outer membrane by other routes. For the former molecules, uptake is limited by the size of the water-filled channels of β-barrel proteins termed porins (58). The total surface area of the outer membrane that is occupied by such channels has been estimated as approximately 0.6% in *E. coli*, and this together with limited diffusion imposed by frictional interactions between molecules passing through the channel and the amino acids lining the channel wall, severely restricts uptake of hydrophilic molecules especially those like β-lactams, trisaccharides, and tetrapeptides that have sizes that are not much smaller than the restricting diameters of these channels in e.g. *E. coli*. Other bacteria, e.g. *Pseudomonas aeruginosa*, have a much smaller number of channels leading to an overall outer membrane permeability that is only 1–8% that of the *E. coli* outer membrane, even though *P. aeruginosa* has larger-sized channels and a larger exclusion limit. Restricted permeability through the outer membrane clearly contributes therefore to the observation that Gram-negative bacteria tend to have higher intrinsic resistance to most antibiotics than their Gram-positive counterparts, a factor that is a major contributor to the drastic dearth of discovery of new Gram-negative selective antibiotics.

It is worth considering the nature of the "fabric" of the outer membrane molecular sieve. As mentioned above, the outer membrane surface largely contains, as its major lipidic molecule, the highly anionic glycolipid LPS, which is partly neutralized, cross-bridged and thus stabilized by divalent cations, predominantly Mg^{2+} and Ca^{2+}. This surface thus tends to repel neutral and anionic polar molecules, but as described below can actually serve to permit self-promoted uptake of cationic molecules. Further evidence that the outer membrane is a barrier to uptake of hydrophilic antibiotics is seen in the fact that increasing outer membrane permeability by cloning in large, abundant porins leads to increased antibiotic susceptibility in *Pseudomonas aeruginosa* (59), while disrupting the fabric of the outer membrane by removal of divalent cations with chelators like EDTA has a similar effect (60, 61).

3.2 Mycobacteria

Based upon the low susceptibility of mycobacteria to most antimicrobials, it is clear that the cell wall of this organism forms a significant antimicrobial barrier. Indeed, early studies examining the permeability of *Mycobacterium chelonae* showed that it was approximately tenfold less permeable to hydrophilic β-lactam antibiotics than was *P. aeruginosa* (62) (i.e. 100- to 1,000-fold less permeable than the *E. coli* outer membrane).

In contrast to the trimeric general porins of Gram-negative bacteria that have a single pore per monomer, MspA is an octamer of small subunits that assemble to form a single central channel (35), and channel numbers tend to be relatively low. In addition, the MspA pore is much longer than for the general porins, presumably due to the thickness of the mycobacterial cell wall. Therefore, substrate interactions with the channel interior may be more pronounced in mycobacteria and might hinder solute diffusion. Indeed, this appears to be the case as the deletion of MspA from *Mycobacterium smegmatis* results in both increased resistance to hydrophilic antibiotics as well as decreased growth due to lowered permeability to nutrients (62, 63).

3.3 Efflux

Intrinsic antibiotic resistance in Gram-negative bacteria is due to the synergy between low outer membrane permeability that restricts the rate of exposure of the interior of the cell to antibiotics, and the presence of additional resistance mechanisms such as drug modification (e.g. β-lactamases) and multidrug efflux systems. Cytoplasmic membrane-localized efflux pumps are widespread among bacteria and are divided into five major classes on the basis of bioenergetic and structural criteria (64) and it is worth noting that in addition to contributing to antibiotic efflux, many of these pumps also have roles in normal cell physiology (65).

The ATP-binding cassette (ABC) superfamily is an ATP-driven efflux system found in Gram-negative and Gram-positive bacteria, as well as in mycobacteria. The major facilitator superfamily (MFS) is another ancient efflux system that uses chemiosmotic energy and functions as a drug-ion antiporter. The resistance/nodulation/cell division (RND) family and the small multidrug resistance (SMR) family are both proton-driven pumps although the former comprises multi-subunit complexes. The fifth system is the multidrug and toxic compound extrusion family (MATE) and also utilizes the chemiosmotic gradient across the cytoplasmic membrane to energize transport. Gram-positive bacteria often employ MFS efflux pumps such as NorA in *S. aureus* (66) which provide resistance to fluoroquinolones.

In Gram-negative bacteria, the RND (resistance-nodulation-division) family of pump proteins are the predominant class (67) involved in intrinsic resistance. RND transporters are tripartite systems consisting of an outer membrane channel-tunnel, an inner membrane pump and a peripheral cytoplasmic membrane/periplasmic linker protein. A broad range of structurally unrelated substrates are known to be pumped out of bacterial cells including most types of antibiotics, biocides, heavy metals, organic solvents, dyes, and detergents (68). Given the ubiquitous distribution of efflux systems in bacteria, there is much interest in determining the natural and intended substrates of these efflux systems (65). In *E. coli* for example, efflux pumps are capable of shuttling toxic fatty acids and bile salts out of the cell and thus it has been suggested that normal metabolic intermediates and noxious compounds that *E. coli* encounters in the gut during infection may be natural substrates (68).

In many bacteria, the expression of efflux system genes is tightly controlled. Although antibiotic efflux is typically described as an intrinsic resistance mechanism, there are a number of mutational events that can lead to increased expression of efflux systems, and therefore increased resistance. For example, *tetR*, the negative regulator of the MFS tetracycline efflux pump is ordinarily bound to the operator sequence upstream of the efflux genes, preventing expression under normal conditions (69). In the presence of its substrate (i.e. tetracycline) the TetR protein is released from the operator and transcription of the gene(s) involved (*tetK*, *tetL*, and/or *tetB*) proceeds. Thus the bacteria do not become resistant to tetracycline unless tetracycline is actually present.

A similar general principle exists for many RND efflux systems in wild-type bacteria in that expression of efflux pumps is tightly regulated, although some pumps are always expressed at basal levels. However, unlike the situation with the TetR protein described above, the actual efflux genes are often not induced by the known substrates of the particular efflux pump. Rather, what often occurs is that a mutation appears in the regulator of the efflux system following antimicrobial therapy, such that the genes encoding for the pump components are expressed constitutively at higher levels leading to increased resistance to all substrates that the pump can efflux. The mutations are often stable point mutations that reduce the DNA binding affinity of particular repressors for their target regulatory regions within promoters and lead to constitutive expression of efflux components (70). Many clinical isolates of the cystic fibrosis pathogen *P. aeruginosa* have multidrug resistance phenotypes due to regulatory mutations that are probably selected for in the lungs of CF patients who are often on chronic antimicrobial therapy (68).

4 Antibiotic Penetration and Resistance Mechanisms

4.1 Porin Pathway

Porins permit the diffusion of a variety of compounds into the periplasm. There are three classes of porins; general, specific, and gated (Fig. 2). Uptake through general porins is considered passive, as it involves passive diffusion through the aqueous channels of the porin and is dependent only on the physicochemical properties of the solute (that is, size, charge, polarity, and the magnitude of the concentration gradient across the membrane) relative to the side chains of the amino acids lining the pore and especially those side chains found at the most constricted part of the channel. The crystal structures of several general porins have been solved and reveal that they are trimers of 16 stranded anti-parallel β-barrels that enclose a pore lined predominantly with hydrophilic amino acids (71, 74, 75). These β-strands tend to be connected by short (3–4 amino acid) turn regions on the periplasmic side of the porin and much longer loops of amino acids on the external side of the outer membrane. The cross-section of the channel interior somewhat resembles an hourglass and can be conceptually divided into three zones; the external mouth, the constriction zone or eyelet, and the exit. The mouth of the general porin pore acts as a crude filter. This region is rich in charged amino acids and may be somewhat restricted by one or more extracellular loops that fold into it. The purpose of these two features is to constrict the opening, both physically and electrostatically, such that large, hydrophobic, and/or highly charged compounds cannot enter the cell. The eyelet is the narrowest part of the channel, usually formed by a single loop 3 that folds from the external surface back into the porin channel. The size of this eyelet determines the maximum size i.e. the exclusion limit of molecules that can pass through the channel. For the prototypic bacterium, *E. coli*, the exclusion limit determined by the major porins OmpF and OmpC is around 600 Da (equivalent to a trisaccharide or tetrapeptide), although there are subtle differences in channel size for these two proteins. Therefore, for this and other enterobacteriaceae, it is presumed that small, hydrophilic antibiotics such as chloramphenicol, tetracycline, fluoroquinolones and β-lactams (including cephalosporins and carbapenems) might utilize these channels as entry points. This fact has been confirmed by the isolation of mutants, both in the clinic and in vitro, that are resistant to the above-mentioned antibiotics due to either a complete loss of or diminished porin expression (76–78).

Specific porins are similar to general porins with one major exception; they have stereospecific binding sites for their substrates, which are located in part in the eyelet. This specificity narrows the structural range of molecules that can pass through these channels. The crystal structure of the LamB channel of *E. coli* has been solved and shows that this

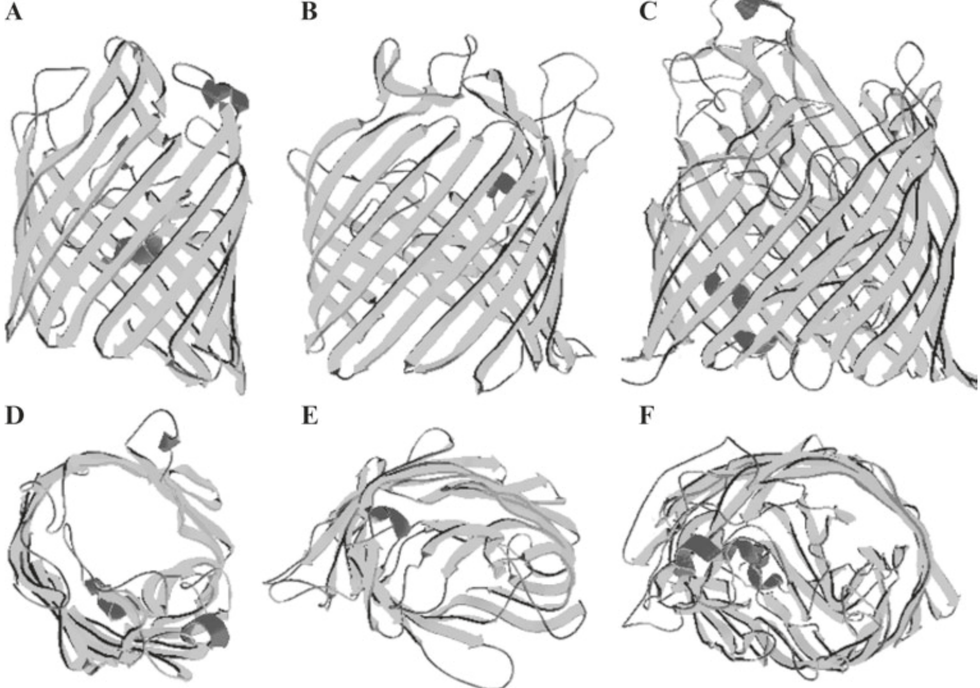


Fig. 2 Representative structures of the porin molecules of *Escherichia coli*. Side (**a**) and top (**d**) view of the OmpF general porin (71). Side (**b**) and top (**e**) view of the maltodextrin-specific channel, LamB (72). Side (**c**) and top (**f**) view of the gated porin FhuA (73). Note the varying degrees of channel constriction imparted in each porin type by the inward folding of various extracellular loops or domains (see text for complete description)

porin is highly specialized for the uptake of maltodextrins (72). The eyelet of this porin is more constrained than in general porins due to the folding of two additional loops into the mouth of the channel. Six contiguous aromatic amino acids (the greasy slide) form a path through the channel, down which the sugar molecules travel. In addition, the channel interior is lined with polar amino acids (the polar track) that stabilize the hydroxyl groups of the sugars (79). An analogous design is found for the phosphate-specific porin OprP of *P. aeruginosa* (80). The channel interior of this trimeric porin is quite constricted and reveals a phosphate-binding "arginine ladder" comprising eight arginine residues that span from the extracellular surface down to a constriction zone where phosphate is coordinated. Lysine residues also coat the inner periplasmic surface of this channel creating an "electropositive-sink" that pulls the phosphates through the eyelet and into the cell.

Due to their specialized nature, the only antibiotics that should be able to penetrate specific porins are those that mimic the channel's natural substrates. Indeed, this is true for the Tsx channel of *E. coli*. Specific for nucleosides, this porin also takes up the structurally related antibiotic albicidin (81, 82). Similarly, the OprD porin of *P. aeruginosa* is specific for the uptake of the basic amino acids arginine and lysine and basic dipeptides, as well as the structurally analogous carbapenem antibiotics imipenem and meropenem (83, 84). Recently we also demonstrated that the tricarboxylate-inducible porin OpdH, a homolog of OprD, appeared to be involved in the uptake of the bulky cephalosporin ceftazidime (85). It should also be noted, that low levels of structurally unrelated compounds can also diffuse through specific porins. This is especially the case for non-fermentative organisms, like *Pseudomonas*, which lack classic general porins. For example, the OprD porin in addition to taking up basic amino acids is the major facilitator involved in the diffusion across the outer membrane of compounds up to 200 Da in mass (59).

Gated porins, also known as TonB-dependent receptors are monomeric proteins consisting of 22-stranded β-barrels, and permit the specific entry of larger compounds such as iron-siderophore complexes into the cell. The mouth of these channels is blocked by a globular domain termed the plug (86, 87). Uptake is initiated once a substrate docks onto a gating porin. This binding, in conjunction with energy input from the TonB energy transducing protein, results in a series of conformational changes in the plug domain that culminate in both the release of the substrate and the revelation of a translocation pathway (73).

Due to their large channel sizes, gated porins may seem like the ideal conduits for antibiotic uptake; however, this use is generally limited by the specificity of substrate docking. It is known that there are certain gated receptors that have somewhat lower selectivity, e.g. Cir and FhuA. However although providing antibiotics with iron binding groups (e.g. catechol or heme groups) can improve uptake across the outer membrane, and consequently lower MICs, none of these substituted drugs have been clinically successful and this may reflect mechanisms of toxicity and/or interference with iron metabolism in the host. Specific antibiotics that can be taken up by ferric-siderophore receptors include albomycin, a structural analogue of ferrichrome, which is taken up by the FhuA gated-porin receptor. Interestingly, rifamycin CGP 4832 (a rifampin derivative), a structurally unrelated antibiotic, is also taken up by FhuA (88). The crystal structures of FhuA in complexes with both of these antibiotics indicate that despite differences in structure, both antibiotics bind to the same residues of the porin (89), indicating that gated porins tolerate some structural flexibility.

As described above, mycobacterial envelopes contain a class of porins that although structurally unrelated to Gram-negative porins, serve as the major pathway for hydrophilic antibiotics. There are two types of mycobacterial porins represented by OmpATb, which is not well-studied, and MspA, which has been crystallized (36). MspA from *M. smegmatis*, the best-characterized mycobacterial porin, is the major route of entry for hydrophilic compounds into this organism (36). However, the medically important mycobacteria, *M. tuberculosis*, and *M. bovis* BCG seem to lack MspA-type porins, and depend exclusively on OmpATb-type porins, an observation that may explain the intrinsically lower susceptibility of these organisms to hydrophilic antibiotics compared to *M. smegmatis*. The diameter of the MspA channel from *M smegmatis* is apparently larger than that of the OmpATb porin from *M. tuberculosis*, which is not well characterized, and cloning of the *M. smegmatis* MspA protein into *M. tuberculosis* increases the sensitivity of *M. tuberculosis* to β-lactams by up to 16-fold (90). Additionally, the growth rate of *M tuberculosis* expressing *M smegmatis* MspA is increased; suggesting that nutrient uptake in this species is also limited by the small pore size of OmpATb. Regardless of which porin proteins a particular strain expresses, the porin pathway seems to be involved in the uptake of pyrazinamide (91) and β-lactams (92).

4.2 Self-Promoted Uptake and Regulatory Mutants

The self-promoted uptake pathway is limited to Gram-negative bacteria and generally pertains to the passage of cationic amphipathic molecules across the outer membrane. Self-promoted uptake involves the interaction of polycations with sites on the surface of the outer membrane at which divalent cations cross-bridge adjacent LPS molecules. Displacement of these divalent cations leads to local distortion of outer membrane structure and this provides sites for

uptake of other polycationic antibiotic molecules; thus these polycations promote their own uptake rather than diffusing across the outer membrane through water-filled channels.

Recently, it has become clear that self-promoted uptake is quite effective in many species of bacteria including *E. coli*, *P. aeruginosa*, *Salmonella enterica* and *Yersinia sp*., which all seem to have the potential to be killed by antibiotics that access the self-promoted uptake pathway (61). Other species such as *Burkholderia cenocepacia* and *Helicobacter pylori* show a significantly lower rate of killing by antibiotics that would normally enter via this pathway (93, 94). For species that are normally sensitive to killing via the self-promoted uptake pathway, the organism in question generally maintains a level of control over the effectiveness and/or accessibility of this pathway (95–98), as discussed in more detail below.

The characteristics of the LPS of a particular bacterial strain primarily determine whether or not a particular bacterium possesses an effective self-promoted uptake pathway. As described in Sect. 3.2, the structure of bacterial LPS is complex and species-specific. The LPS of many bacteria is characterized by a large number (3–12) of negatively charged phosphate groups and anionic sugars (e.g. KDO) in the core oligosaccharide and usually two additional phosphates attached to the Lipid A moieties of the LPS (28). These negatively charged groups are ordinarily bridged by divalent cations, which serve to stabilize the outer membrane by preventing the LPS molecules from repelling one another. Studies carried out with chelators of divalent cations, such as EDTA, have shown that when the cell is rapidly depleted of the divalent cations bound to the LPS, there is a massive disruption in outer membrane integrity, with a concomitant loss of ~50% of the LPS (61). Thus, these divalent cations are an integral component required for maintenance of outer membrane structure.

Cationic antibiotics and the cationic antimicrobial peptides can also disrupt the bacterial outer membrane. The cationic peptides are ubiquitous in nature and form an important component of the human innate immune system (99). Basically, these are small peptides that have a net positive charge due to the presence of a number of lysine or arginine residues in their sequence. Soil-dwelling bacteria, lactic acid bacteria, plants, insects, fish, birds, amphibians, and other animals also produce cationic peptides. Studies with the cationic lipopeptide antibiotic polymyxin B showed that when bacteria are exposed to this antibiotic the integrity of the bacterial outer membrane is rapidly destroyed, indicating that the outer membrane might be a primary determinant by which these compounds gained access to Gram-negative cells (100, 101). Cationic antimicrobial peptides have a number of physical properties that are important for their activity. As suggested by their name, the cationic nature of the molecule is very important and substituting uncharged for the charged amino acids severely impairs their antimicrobial ability. Additionally these peptides usually contain up to 50% hydrophobic amino acids and consequently can insert into membranes while folding into an amphipathic structure that contains both a highly polar face and a hydrophobic face.

Regulation of self-promoted uptake has been studied in a number of organisms including *E. coli. enterica* and *P. aeruginosa*. The genetics of resistance are perhaps best understood in *E. coli* and *S. enterica* and these systems will serve as the model for the remainder of this discussion, with important exceptions being highlighted where applicable. Early work in *S. enterica* showed that there were two loci responsible for increased resistance to polymyxin B and other cationic antimicrobial peptides and that these mapped to two systems named *pmrAB* (*p*oly*m*yxin *r*esistance gene *A* and *B*) and *phoPQ* as reviewed elsewhere (102). Both of these systems are two-component regulatory systems that normally turn on genes in response to a given environmental condition, limiting concentrations of divalent cations for the *phoPQ* system (103), and high concentrations of ferric iron in the case of the *pmrAB* system of *S. enterica* (104). *S. enterica* are intracellular pathogens that encounter limiting divalent cation concentrations and high concentrations of antimicrobial peptides when engulfed by the host cell. Thus the bacterium senses the limiting divalent cation concentration and responds in a way that makes it more resistant to cationic peptides. Alternatively it was recently demonstrated that cationic peptides can bind directly to PhoQ and regulate their own resistance (105). Although the precise mechanism underlying signalling by cationic peptides is not completely defined, it appears to involve interaction with a cytoplasmic-membrane-facing polyanionic domain of PhoQ. Clearly, direct regulation by a host molecule would appear to provide a distinct advantage to the bacterium in a host at a site where Mg^{2+} is not limiting and where the concentration of antimicrobial peptides is very high, such as for example the granules of cells or the lumen of the lung. When these systems are turned on by any of the mentioned conditions, the expression of a number of genes is modified, including those that affect susceptibility to cationic peptides that are taken up by self-promoted uptake.

To decrease susceptibility to agents taken up by self-promoted uptake, bacteria regulate gene sets, through PhoPQ or PmrAB or both, that alter their LPS in a number of important ways. The most important is reduction of the requirement for divalent cation cross-bridging of the LPS. Bacteria accomplish this by masking the negatively charged groups via the synthesis and addition of N_4-aminoarabinose and phosphoethanolamine to the Lipid A phosphates (106). In addition to this modification, activation of the *phoPQ* system leads to increased expression of the *pagP* gene. The PagP protein catalyzes the addition of an extra acyl chain to the

hydrophobic portion of lipid A (107). The addition of this extra fatty acid increases the amphipathicity of the Lipid A, thereby making the outer leaflet more stable in the presence of bulky cationic peptide molecules. Both of these additions lead to substantially increased resistance to molecules that utilize the self-promoted uptake pathway. The PhoPQ system in *Salmonella* also regulates the production of an outer membrane protease, PgtE (108). When this protein is expressed, it is capable of degrading certain cationic peptides that access the cell via the self-promoted uptake pathway, thus providing another way of reducing influx of the antibiotic.

Although the system described above is essentially conserved for Enterobacteriaceae, there are major differences in other organisms. In *P. aeruginosa* for example, LPS modification genes responsible for the addition of N_4-aminoarabinose are also regulated by sub-inhibitory concentrations of cationic antimicrobial peptides, but this regulation is independent of either the PmrAB or the PhoPQ systems (109). Additionally, in *Pseudomonas* the PmrAB system is regulated by the presence of limiting divalent cation concentrations, similar to PhoPQ (109), in contrast to *E. coli*, *Salmonella* and *Erwinia* where it is regulated by high concentrations of Fe^{3+}. Although the precise mechanism by which this signalling takes place is ill-defined, it would appear to provide a distinct advantage to the bacterium in the CF lung, where Mg^{2+} is not limiting and where the concentration of antimicrobial peptides is very high. Overall these systems seem to be arranged in such a way as to limit bacterial susceptibility to self-promoted uptake in environments where the bacterium is likely to encounter cationic antimicrobial peptides or limiting divalent cation concentrations.

As Gram-positive bacteria do not possess outer membranes they utilize other mechanisms for decreasing uptake into the cell and consequently have different resistance mechanisms for cationic peptides. These include the modification of peptidoglycan or lysinylation of phosphatidylglycerol in *S. aureus* (110). The general principle appears to be the same however, in that by decreasing the affinity of envelope components for catonic peptides, resistance is promoted.

4.3 Hydrophobic Pathway

As suggested by the name, the hydrophobic pathway involves the passage of antimicrobial compounds through the hydrophobic interior of the lipid bilayer. The hydrophobic pathway of antimicrobial uptake tends to be more important in Gram-positive bacteria than it is in Gram-negative bacteria, since slowed hydrophobic passage through the Gram-negative outer membrane can be counteracted by active efflux through RND efflux systems. In contrast, the peptidoglycan layer of Gram-positive bacteria has a diffusion limit of approximately 50 kDa and decreased uptake very seldom contributes to resistance. The hydrophobic pathway is especially important for molecules that are active on intracellular targets, but that do not access a specific transporter. In Gram-positive bacteria, this includes many commonly used antibiotics including fluoroquinolones (which can be present at low concentration in an uncharged form), and macrolides.

As mentioned above, bacterial outer membranes have somewhat diminished hydrophobic uptake through the outer membrane bilayer primarily due to the reduced fluidity of the LPS monolayer compared to the cytoplasmic membrane. However, certain mutants that affect LPS core biosynthesis, e.g. *lpxA* and *lpxD*, exhibit up to 1,000-fold increased sensitivity to hydrophobic antimicrobials (111), largely by increasing uptake to an extent where it overwhelms efflux systems. In addition, a study with a series of isogenic LPS mutant strains of *E. coli* and *Salmonella enterica* demonstrated that the susceptibility of each mutant to hydrophobic antibiotics increased as the length of the LPS decreased (112). This study further supports the role of the LPS of Gram-negative bacteria as major determinant of reduced permeation of hydrophobic antibiotics.

Although the porin-mediated pathway described above is somewhat important in mycobacteria, it is believed that many clinically relevant antibiotics used for anti-mycobacterial therapy access the cytoplasm via the hydrophobic pathway. The general rate of diffusion across the mycobacterial envelope is slower due to the high rigidity of the mycolate bilayer, but does not seem to be reinforced by a broad spectrum efflux system that pumps out hydrophobic compounds as in Gram-negatives. Consequently, rifampin, isoniazid, and hydrophobic fluoroquinolones are thought to access the cell via the hydrophobic pathway (113).

4.4 Inner Membrane Transporters

A small number of antibiotics use specific membrane transporters to get across the cytoplasmic membrane, leading generally to a requirement that cells be energized for uptake. Usually this involves structural features that are conserved between the antibiotic and the normal substrate for the transporter. Thus, the antibiotic D-cycloserine is transported across the bacterial cytoplasmic membrane via the D-alanine transport system in a manner that is dependent upon the proton motive force (114). Fosfomycin, an antibiotic that inhibits the biosynthesis of peptidoglycan, crosses the cytoplasmic membrane using the glycerol-3-phosphate or hexose phosphate transporters (114). The antibiotic streptozotocin is also taken across the inner membrane via an active transport process involving the phosphoenol-pyruvate phosphotransferase system.

Aminoglycoside antibiotic uptake is still fairly poorly characterized. The drugs are taken up in a three-step process whereby the first step involves electrostatic LPS interactions on the surface followed by two energy-dependent phases of uptake (EDP I and EDP II) (115). EDP I is believed to represent the initial stages of aminoglycoside passage across the cytoplasmic membrane and binding to the ribosome. It is thought that some aspect of electron transport drives the vectorial transport of aminoglycosides across the cytoplasmic membrane during EDP I, possibly the shuttling of ubiquinones across the membrane (116). At this point the aminoglycoside triggers an event that initiates cell death and at the same time promotes an acceleration of energy dependent aminoglycoside uptake in the EDP II. Many aminoglycoside resistant mutants are altered in the energization of uptake, while a very common mechanism known as impermeability type resistance has been associated with disregulation of RND efflux pumps in *P. aeruginosa* (117).

5 Synergy

Synergy between antimicrobials is a common theme that is clinically utilized in the treatment of complicated infections. Often this is stated to be because one antibiotic assists the uptake of another. In many instances there is little direct evidence for this. However it should be noted that it has been well established that those molecules that access self-promoted uptake and act by increasing outer membrane permeability, also have the capability to increase permeability to other antibiotics. Deacylated polymyxin B is the prototype for such molecules (118) and it has also been shown that cationic peptides have this property as do other polycations and divalent cation chelators (61).

6 Conclusions

It is now well established that decreased outer membrane permeability is a common mechanism leading to clinical resistance. Because in Gram-negative bacteria this often involves uptake pathways of broad significance, these mutants tend to be cross-resistant to several antibiotic classes. While we still have exploitable mechanisms (e.g. self-promoted uptake) that can be manipulated to increase uptake in poorly susceptible bacteria, a recent meta-analysis has described an increase in the rates of resistance to polymyxin B in MDR isolates of *P. aeruginosa*, *Acinetobacter baumanii* and *Klebsiella pneumoniae*, suggesting that even these drugs of last resort may become decreasingly effective as their use becomes more widespread (119). Only through continued research will we be able to overcome these setbacks and effectively exploit the uptake systems described in this review.

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Chapter 10
Transport Mechanisms of Resistance to Drugs and Toxic Metals

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1 Introduction

This chapter discusses the types of transport systems that confer resistance to antibiotics, antimicrobial drugs, and toxic metals. A number of these are discussed in detail in other chapters, so here we focus on the ways in which microorganisms have evolved to use transporters to evade the toxic effects of drugs and metals.

Resistance to therapeutic drugs and toxic metals encompasses a diverse range of biological systems, all of which have an impact on humans. From the relative simplicity of bacterial cells, fungi, and protozoa to the complexity of human cancer cells, resistance has become problematic. One of the most frequently employed strategies for resistance to cytotoxic compounds and elements in both prokaryotes and eukaryotes is extrusion from the cell catalyzed by membrane transporters. These efflux proteins reduce their intracellular concentration to subtoxic levels (1). Although some of these transporters extrude specific drugs and metals, others can extrude a wide range of structurally dissimilar drugs. Currently, much research is directed toward understanding the molecular mechanisms of these transport proteins. Potential clinical applications include the design of inhibitors that block these efflux systems. Clinically useful inhibitors could allow a renaissance for drugs rendered obsolete by the development of efflux systems in both prokaryotes and eukaryotes.

Operationally, two primary types of drug and metal extrusion systems have been identified: secondary carriers such as H^+ (or Na^+)-drug antiporters, where ion gradients provide the driving force, and primary pumps such as ABC (adenosine binding cassette) or P-type ATPases, where extrusion is coupled to ATP hydrolysis (Fig. 1). In some cases, carriers and pumps are responsible for drug uptake, and loss-of-function mutations confer resistance. Until recently, though, there was little knowledge of the molecular structure of these transporters; importantly, how some transporters can accommodate a wide range of structurally dissimilar drugs was not understood. Recently, the determination of the structures of several bacterial transporters has given insight into the mechanisms of action of drug efflux proteins.

2 H^+/Na^+-Driven Antiporters

These secondary drug exchangers fall into several evolutionarily unrelated families: the major facilitator (MF) family, the resistance-nodulation-cell division (RND) family, and the small multidrug resistance (SMR) families that are the H^+-driven, and the Na^+-driven multidrug and toxic compound extrusion (MATE) family (1). These transporters range in size from about 1,000 amino acid residues for the RND transporters, through 400–450 for the MF and MATE transporters, and down to 100 amino acids for the SMR transporters. Most members of the RND, MF, and MATE families are thought to have 12 transmembrane α-helices (TMs), with the RND transporters possessing large extracytoplasmic domains between helices 1 and 2, and between helices 7 and 8, which are not present in MF or MATE transporters. The SMR transporters are thought to adopt a 4-TM structure, but function as oligomers, probably dimers. More elaborate drug efflux systems exist in Gram-negative bacteria, in which an inner membrane transporter interacts with an outer membrane channel-like protein to facilitate the extrusion of drugs across both membranes (Fig. 1). Presently, considerable research effort is directed toward understanding the structure and function of these transport proteins.

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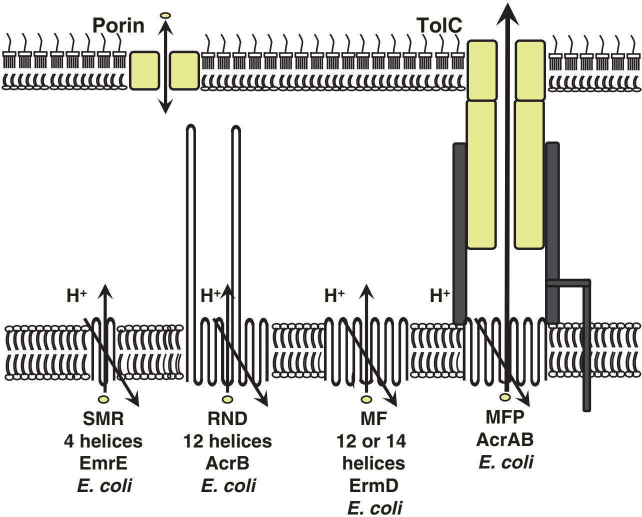

Fig. 1 Simplified representation of proton-coupled antiport systems involved in multidrug efflux. Many drugs enter the periplasm through outer membrane porins. They are extruded through the inner membrane by members of the small multidrug resistance (SMR) (ErmE), major facilitator (MF) (ErmD) and resistance-nodulation-cell division (RND) families. In Gram-negative bacteria, drugs are extruded through both the inner and outer membrane by tripartite complexes, which can utilize either MF or RND inner membrane transporters. For example, when part of a tripartite complex, AcrB, interacts with the outer membrane protein, TolC, in a complex stabilized by the membrane fusion protein (MFP) AcrA

3 Structural Analysis of Antiporters

Structures have been determined for several drug resistance proteins belonging to the MF family, e.g. EmrD (2), the RND family, e.g. AcrB (3), and the SMR families, e.g. EmrE (4), confirming that MF, RND, and SMR transporters are composed of 12, 12, and 4 helices, respectively. The structure of EmrE reveals that it is a homodimer, suggesting that the functional unit has eight transmembrane helices. The structure of EmrD, an MF transporter for multidrugs from *E. coli*, has been determined to a resolution of 3.5 Å (2), revealing a compact 12-helix structure with an interior that is composed mostly of hydrophobic residues, consistent with a role in transporting amphipathic molecules. Two long loops, which connect helices 4 and 5 and helices 10 and 11, are located on the cytoplasmic side that could serve to recognize and bind substrates from the lipid bilayer.

The structures of two other MF transporters, LacY (5) and GlpT (6), which transport lactose and glycerol-3-phosphate, respectively, have been determined. While neither LacY nor GlpT are drug transporters, their structures suggest how related proteins that extrude drugs might function. In contrast to EmrD, both of these transporters assume a V-shaped configuration, in which the more open end faces the cytoplasm, exposing a hydrophilic internal cavity. The structure determined for EmrD may represent an intermediate form between the inward-facing and outward-facing conformations. The structure of LacY has been determined as both acidic and neutral pH, giving an insight into its mode of operation (7). The sugar binding site in LacY does not exist in the absence of the sugar, which is important for understanding how drug binding sites might form. The initial interaction with a tryptophan residue induces a conformational change that positions the residues that interact with the sugar, while causing a key protonated glutamate residue to move from a hydrophobic environment to one in which it can form a salt-bridge as it is deprotonated. In other words, the binding of the sugar drives the deprotonation of this glutamate residue, the initial step in H^+-translocation.

The structure of AcrB, an RND multidrug transporter from *E. coli*, has been determined to a resolution of 3.5 Å (3), revealing a trimer with a jellyfish-like appearance that comprises a TM domain of dimensions 70 Å by >80 Å, and a periplasmic headpiece of dimensions 50 Å by >100 Å, formed from the loops connecting helices 1 and 2 and helices 7 and 8. It has been co-crystallized with four of its known substrates, and these drugs have been shown to bind to a series of overlapping sites within a central cavity between the protomers at the interface of the periplasmic and membrane domains. The existence of multiple sites partially explains AcrB's ability to bind and confer resistance to multiple drugs (8). In this position, the drugs would be poised for delivery to the outer membrane protein, TolC (9). Although the pathway for the delivery of drugs to this cavity has not been elucidated, it seems likely that each TM domain is capable of drug translocation. Consistent with this view, LacY has been shown to have a constriction of the α-helices toward the periplasmic side, which forms an internal cavity to which substrates bind (7). It has been proposed that a change in the constriction from the periplasmic to the cytoplasmic side is responsible for substrate translocation. Further support for such a model comes from the structure of EmrE that, interestingly, was crystallized as a homodimer in which the monomers have opposite orientations (4). Each monomer has a cone-shape due constriction of the α-helices on one side. The two cones fit together, and the drug TPP is observed bound at the interface of the cones, about halfway down and buried within the α-helical structure. The drug-binding pocket is considerably larger than TPP, explaining its ability to bind a wide range of substrate sizes and shapes.

4 Tripartite Pumps

The inner membrane protein, AcrB, interacts with the outer membrane protein (OMP), TolC, to transfer drugs across these membranes, so as to extrude them from the cell. This interaction is transiently coupled by the periplasmic protein AcrA, a membrane fusion protein (MFP) that is anchored to the inner membrane by a lipid moiety. The structure of TolC (9) and of two homologues, OprM (10) and VceC (11), that function as part of the MexAB-OprM and VceABC multidrug pumps from *Pseudomonas aeruginosa* and *Vibrio cholerae*, respectively, have been determined. These OMPs are homotrimers that form a cylindrical channel composed of an outer membrane β-barrel and a periplasmic α-helical barrel. In vivo cross-linking studies show that the periplasmic domain of TolC is long enough to directly contact the periplasmic domain of AcrB (12). However, the crystal structure of TolC indicates that the periplasmic end of the channel is constricted (9), suggesting that interactions with AcrA are necessary to induce conformational changes that open the channel to enable drug transfer from AcrB. Opening of the OMP is proposed to occur by an iris-like movement of the helices, which move between states that are stabilized by different sets of H-bonding and ionic interactions. Recently, the structure of AcrA (13), and previously of the homologue MexA (14), have been determined. Both proteins have a pair of long α-helices that form a coiled-coil sandwiched by β-strands. In the case of MexA, the monomers are arranged into a multimer that appears as a woven rattan cylinder, which might suggest that it could form a passive connecting channel between the MFP and OMP. However, the fact that AcrB can connect with TolC would argue against such a structure, and an alternate model of the AcrB-AcrA-TolC complex has been proposed, in which three AcrA molecules are predicted to fit simultaneously in the inter-protomer grooves of TolC and AcrB. Moreover, a prediction of the model is that AcrA preferentially interacts with and stabilizes the 'open state' of TolC. Four conformational states of AcrA have been observed, providing evidence for a hinge-like conformational flexibility at the base of the coiled-coil domain. This would allow it to move through an angle of 15° at a distance of 21 Å from the tip. By undergoing such movement as it interacts with TolC, AcrA could be used to drive the transition between the closed and open states of TolC. Recently, the structures of a number of different confirmations of AcrB have been determined that support a mechanistic model in which there is a functional rotation in the periplasmic domains, which can accept drugs from the periplasm, driving transfer of the drugs into TolC (14a, 14b). In contrast to TolC, the surface loops of the β-barrel domains of OprM and VceC are larger and are folded down, closing the channel at the outside of the cell. It is not known whether opening of the β-barrel pore occurs as a natural consequence of thermal fluctuations in conformation, or whether this is induced in response to drug binding. If they open in response to drugs, most of which are highly hydrophobic, how are these drugs expelled from the channel into an aqueous environment, or how are they delivered to the outer-leaflet of the bilayer? A recent study has shown that hemolysin, a substrate of TolC, is found in TolC-containing vesicles released from cells (15). Could the OMP act as a 'sink' for drugs, which is subsequently released with its cargo as vesicles? Although the MFP may connect to the OMP by sitting across the surfaces of the periplasmic domain of RND transporters, this situation would not prevail in tripartite pumps that incorporate an MF transporter, because these do not have extensive periplasmic domains. Perhaps there is a requirement for these MFPs to form a cylindrical connecting channel through which the transported substrates can be transferred between the IMP and the OMP?

5 ABC Transporters

The superfamily of ABC ATPases forms the largest group of ATP-coupled pumps. Its best-known members are the human multidrug resistance P-glycoprotein (P-gp) (16) and the multidrug resistance-associated protein (MRP) (17), both of which confer resistance to anti-cancer drugs. Related transporters are found in a number of pathogenic fungi and parasitic protozoa, where they confer resistance to antimicrobial drugs: for example, Candida drug resistance (CDR) 1 and 2 confer resistance to azole drugs in *Candida albicans* (18), whereas PgpA, an MRP homologue, is an arsenic/antimony pump that is responsible for resistance to the antimonial drug Pentostam in *Leishmania* (19). There are also bacterial homologues, including the LmrA (20) and VcaM (21) multidrug transporters from *Lactococcus lactis* and *Vibrio cholerae*, respectively, the DrrAB (22) doxorubicin/daunorubicin transporter from the anthracycline-producing actinomycete *Streptomyces peucetius*, and the MacB macrolide transporter that is found in a number of bacteria (23). In addition to extruding drugs, ABC transporters frequently transport other substrates, including dyes, ionophoric peptides, lipids, and steroids.

Generally, ABC proteins have homologous halves, each containing two parts: a trans-membrane domain (MD) arranged into six TMs, and a nucleotide-binding domain (NBD) (Fig. 2) (25). In most cases, a single gene encodes the four domains: for example, a single gene encodes P-gp, which has a MD-NBD-MD-NBD structure. Although a single gene also encodes MRP, it differs from P-gp in having an additional α-helix membrane domain at the N terminus. On the other hand, the *lmrA* and *vcaM* genes encode proteins

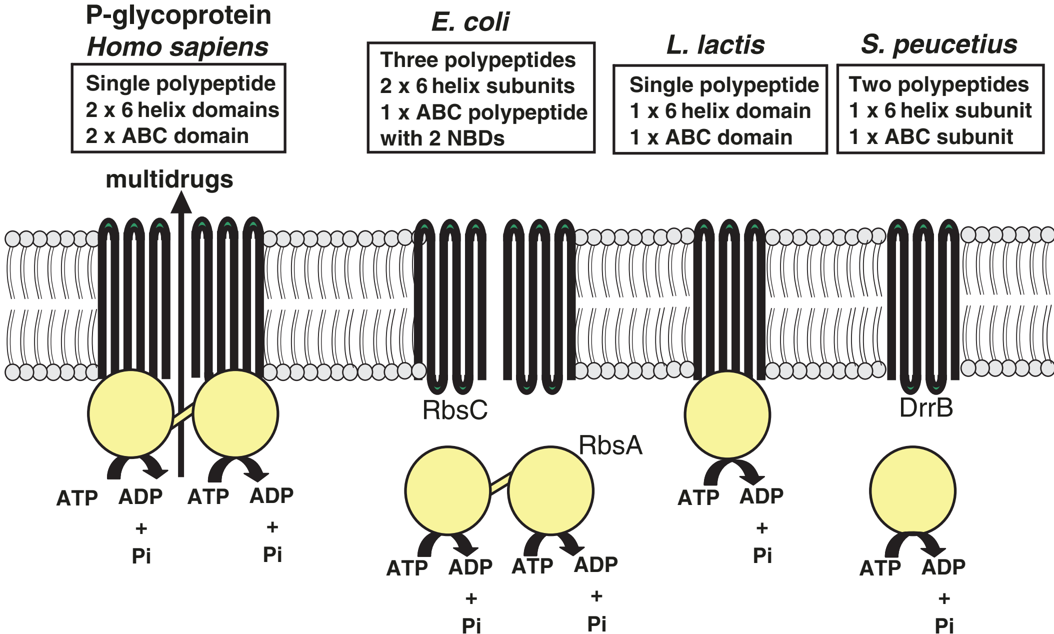

Fig. 2 The ABC superfamily of transport ATPases. Most ABC transporters have four elements: two NBDs, and two groups of six membrane-spanning α-helices. The four elements can be in a single polypeptide, encoded by a single gene such as the P-glycoprotein (16). The complex can be a dimer of a half-sized protein such as LmrA (20), or a tetramer composed of two types o subunits, a membrane domain and a NBD, as for DrrAB (22). More complicated situations exist with multiple genes for the various subunits such as the ribose transporter, RbsAC, which also works in conjunction with a periplasmic binding protein, RbsB, which sequesters ribose in the periplasm and docks with the transporter, delivering sugar for transport (24)

with a single membrane and nucleotide-binding domain, and the *drrA* and *drrB* genes encode the nucleotide-binding and membrane domains, respectively, as individual proteins. However, LmrA and other 'half ABC-transporters' are thought to function as homodimers (20) and Drr as a tetramer (26), with two DrrA and two DrrB subunits, suggesting that these transporters have an analogous topology to 'full-size ABC transporters'. For full-size ABC transporters, the two halves of the protein resemble one another, suggesting that they have arisen by gene duplication. Although MacB is a transport ATPase, it has a different structure to the aforementioned ABC transporters in that it is has three domains, a cytoplasmic NBD, a transmembrane domain that is predicted to have four α-helices, and a periplasmic domain. In *E. coli*, this inner membrane protein forms a tripartite pump in association with the MFP MacA and the OMP TolC. Even in Gram-positive bacteria, MacB is invariably associated with MacA, suggesting that the MFP may play a regulatory role in addition to its structural role in forming the tripartite complex in Gram-negative bacteria. Indeed, *Staphylococcus aureus* MacA has a large deletion that corresponds to the coiled-coil domain that would normally be involved in bridging the IMP and OMP.

6 Structural Analysis of ABC ATPases

The first structure of an ABC transporter of multidrugs was that for Sav1866 from *Staphylococcus aureus* (27, 28). The structure revealed a V-shaped homodimer in which each monomer contributes six α-helices: towards the middle of the membrane, bundles of these helices diverge into two discrete 'wings' that point away from one another towards the cell exterior, thus providing an outward-facing conformation; whilst the nucleotide binding domains, with bound nucleotide, are in close contact with one another in the cytoplasm. Rather than representing individual monomers, each 'wing' consists of helices 1-2 from one subunit and helices 3-6 from the other subunit. The helices are connected by long intracellular (ICLs) and short extracellular (ECLs) loops, with the ICLs extending the helical secondary structure beyond the lipid-bilayer, so that they protrude approximately 25 Å into the cytoplasm. Conversely, the structure of the *Archaeoglobus fulgidus* molybdate ABC transporter ($ModB_2C_2$), a tetramer composed of two 6-helix transmembrane subunits (ModB) and two nucleotide binding subunits (ModC), revealed that the wings point away from one another on the cytoplasmic, rather than extra-cellular, side of the membrane and that

the NBSs had relatively little contact (29). This structure suggests that the transporter adopts an inward-facing conformation and since this was determined in the absence of nucleotides, that nucleotide binding induces closure of the NBSs that would induce the transporter to adopt the outward-facing conformation (30). Indeed, it has been proposed that the binding of ATP induces dimerization of the NBDs, which can freely rotate about the membrane domains, burying the nucleotide at the interface of the NBDs; and that these rearrangements close the membrane domain around the substrate (31). There is evidence to support such a mechanism, such as the ATP-induced association of NBDs in the MalK ABC transporter (32, 33).

In the case of drug pumps such as P-gp and LmrA, there is evidence to suggest that the NBDs function by an alternating-site mechanism, in which binding and hydrolysis of ATP by one site prevents hydrolysis of ATP at the other site (34). Disruption of either of the NBD of an LmrA-LmrA fusion protein inhibited the ATPase activity and abolished transport (35). Additionally, there are high- and low-affinity drug-binding sites on the intracellular and extracellular sides of the membrane, respectively, which are also alternately exposed to facilitate drug translocation. One possibility is that the alternate exposure of the high- and low-affinity drug-binding sites is driven by ATP hydrolysis, but another is that binding of ATP is itself sufficient, and that ATP hydrolysis by the second site is required to reset the transporter in its original conformation (36). Possibly, the transport cycle is initiated by the binding of the substrate to the transporter, triggering conformational changes that modulate the interaction of the NBD with ATP. In the case of P-gp and LmrA, the results of vanadate-trapping experiments indicate that the ADP-Pi transition-state has reduced capacity for substrates, which might be attributable to occlusion of the binding-site. Presumably, in the presence of substrate, binding and hydrolysis of ATP occludes the substrate as the translocation gate moves from in front to behind the substrate. This would convert the intracellular high-affinity binding site to an extracellular low-affinity binding-site, facilitating release of substrate from the cell.

Binding of ATP, but not ADP, triggers dimerization of the ATPase subunits of MJ0796, suggesting that binding of ATP drives interaction of the two ATPase subunits. A comparison of the structures of the ADP-bound monomer with that of the ATP-bound dimer of E179Q MJ0796 indicates that reorientation of the α-helical subdomain is required to stabilize binding of ATP. Potentially, the transmembrane domains control the reorientation of the α-helical sub-domain, with substrate binding to the transmembrane domains inducing the ATPase subunits to adopt the ATP-binding state. One can imagine that as the two NBDs interact, there is a potential to bury the nucleotide-binding sites, leading to occlusion or trapping of ATP at one NBD and preventing ATP binding to the other nucleotide-free NBD, as implicit in the alternating site model. However, the structure of E171Q MJ0796 was determined with two bound ATP molecules, suggesting that both nucleotide-binding sites can simultaneously bind ATP (37). Perhaps the apparent use of alternate nucleotide-binding sites of ABC transporters is attributable to a requirement for substrate binding to the transmembrane domains to activate both NBDs? If so, the ATPase activities of the two NBDs could be used to drive different events. For example, ATP hydrolysis at one NBD could be used to open the substrate-binding site at the alternate face of the membrane, while ATP hydrolysis at the other site could be used to reset the transporter by driving the NBDs apart. There is evidence to suggest that NBD1 and NBD2 of the cystic fibrosis transmembrane conductance regulator (CFTR) stably bind and hydrolyze ATP, respectively (38). Perhaps one NBD controls dimerization and channel opening, while the other controls channel closure?

7 Metal Efflux Systems: P-Type ATPases, RND Transporters and the Arsab Pump

While this book is concerned mainly with drug resistance mechanisms, this chapter includes information about resistance to transition and heavy metals as well. On the one hand, the mechanisms by which cells evade the toxic effects of metals and metalloids such as copper, zinc, cobalt, platinum, lead, cadmium, silver, mercury, arsenic, and antimony provide excellent models for drug resistance systems. On the other hand, metals have traditionally been used, and are still used today, to treat infectious diseases and as anticancer drugs.

Members of the superfamily of P-type ATPases include pumps that extrude toxic metals from microorganisms, conferring resistance. P-type ATPases form an acylphosphate intermediate with a conserved aspartate residue during the catalytic cycle, hence the 'P' (39). Many P-type ATPases transport alkali (protons, sodium, potassium) and alkaline earth (magnesium, calcium) metal ions. A number of crystal structures of the SERCA1 sarcoplasmic reticulum calcium pump have been solved (40, 41). These represent nearly each step in the catalytic cycle, and are the best structural characterization of a transport protein. Ions such as Cu(I) and Zn(II) are required, but are toxic in excess. Ions such as Cd(II) and Pb(II) have no biological function, and are only toxic. Genes for metal resistance P-type ATPases are located both on plasmids and in chromosomes of bacteria, archaea, and eukaryotic microbes (42). In *E. coli*, for example, there are two chromosomally encoded P-type ATPases, CopA (43) and

ZntA (44), that catalyze efflux of and confer resistance to monovalent (Cu(I)/Ag(I)) and divalent (Zn(II)/Cd(II)/Pb(II)/Co(II)) soft metal cations, respectively (Fig. 3). These pumps have two regions characteristic of that branch of the family: one to six metal binding domains (MBDs) with Cys$(X)_2$Cys motifs, a CysProCys motif in the sixth transmembrane helix (TM6), and a conserved HisPro located 34–43 amino acids carboxyl-terminal to the CPC motif. Some pumps have a histidine-rich N-terminal MBD instead of the cysteine-rich motif, and some have CysProHis or CysProSer in TM6. While SERCA1 has ten TMs, the pumps for toxic metals have eight TMs (47–49). Six of the TMs form a common core in all P-type ATPases. These include the first six of SERCA1 and the last six of toxic metal pumps. Thus, while the two branches of this family are related, they have significant differences in primary structure. However, there are no crystal structures of the pumps for toxic metals, so we can only speculate how these compare with the structure of SERCA1.

Resistance-nodulation-cell division (RND) complexes, discussed above as drug resistances, can also confer metal ion resistance. The best characterized example is the CzcCBA complex of *Cupriavidus metallidurans* (formerly, *Ralstonia metallidurans*), which gives resistance to cadmium, zinc, and cobalt (50). CzcCBA is proposed to pump metals from the periplasm to the medium, and works in concert with P-type ATPases that pump the metals from the cytosol to the periplasm (50). In *E. coli*, the homologous system is encoded by the *cusCFBA* operon (46). CusF is proposed to be a periplasmic chaperone, ferrying copper from the inner membrane CopA copper pump to the tripartite CusCBA complex that extrudes the copper out of the periplasm (Fig. 3).

A novel resistance efflux pump is the ArsAB ATPase, which confers resistance to trivalent arsenic and antimony (45). This pump is similar to ABC ATPases in that it has a membrane subunit, ArsB, with 12 TMs and a catalytic subunit, ArsA, with two NBDs (Fig. 2). However, it is more closely related to ATPases such as ParA, NifH, and MinD (51).

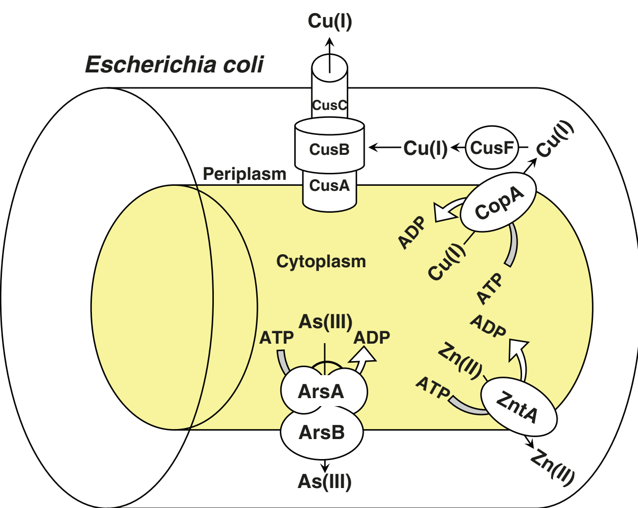

Fig. 3 Metal resistance ATPases of *E. coli*. *E. coli* has three major types of ATPases for extrusion of and resistance to toxic metals. As(III) and Sb(III) are metalloids, and arsenic detoxification systems have been found in the genomes of every organism sequenced to date. In *E. coli*, these trivalent metalloids are extruded from cells by a plasmid-encoded ArsAB As(III)-translocating ATPase (45). Although zinc ions are required for growth, too much zinc is toxic. In *E. coli*, excess Zn(II) is extruded by the chromosomally-encoded ZntA P-type ATPase (44). ZntA also pumps and confers resistance to Pb(II) and Cd(II). Similarly, copper is required by all cells, but is toxic in excess. The CopA Cu(I)-translocating P-type ATPase, encoded chomosomally in *E. coli*, pumps Cu(I) into the periplasm (43). CopA pumps Cu(I) into the periplasm. CusBAC is a tripartite copper exporter composed of an RND inner membrane protein, CusA, a membrane fusion protein, CusB, and an outer membrane protein, CusC (46). The 10-kDa CusF protein, also encoded by the *cusCFBA* operon, may serve as a periplasmic copper chaperone between CopA and CusBAC

8 Drug Resistance Can Result from Decreased Uptake

The site of action of most drugs is intracellular, and their effectiveness depends on concentration within the cell. Efflux systems confer resistance to drugs and metals because they reduce their intracellular concentration. Another way to produce drug resistance is to limit uptake. For example, D-cycloserine is transported into *E. coli* by the chromosomal *cycA* gene product, and resistance is exclusively due to mutations in the CycA transporter (52). The P-type ATPase LdMT, which is related to phospholipid translocases, is responsible for uptake of and sensitivity to the drug miltefosine in *Leishmania donovani* (53). Mutations in LdMT produce miltefosine resistance, and transfection with a wild-type gene restores sensitivity. The trivalent forms of the metalloids arsenic and antimony are taken into cells of prokaryotes and eukaryotes by aquaglyceroporin channels (AQPs), and disruption of the genes for AQPs results in resistance to trivalent metalloids (54, 55). Trivalent arsenic (Trisenox) is used clinically as a chemotherapeutic drug for the treatment of acute promyelocytic leukaemia, and transfection of cultured leukaemia cells with the human AQP9 gene makes those cells hypersensitive to the metalloid drug (56). Antimony (Pentostam) is used as a drug of choice for the treatment of leishmaniasis, and antimonial resistance is a serious clinical problem (57). In *Leishmania major*, uptake of the active trivalent form of the drug is via an aquaglyceroporin LmAQP1 (58). Downregulation of AQP1 expression results in Pentostam resistance (59).

9 Circumventing Drug Efflux

The development of clinically useful inhibitors that reduce the effectiveness of efflux proteins would represent a significant advance in our ability to treat drug-resistant diseases. Until recently, rational drug design was hindered by the paucity of structural information about drug transporters. Attempts at drug discovery focused primarily on screening of chemical libraries for inhibitors that prevent accumulation of radiolabeled drugs or ATP hydrolysis. Other inhibitors were identified from changes in fluorescence upon entering the cell. Inhibitors of microbial drug resistances could be identified from reduction in the minimal inhibitory concentration of specific antimicrobial pump substrates. Increasing uptake of a drug is another way to reverse resistance. For example, there are many ways in which *Leishmania* can become resistant to Pentostam, but of considerable relevance is the observation that expression of the LmAQP1 overcame resistance in every resistant strain examined, even field isolates from West Bengal, India (58). This illustrates an important point: much of the time, drug resistance can be overcome simply by flooding the cell with the drug. If pharmacological agents that increase expression of or activate drug uptake systems could be developed, they might be used in combination therapy with the drugs themselves.

10 Reversing P-Glycoprotein-Mediated Multidrug Resistance

P-gp-mediated multidrug resistance is a significant clinical problem, which has led to an intensive quest for reversal agents. Chemical modulators that inhibit the action of P-gp can be classified in three generations. First-generation inhibitors are pharmaceuticals that are already in use for other treatments, and include calcium channel blockers such as verapamil, immunosuppresants such as cyclosporine A, antihypertensives such as reserpine, quinidine, and yohimbine, and anti-estrogens such as tamoxifen and toremifena. However, the clinical efficacy of these compounds is limited by their toxicity (60). Second-generation P-gp modulators retain the reversal properties of first-generation modulators but are significantly less toxic. However, even though these second-generation modulators possess greatly reduced toxicity compared with their first-generation counterparts, they can produce deleterious effects in vivo (60). Second-generation inhibitors can alter the pharmacokinetic and distribution properties of co-administered anticancer drugs, which can result in toxic accumulation of anticancer drugs in blood plasma, or in organs such as the liver, kidney, or intestine (60). Several third-generation modulators that are highly potent, selective inhibitors of P-gp are currently under development (61). To date, co-administration of P-gp modulators and anticancer drugs has had disappointing limited clinical value (61).

11 Reversal of Bacterial Multidrug Resistance

Several potentially useful bacterial efflux protein inhibitors have been discovered. Quinolone derivatives inhibit bacterial antibiotic efflux systems, for example, the alkylaminoquinoline 7-nitro-8-methyl-4[2′-(piperidino)ethyl] aminoquinoline inhibits the AcrAB-TolC efflux complex (62), and alkoxyquinoline derivatives can restore drug susceptibility to clinically resistant strains of bacteria (62). Semi-synthetic analogues of tetracycline have been shown to inhibit tetracycline efflux in *E. coli* (63). A series of inhibitors, exemplified by L-Phe-L-Arg-β-naphthylamine (MC-207 110) and D-Orn-D-hPhe-3-aminoquinoline (MC-02 595), have been identified as inhibitors of RND efflux complexes in *P. aeruginosa*, *Enterobacteriacea*, *Haemophilus influenzae*, and *Stenotrophomonas maltophiliam*, potentiating the activity of fluoroquinolones such as levofloxacin (64, 65). Additionally, a synthetic inhibitor of P-gp inhibits NorA from *Staphylococcus aureus* (66). The first-generation P-gp inhibitor, verapamil, inhibits the lactococcal transporters LmrP and LmrA, and has also been shown to act synergistically with tobramycin against *Burkholderia cepacia* (67). The major catechin from green tea extracts, epigallocatechin-gallate, appears to inhibit the tetracycline transporter in clinical isolates of *Staphylococcus* (68). The development of similar clinically useful compounds could provide novel therapies in the treatment of bacterial infections.

12 The Future of Multidrug Resistance Inhibition

The availability of several efflux transporter structures offers the potential to develop novel rationally designed agents that can reverse resistance. However, their development remains in their infancy, and obstacles remain. First, the number of structures of efflux transporters remains in single figures. Second, the structure may not reflect a transport-competent conformation, but could be a dead-end conformation or even a crystallographic artifact. Third, a single crystal structure provides only a static representation of the transporter, equivalent to a single frame of a movie. Multiple structures are required for a complete understanding of

drug/transporter interactions and transporter conformational changes associated with drug translocation. Structures of transporters co-crystallized with various substrates in different conformations that represent different steps in the catalytic cycle will be required. A glimpse of how this might affect future development of efflux inhibitors arises from the co-crystallization of AcrB with several of its transported substrates (69). In this study, co-crystallization of AcrB with dequalinium, ciprofloxacin, ethidium bromide, and rhodamine 6G showed that they all bind the transporter in its central cavity, but each interacts with a different range of residues. This suggests that an effective broad-spectrum efflux inhibitor may have to disrupt drug/transporter interactions at a variety of points over a relatively large area. By implication, it also suggests that efflux inhibitors that prevent the transport of single drugs may be a more feasible option.

Efflux inhibitors do not necessarily have to target the actual transporter/drug complex. Inhibition of efflux can also be achieved by preventing assembly of drug transporter complexes, such as the tripartite systems typical of Gram-negative bacteria. Again, this sort of drug development requires detailed structural information on all of the components of the complex. An additional target is the transcriptional regulators that modulate expression of efflux systems. Transcriptional repressors/activators respond to the levels of extracellular drug to regulate expression of the components of the efflux systems (70). The BmrR multidrug regulator from *Bacillus subtilis* was co-crystallized with tetraphenyl phosphonium (71), and the QacR efflux pump regulator from *S. aureus* was co-crystallized with six structurally diverse substrates (72). These studies, along with the AcrB/substrate co-crystallization, are providing important information on the organization of the multidrug binding pocket. Additionally, detailed information on the drug/regulator interaction might provide potential targets for development of agents that can reverse resistance by attenuating transcription of the transporter genes. Other potential non-transporter targets exist. Recent reports suggest that the expression of certain bacterial multidrug transporters is controlled by two-component signal transduction systems (73, 74) that might represent novel targets for compounds that reduce expression of the resistance complexes.

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Chapter 11
The Functional Resistance of Bacterial Biofilms

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1 Pathogenic Bacterial Communities

There is intellectual coherence when a physician must tell patients that the bacteria causing their infection have tested resistant to the empiric antibiotic therapy, and that an alternative drug must be used. In this chapter, we will concern ourselves with the growing number of bacterial infections in which antibiograms of the causative organism show sensitivity to standard antibiotics in readily attainable concentrations, but the infection fails to be cleared. This discrepancy is troubling and frustrating for patients, physicians, and diagnostic laboratories alike, but it can now be resolved by concepts that have become widely accepted in microbial ecology.

Microscopic observations of natural ecosystems have shown that more than 99.9% of bacteria grow in slime-enclosed, surface-adherent biofilms, while only a minority exists as the free-floating planktonic cells we grow in laboratory broth cultures (1). Most cells within sessile biofilm communities show reduced metabolic rates and radically (more than 50%) different protein expression patterns compared to planktonic cells (2). One of the many consequences of these phenotypic alterations is their tolerance to almost all of the adverse factors (dehydration, antibiotic exposure, and the predation by amoebae) that readily kill their planktonic counterparts (1).

Using the same microscopic technologies, biofilm communities have been discovered in device-related and chronic infections. A pivotal report in 1982 documented large numbers of sessile, slime-embedded *S. aureus* on a pacemaker lead, which caused a systemic infection (Fig. 1a) (3). The biofilm had formed as a result of bacteremia secondary to an olecranon bursitis, and it drew considerable clinical attention because it resisted weeks of high-dose antibiotic therapy. Since then, biofilms have been revealed in an increasing variety of diseases (Table 1, Fig. 1a–f). As many as 60% of bacterial infections currently treated by physicians in the developed world are considered to be related to biofilm formation (4).

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Biofilm infections are especially frequent in the presence of foreign-body materials. Biofilms on intracorporeal devices mostly originate from perioperative contaminants; transcutaneous catheters become colonized by exponents of the skin flora within days after catheter insertion (5). A fragile balance between colonization and infection is often maintained for months. Host defenses control the shedding of planktonic bacteria and toxins and thereby prevent clinical symptoms, but they are unable to clear the biofilm. Episodes of acute inflammation, caused by the breakthrough of planktonic cells, can be successfully treated with antibiotics. Because short-term therapies usually fail to sterilize biofilms, however, flare-ups after treatment termination are frequent.

2 Stealthy Infections: Flying Below Our Radar

The diagnosis of biofilm infections is difficult. The biofilm mode of growth can delay overt symptoms for months or years. Diagnostic aspirates or swabs are often falsely negative, possibly because the microorganisms persistently adhere to a surface, but not in planktonic form. Individual biofilm fragments with hundreds of slime-enclosed cells may yield only a single colony when plated on agar, or may fail to grow at all because of the dormant state (as explained below) of the embedded bacteria. Consistently, the sonication of removed implants and PCR amplification techniques have shown increased sensitivity in the detection of bacteria sequestered in biofilms (6). Furthermore, many biofilm pathogens are skin organisms that may be dismissed as contaminants.

Culture-independent diagnostic techniques have revealed that several diseases associated with a presumably sterile inflammatory process are indeed bacterial infections that escape culture because of their biofilm mode of growth. For both culture-negative chronic otitis media with effusion (7, 8) and chronic prostatitis (9), a bacterial etiology has been evidenced by the detection of bacterial DNA and mRNA, as well as by electron and confocal scanning laser microscopy

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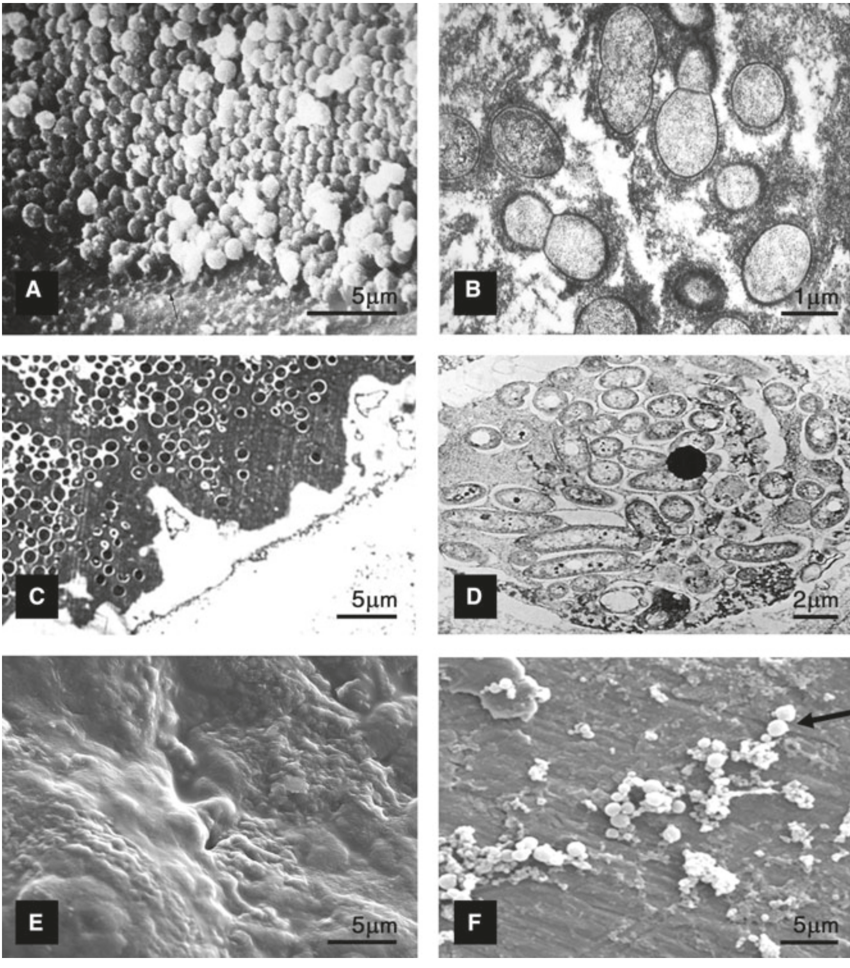


Fig. 1 Biofilms are increasingly recognized as a cause of chronic and device-related infections. Electron microscopy has documented surface-adherent bacteria embedded in extracellular slime on pacemaker leads (**a**), in endocarditis vegetations (**b**), on bone sequestrae in osteomyelitis (**c**), or chronic pneumonia in patients with cystic fibrosis (**d**). The microscopic detection of biofilms in culture-sterile samples of chronic otitis media with effusion (**e**) and aseptic prosthesis loosening (**f**) suggests an infectious etiology in these inflammatory states. The biofilm matrix in these images is reduced due to the dehydration process necessary for electron microscopy

Table 1 Partial list of human infections involving biofilms (adapted from (4))

Infection or disease	Common bacterial species involved
Dental caries	Acidogenic Gram-positive cocci (*Streptococcus sp.*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypeable *Haemophilus influenzae*
Chronic tonsillitis	Various species
Cystic fibrosis pneumonia	*Pseudomonas aeruginosa, Burkholderia cepacia*
Endocarditis	Viridans group streptococci, staphylococci
Necrotizing fasciitis	Group A streptococci
Musculoskeletal infections	Gram-positive cocci
Osteomyelitis	Various species
Biliary tract infection	Enteric bacteria
Infectious kidney stones	Gram-negative rods
Bacterial prostatitis	*Escherichia coli* and other Gram-negative bacteria
Infections associated with foreign body material	
Contact lens	*P. aeruginosa*, Gram-positive cocci
Sutures	Staphylococci
Ventilation-associated pneumonia	Gram-negative rods
Mechanical heart valves	Staphylococci
Vascular grafts	Gram-positive cocci
Arteriovenous shunts	Staphylococci
Endovascular catheter infections	Staphylococci
Peritoneal dialysis (CAPD) peritonitis	Various species
Urinary catheter infections	*E. coli*, Gram-negative rods
IUDs	*Actinomyces israelii* and others
Penile prostheses	Staphylococci
Orthopedic prosthesis	Staphylococci

(Fig. 1e). We are currently investigating acetabular cup prostheses that had been removed because of "aseptic loosening" (Maale, Costerton et al., unpublished data). Preoperative synovial fluid aspirations and conventional cultures of the explanted prostheses had all yielded negative results. Direct microscopy and fluorescent in situ hybridization (FISH), however, have revealed extensive *Staphylococcus epidermidis* biofilms in eight out of ten samples tested to date (Fig. 1f).

3 Biofilm Structure and Physiology

Biofilm formation is a sequential process of microbial attachment to a surface, cell proliferation, matrix production, and detachment (2). This process involves a coordinated series of molecular events, which are partially controlled by quorum sensing, an interbacterial communication mechanism dependent on population density (10). As schematized in Fig. 2, mature biofilms demonstrate a complex 3-dimensional structure containing functionally heterogeneous bacterial communities. Embedded bacteria occupy numerous microenvironments differing in respect of osmolarity, nutritional supply, and cell density. This heterogeneity produces a variety of phenotypes within one biofilm – a single specific "biofilm phenotype" does not exist.

Biofilm-imaging using microsensors, fluorescent probes, and reporter gene technologies have allowed the correlation of the spatial distribution of nutrients with metabolic activity (Fig. 3) (11, 12). Both oxygen and glucose were completely consumed in the surface layers of the biofilms, leading to anaerobic, nutrition-depleted niches in the depths (13). Areas of active protein synthesis were restricted to surface layers with sufficient oxygen and nutrient availability (12, 14).

4 Resisting Host Defense

The biofilm mode of growth provides a variety of defense strategies against the host immune system. Phagocytes have a reduced efficacy in ingesting sessile bacteria and biofilm clumps. Biofilm fragments of eight to ten cells survived pulmonary host defenses, even when deposited into the lungs of healthy animals (15). Furthermore, large amounts of extracellular polymeric slime are believed to hinder the penetration of leucocytes into biofilms (16).

Perhaps the most invidious characteristic of biofilm infections, however, is not their resistance, but the induction of deleterious immune responses. Biofilms stimulate the production of antibodies and cytokines (16). Ensuing immune-complex deposits and the oxidative burst of macrophages, however, cause greater collateral damage to the host than to the slime-embedded biofilm (17). The destruction of heart valves in bacterial endocarditis, the de-ossification adjacent to infected joint prostheses, and the progressive fibrosis in cystic fibrosis lungs provide proof of these deleterious effects. In cystic fibrosis, progression toward chronic pneumonia has been associated with an immunologic shift toward a Th2 response (18).

5 Why Antimicrobials Fail: Learning from Planktonic Cultures

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) assess the effect of antibiotics against planktonic organisms in the exponential phase of growth. The physiology of these cells resembles that of rapidly dividing planktonic bacteria in acute infections such as septicemia. It is therefore no surprise that antibiotic efficacy against acute infections in vivo can be predicted from MIC and MBC measurements in vitro. On the other hand, MBCs of the same bacteria grown as a biofilm may be three orders of magnitude higher (19–21). What makes strains that are susceptible in exponential planktonic cultures turn highly tolerant to the very same antibiotic when grown as a biofilm? Nutritional depletion? High bacterial density? Both hypotheses can be tested by comparing exponential planktonic cultures with stationary phase planktonic cultures, because the latter contain high concentrations of starved bacteria.

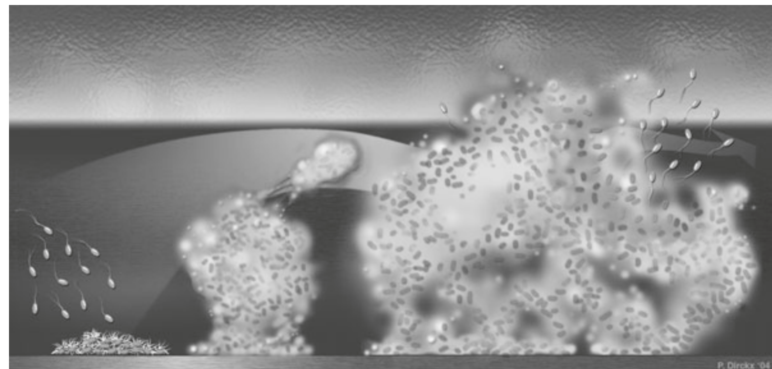
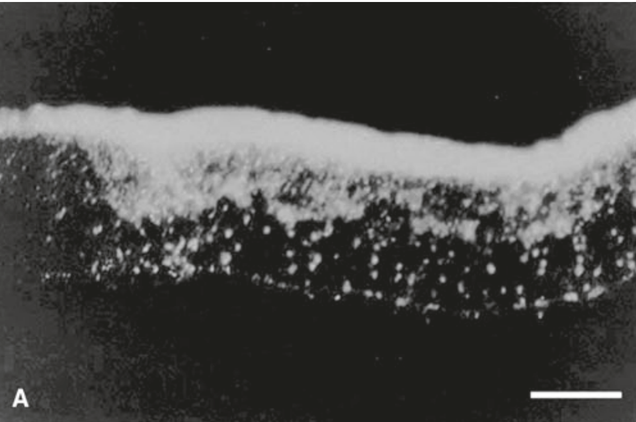
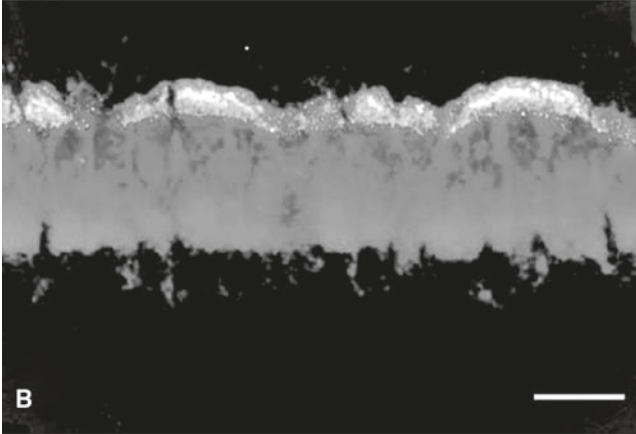


Fig. 2 The structural heterogeneity of biofilms is the product of continuous growth and detachment. This cartoon illustrates the various mechanisms involved in this process. P. Dirckx, Center for Biofilm Engineering





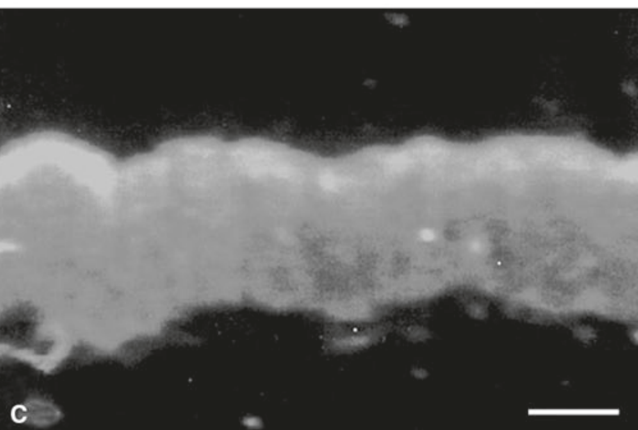


Fig. 3 Visualization of the spatial heterogeneity of respiratory activity, protein synthesis, and bacterial growth by epifluorescent microscopy. A *P. aeruginosa* biofilm was grown on a surface (*bottom*) covered by bulk fluid containing nutrients. (**a**) CTC-staining (*bright*) indicating respiratory activity. (**b**) Fluorescent staining of alkaline phosphatase (*white*) showing de novo protein synthesis under phosphate starvation; counterstaining of alkaline phosphatase-negative cells with propidium iodide (*grey*). (**c**) Biofilm section hybridized with a eubacterial oligonucleotide probe. The more intense staining near the bulk fluid suggests a higher rRNA content, and thus a more rapid growth rate than in the interior of the biofilm. Bar 50 µm. Adapted from (11) with permission of the publisher

Antimicrobials are more effective in killing rapidly growing cells rather than in stationary cells (22). While some antibiotic classes such as fluoroquinolones may kill non-growing cells, beta-lactams have an absolute requirement for cell growth in order to kill (23). Consistently, the rate of survivors in planktonic bacterial cultures challenged with antibiotics increased exponentially during logarithmic growth, to plateau in the stationary phase, with 100% survivors for a betalactam and 0.1–1% for quinolones (23, 24). Repeated re-inoculation of a culture to maintain it in the early exponential state eliminated any survivors, suggesting that antibiotic tolerance does not arise in the early logarithmic phase, but depends on the few cells remaining in the phenotype they expressed in the stationary phase (24). These survivors – alternatively known as "persisters" – were tolerant to immediate challenge with any of the antibiotic agents tested, but regained full antibiotic susceptibility after dilution in fresh medium (25). This observation suggests that persistence reflects an expressed phenotype rather than individual resistant clones, and that this phenotype can be overcome by nutritional stimulation and dilution. Interestingly, bacteria in high density (10^9–10^{11} CFU/mL as compared to 10^5 CFU/mL) remained tolerant to antibiotics despite transfer to fresh medium (23). Similar findings in rhizobium, where a greater percentage of cells survived in the stationary phase if cells were starved at high density (26), support the hypothesis that quorum sensing influences the proportion of survivors.

Based on elegant batch culture assays, Gilbert and coworkers directly related antibiotic tolerance to growth rate (25). They reduced bacterial growth rates by starvation, to the extent that bacteria were not susceptible to antimicrobials while still replicating fast enough to compensate for washout in a continuous culture system. Thus, bacteria did not need to be totally dormant in order to become persisters. Furthermore, they demonstrated that growth rates within a planktonic culture were strikingly heterogeneous. Mean doubling times of individual clones derived from late logarithmic culture varied between 500 and 45 min. For any time point between the lag phase and the stationary phase, a specific proportion of clones with maximum growth rates beneath the levels required for antibiotic susceptibility, i.e., survivors, could be determined. Any sample – irrespective of its proportion of replicating and dormant cells – repeated the general distribution pattern of active and inactive cells when diluted and re-grown in fresh medium. This again suggests that the distribution in active and susceptible versus inactive and tolerant cells is merely functional, and is an effect of alterations in growth medium and cell density.

The physiology of stationary phase planktonic bacteria is similar to biofilm-embedded cells. Both are affected by nutrient limitation and high cell densities. Both express similar degrees of antibiotic tolerance (Fig. 4) (21, 23). Like their planktonic counterparts, biofilm cells rapidly regained their

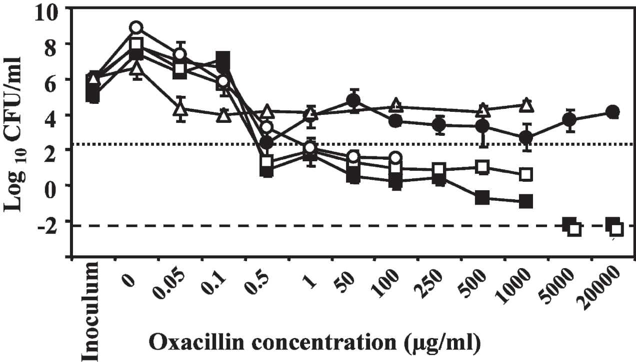


Fig. 4 Log reduction of viable cells in response to increasing oxacillin concentrations. The dotted line marks a 3-log reduction in CFU, and therefore indicates the minimal bactericidal concentration (MBC). Intact biofilm clumps tested in fresh medium (*filled circle*) and stationary phase planktonic cultures tested in spent medium (*open triangle*) were highly tolerant to antibiotics. Mechanically disrupted large clumps (o) regained their antibiotic susceptibility. Exponential phase planktonic cultures (*open square*) and stationary phase planktonic cultures in fresh medium (*filled square*) showed a conventional MBC of 0.5 µg/mL. The detection limit is represented by the *dashed line*. Error bars = 1 SD. Reprint from (21) with permission of the publisher

antibiotic susceptibility after mechanical disruption of the biofilm architecture and dilution in fresh medium (Fig. 4) (13, 21, 27). Disruption of the biofilm may provide cells, previously starving in deep layers, with new access to nutrients, which brings them back to the susceptible state of exponential growth. Alternatively, loss of tolerance may be explained by the dilution of protective cell signals – just as had been suggested for high-density planktonic cultures. The exponential increase in persister cells in planktonic cultures over time may mirror the increase in the number of dormant cells as we progress from the biofilm surface into its depths. Persisters in planktonic cultures may represent the viable but non-culturable bacteria found in many biofilm infections.

Furthermore, the patchy distribution of growth rates within any culture at any growth phase could explain why small pockets of surviving cells can be detected on the periphery of biofilms, where exposure to antibiotics and nutrients is unrestricted (25). As the availability of nutrients decreases into the depths of a thick biofilm, the density of bacteria growing at less than the critical growth rate necessary for antibiotic-mediated killing would increase.

How persisters survive. Persisters resist killing while remaining fully susceptible to growth inhibition (i.e., without changes in MIC) (24). Their phenotype is generally explained by reduced metabolic activity or even a dormant state. In addition, a variety of stress response systems are turned on once bacteria reach stationary growth phase, especially when stimulated by environmental stresses (such as alterations in nutritional quality, temperature, pH, or osmolarity) (28–30). Stress response genes protect bacteria from killing by antibiotics, the host immune system, and environmental toxins (29). Improved survival may be explained by an altered reaction to cell damage. For example, the SOS DNA-repair system, though not specifically reported in biofilms, is induced in ageing colonies on agar plates (31).

Stress response genes are regulated by a network of interacting signals, such as quorum-sensing, (p)ppGpp, or poly P kinase (PPK). In *E. coli*, expression of the *hipA* gene increased tolerance, probably by inducing (p)ppGpp synthesis, which potentiates the transition to a dormant state upon application of stress (32). Knock-out mutants for *hipA* contained 10–10,000 times more persisters during exponential growth than the wild-type (24). A *P. aeruginosa* PPK mutant showed inhibited quorum sensing, and failed to form thick, differentiated biofilms (33). Similar mutants of *E. coli* were unable to adapt to nutritional stringencies and environmental stress, which was attributed in part to their failure to express *rpoS* (34).

Sigma factors are key elements in general stress response. Bacteria lacking the sigma factor S had an increased susceptibility to oxidative stress during the stationary phase (30). *RpoS*, a sigma factor expressed in Gram-negative bacteria during the stationary phase, has been detected in *P. aeruginosa* biofilms in vitro (35) as well as in the sputa of CF patients (36). Whereas *rpoS* mutant *Escherichia coli* were dramatically impaired in biofilm growth (37), *rpoS* mutant *P. aeruginosa* grew thicker biofilms and showed higher antimicrobial tolerance (38, 39). Therefore, the role of *rpoS* in biofilm formation remains unclear, but may depend on strain-specific cofactors and specific growth conditions.

6 Biofilm-Specific Resistance

Metabolic dormancy and general stress responses are of crucial importance for phenotypic antimicrobial tolerance, both in planktonic and biofilm-grown bacteria. In addition, several biofilm-specific mechanisms of tolerance have been evaluated. They range from preventing antibiotics from reaching their site of action to reducing the susceptibility of embedded bacteria as a result of their biofilm mode of growth.

Impenetrable biofilms. The diffusion of antibiotics through biofilms has been assessed by concentration measurements and the visualization of bactericidal effects in the depths of in vitro biofilms (13, 19). While most studies have documented unimpaired antimicrobial penetration (19, 40), three exceptions have been noted: In a betalactamase-positive *Klebsiella pneumoniae* biofilm, betalactam antibiotics were deactivated in the surface layers more rapidly than they diffused (13). Second, biofilm penetration of positively charged aminogylcosides is retarded by binding to negatively charged matrices, such as the alginate in *P. aeruginosa* biofilms (14, 41). This retardation may allow more time for bacteria to implement adaptive stress responses. Third, extracellular slime derived from coagulase-negative

staphylococci reduced the effect of glycopeptide antibiotics, even in planktonic bacterial cultures (42, 43).

Once the antibiotic has successfully reached the bacterium, it may be inhibited from penetrating or may be shifted back out again by efflux pumps. A recent study identified a mutant of *P. aeruginosa* that formed biofilms in characteristic architecture, but did not develop tolerance to three different classes of antibiotics (44). As the mutant lacked periplasmic glucans, which were shown to bind tobramycin, tolerance was attributed to the sequestration of antimicrobial agents in the periplasm. Efflux pumps provide resistance to several antibiotic classes, including tetracyclines, macrolides, beta-lactams, and fluoroquinolones (45). Therefore, their upregulation seemed to be an attractive hypothesis to explain the class-independent tolerance of biofilms. However, current evidence cannot relate reduced biofilm susceptibility to an increased expression of these pumps. Temporal and spatial analyses in a developing *P. aeruginosa* biofilm revealed that the four multidrug efflux pumps decreased over time, with maximal expression occurring at the biofilm–substratum interface (46). Interestingly, quorum-sensing molecules are an alternative substrate for efflux pumps, and have been shown to accumulate when pumps are inactivated (47). In this context, a reduced pump activity within mature biofilms might contribute to biofilm tolerance through mechanisms related to cell density rather than to drug efflux per se.

Phase variation. While the transcription control of most bacterial genes permits a gradual response, phase variation constitutes an "all-or-none" mechanism. The high-frequency ON-OFF switching of phenotype expression is basically random, but modulated by environmental conditions (48). Phase variation has been discovered in a variety of bacterial species (48, 49). In *P. aeruginosa*, phenotypic variation to small colony variants occurred under the influence of antibiotics, both in vitro and in the lungs of patients with cystic fibrosis (48). Remarkably, small colony variants exhibited increased biofilm formation and antimicrobial tolerance. This first report certainly needs confirmation, but suggests therapeutic initiatives. The specific gene product that modulates the phenotypic "switch" from small colony variants back to the susceptible phenotype, for example, presents a promising target (48).

Quorum sensing. Many bacteria communicate via the production and sensing of autoinducer "pheromones" in order to control the expression of specific genes in response to population density. This so-called quorum-sensing (QS) coordinates gene expression within and among species (50). Given the tremendous changes associated with the switch from planktonic growth to growth within a mature biofilm community, it seems reasonable that cell–cell signaling regulates biofilm formation. As a matter of fact, planktonic *P. aeruginosa* depended on QS signals to form a differentiated, 3-dimensional biofilm structure under static conditions (10). Under flow conditions, however, biofilms of QS mutants and wild-type bacteria were exactly alike (51), indicating that, although important, QS is not indispensable. Many Gram-negative bacteria utilize *N*-acyl homoserine lactone (AHL)-dependent QS systems. These signals are involved in virulence gene expression and biofilm formation (10, 52). In vivo, AHLs have been detected in the urine of patients with catheter infections (53) and in the lungs of patients with cystic fibrosis, thereby coinciding with the development of respiratory biofilms (54). Two recent studies used microarray analysis to identify QS controlled genes in *P. aeruginosa* (55, 56). The QS regulated genes represented 6% (55) and more than 10% (56) of the genome, respectively.

The seaweed *Delisea pulchra* utilizes halogenated furanones to discourage bacterial colonization by blocking bacterial cell–cell communication (57). In vitro, similar compounds affected the architecture and enhanced the detachment of a *P. aeruginosa* biofilm (58), but also inhibited growth, motility, and biofilm formation of *Bacillus subtilis* (59). Possible strategies to influence QS were extensively reviewed by Camara and coworkers (50). Although promising, manipulation of QS is still a long way from clinical practice.

The "biofilm gene". Several studies have documented antimicrobial tolerance in biofilms too thin to pose a barrier to the diffusion of metabolic substrates (60, 61), thus arguing against starvation-induced dormancy as the only reason for antimicrobial tolerance of biofilms. This observation led to the hypothesis of a genetically controlled, biofilm-specific phenotype. Expression of a "biofilm gene" would lead to the cooperative development of a characteristic architecture, and to the expression of specific antimicrobial tolerance. This concept is of particular interest, as the control of key biofilm genes would offer excellent options to overcome tolerance. Biofilm-specific epitopes could further be used for diagnostic tests and vaccinations.

When assessed by DNA microarrays, gene expression in biofilms differed from planktonic cultures by 6% in *B. subtilis* (as assessed after 24 h) and 1% in *P. aeruginosa* (assessed after five days of culture) (39, 62). In *B. subtilis*, the transition from a planktonic to a biofilm state involved several transcription factors (62). Most were maximally active after eight hours of culture, when only 7% of the bacteria grew as a biofilm. Their increased activity under anaerobiosis, starvation, and high cell density suggest that these growth conditions stimulate biofilm formation. On the other hand, biofilm formation was inhibited by high glucose concentrations through the accumulation of an inhibitory catabolite in a phenomenon known as catabolite repression (62).

Staphylococcal biofilm formation is mediated by the polysaccharide intercellular adhesin PIA, a product of the *ica*ADBC gene cluster (63, 64). Ziebuhr et al. detected the *ica* locus in 85% of coagulase-negative staphylococci causing invasive infections, but only 6% of contaminating strains, and proposed targeting the *ica*-locus as a diagnostic marker for pathogenicity in staphylococci (65). This power to discriminate between invasive and non-invasive coagulase-negative staphylococci, however, could not be

confirmed (66). Knobloch and coworkers reported that virtually all *S. aureus* strains contain the *ica* gene cluster, but do not necessarily produce biofilms, thus stressing the importance of the control of gene expression (67). In 44% of the tested strains, biofilm formation was only seen in certain media. In addition, PIA synthesis was altered by subinhibitory antibiotic concentrations (68), phase variation (69), quorum sensing (70), or *icaR* (71), a transcriptional repressor of *ica* expression under environmental control (72). Despite the apparent relevance of the *ica* gene cluster and PIA for biofilm formation, no diagnostic or therapeutic targets have been found so far, the search being complicated by the vast number of co-variables.

The remainder of the differentially expressed genes and proteins identified so far in biofilms are involved in (mainly anaerobic) metabolism, the regulation of osmolarity, the production of extracellular polymeric slime, cell–cell signaling, and motility (2, 39, 73–75). Finelli et al. described five "indispensable" genes for *P. aeruginosa* biofilm formation (74). They include genes for aerobic and anaerobic metabolism, osmoregulation, a putative porin, and a gene thought to be involved in carbon metabolism, the production of virulence factors, and the response to environmental stresses. In *S. aureus* biofilms, five genes were identified as being upregulated compared to planktonic cultures, encoding enzymes needed for glycolysis, fermentation, and amino acid metabolism, as well as a general stress protein (73). Yet, none of these differentially expressed genes and proteins were irreplaceable in their function or reproducibly found among various species, and therefore do not promise diagnostic or therapeutic potential.

7 Trading Posts for Resistance Genes

Besides providing antimicrobial tolerance for embedded cells, biofilms promote the propagation of antibiotic resistance and virulence genes among the bacterial community by horizontal gene transfer. Competence factors and plasmids are key players not only in horizontal gene transfer, but also in biofilm formation. In *Streptococcus mutans*, a quorum-sensing system was found to propagate structural biofilm differentiation and genetic competence (76). Its activation altered biofilm architecture, and increased transformation frequencies in biofilm-grown bacteria by 10–600 times compared to planktonic cells.

The capacity of *E. coli* K12 to form biofilms dramatically improved upon the acquisition of a plasmid (77). The expression of conjugative pili thereby seemed to boost the formation of a 3-dimensional biofilm architecture. Biofilms, in their turn, provide a sufficient density of bacterial recipients to assure high transfer rates of plasmids (77). The high expression level of prophages found in Gram-negative (39) and Gram-positive biofilms (62) is another indicator of a very active transfer of mobile genetic elements within biofilms.

From an epidemiological point of view, horizontal gene transfer is especially important within polymicrobial biofilms formed by the oral and intestinal flora (78, 79). In that environment, resistance genes can be transferred from apathogenic to highly virulent strains, both within and beyond species borders (78, 80). Considering that, for example, only 5% of the oral flora are detected by routine culture techniques, this gene pool available for horizontal transfer may still be profoundly underestimated.

All in all, biofilms play a triple role in the spread of antibiotic resistance: First, the treatment of biofilm-related infections requires long-term (and often recurrent) antibiotic therapy, exposing colonizing bacteria to prolonged antibiotic selection pressure. Second, biofilm physiology enables embedded bacteria to survive antibiotic exposure long enough to acquire specific resistance to the drug. Finally, the high cell density and the accumulation of mobile genetic elements within biofilms provide an ideal stage for efficient horizontal gene transfer.

8 Treating Biofilm Infections

Current therapeutic strategies are based on two pillars: (19) high-dose, long-term antibiotic therapy and (13) the removal of infected foreign-body material and any necrotic tissue. In bacterial endocarditis, for example, antibiotic treatment was shown to be more successful when serum antibiotic levels were held at least tenfold above the MBC (81). But even with 8 weeks of parenteral antibiotic treatment, few patients with prosthetic heart valve endocarditis have been cured by antimicrobial therapy alone (82).

The sterilization of a biofilm infection is highly demanding, both for the patient and the treating physician. The patient may face recurrent surgery, prolonged hospitalization for intravenous therapy, adverse drug reactions to the antibiotic agent(s), infectious complications related to intravascular devices, the disturbance of the colonizing flora, and tremendous costs. From an epidemiological point of view, any prolonged exposure to antibiotics selects for resistant organisms within the bacterial flora, and represents another step toward the postantibiotic era. Considering these risks and the considerable failure rate of current strategies, it cannot be stressed enough that any therapy should be based on a thorough diagnostic workup and treatment plan. Advances in molecular biology make culture-independent diagnostic strategies (such as the detection of bacterial 16S ribosomal DNA by polymerase chain reaction, or the detection of specific organisms with FISH-probes) available for clinical practice. Selan and coworkers have recently developed a non-invasive test for endovascular staphylococcal biofilms

that detects IgM antibodies directed against an epitope that is exclusively expressed on staphylococci growing in a biofilm (83). However, all these new techniques cannot provide the antibiogram of infecting organisms – a major shortcoming for the treatment of a smoldering chronic infection, where treatment success or failure may not be evident for weeks.

Therapeutic approaches for specific biofilm infections have been reviewed elsewhere (84–86). They have lately been complemented by new experimental approaches, such as the exposure of biofilms to ultrasound, or to an electrical field to facilitate matrix penetration or disturb the integrity of bacterial membranes (87, 88). We will attempt to crystallize the discussion of the clinical management of biofilm infections by focusing on two classical biofilm diseases: hip prosthesis infection and central venous catheter infection.

When dealing with infected prostheses, acute exacerbations respond well to antibiotic therapy, but sterilization is difficult. Debridement without removal of the implant, combined with 4–6 weeks of intravenous antibiotic treatment and subsequent long-term oral therapy, has a failure rate between 32 and 86% (86). Successful prosthesis sterilization relies upon intact surrounding host tissue, vigorous debridement surgery, and antibiotics with sufficient efficacy against surface-adhering, metabolically inactive microorganisms. Such antibiotics include rifampicin combined with quinolones, fusidinic acid or cotrimoxazole for staphylococci, and quinolones for Gram-negative rods (89–92). For microorganisms like enterococci, quinolone-resistant *P. aeruginosa*, or any type of multi-resistant bacteria, there are no potent oral antimicrobial agents. These cases require the removal of any foreign body material for a definitive cure (86).

The sterilization of infected central venous catheters with systemic antibiotic therapy failed in 33.5% of 514 published cases (85). One reason for treatment failure is insufficient local antibiotic concentrations to sterilize biofilms. This obstacle can be overcome for endoluminal catheter infections by periodically filling the catheter with pharmacological concentrations of antibiotics (i.e., 1–5 mg/mL). This "antibiotic lock" – with and without systemic antibiotic therapy – has been successful in 82.6% of 167 selected episodes (85).

9 Conclusion

In the industrialized world, acute bacterial infections caused by rapidly proliferating planktonic cells (e.g., *Salmonella typhi*) have been gradually replaced by chronic infections due to environmental organisms (e.g., *Staphylococcus epidermidis*) growing in biofilms. Biofilm eradication requires the elimination of all bacteria, otherwise infection recurs and its chronicity established. Current antimicrobial therapies are not aimed at growth-restricted bacteria protected by a biofilm mode of growth. To clear the residual fraction of dormant cells, we need antibiotics reaching far beyond the MBC definition of killing (≥3log) and the design of what we could call "antipathogenic" drugs. The latter may interfere with bacterial signaling or the expression of specific effector genes in order to convert resistant and virulent phenotypes into susceptible commensal organisms. Modulation of the host response is another strategy to promote biofilm clearance. Reviewing the redundancy of strategies providing tolerance within biofilm communities, the discovery of a single ON/OFF-switch for biofilm formation seems unlikely. Rather, biofilm eradication may depend on combined treatments.

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Section C
Bacterial Drug Resistance – Mechanisms

Chapter 12
The Importance of β-Lactamases to the Development of New β-Lactams

Karen Bush

1 Introduction

β-Lactams are considered to be among the safest, most efficacious, and most widely prescribed antibiotics for the treatment of bacterial infections. Their therapeutic use began with the introduction of benzylpenicillin (penicillin G) during World War II (1, 2), and continues with the development of newer cephalosporins and carbapenems for antibiotic-resistant infections. These agents act by inhibiting bacterial cell wall synthesis, as a result of their strong covalent binding to essential penicillin binding proteins (PBPs) that catalyze the last steps of cell wall formation in both Gram-positive and Gram-negative bacteria (3, 4). However, resistance to these agents has been a major concern to all who use, or have used, β-lactams therapeutically.

Resistance mechanisms associated with β-lactams include modification or acquisition of a low-affinity bacterial target (i.e., a PBP); inactivation of the antibiotic by β-lactamases; and decreased concentration of the β-lactam at the site of the target, due to increased efflux or decreased entry of the drug (5–7). In Gram-positive bacteria, especially staphylococci, low-affinity PBPs now represent the most important β-lactam resistance mechanisms (8), in contrast to the selection of penicillin-resistant staphylococci due to increasing numbers of strains that began producing penicillinases soon after the therapeutic introduction of penicillin G (9, 10). In Gram-negative bacteria, the appearance of β-lactamases with increased catalytic efficiency for β-lactams of multiple classes has remained the major resistance mechanism (11). However, the combination of increased β-lactamase production with decreased β-lactam concentrations within the periplasm results in perhaps the most effective β-lactam resistance mechanism (12).

Because the most common β-lactam resistance mechanism overall is β-lactamase production, it is no coincidence that the emergence of new β-lactamases can be correlated with the introduction of new β-lactam molecules into clinical practice. In this chapter, the origin and hydrolytic action of β-lactamases will be described, together with the most common classification schemes. In addition, the identification of new enzymes will be shown to have a close relationship with recently developed antibacterial drugs and their increased use as therapeutic agents.

2 Hydrolytic Activity

All PBPs and β-lactamases interact with β-lactam antibiotics in reactions that result in the hydrolysis of the antibiotic to form an inactive chemical substance no longer possessing antibacterial activity. The reaction can proceed by at least two separate mechanisms, dependent upon the characteristics of the active site of individual enzymes. All known PBPs react with β-lactams via a conserved active-site serine (13). However, β-lactamases belong to families of enzymes that can utilize either an active-site serine or a metallo (zinc) ion to mediate hydrolysis (14).

PBPs and serine β-lactamases hydrolyze β-lactams by forming an acyl enzyme complex via the active-site serine residue (see Fig. 1). In this scheme, acylation and deacylation occur at different rates for the two sets of enzymes, with their classification as a PBP or β-lactamase based on the rates at which each step occurs. Thus, for PBPs, acylation may be rapid, but deacylation must be quite slow, to allow the enzyme to remain inactive during at least one cell division cycle (15, 16). For β-lactamases, both acylation and deacylation are generally rapid, with k_{cat} values approaching the limit for a diffusion-controlled reaction (17, 18).

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Fig. 1 Comparative acylation and deacylation rates for PBPs and β-lactamases

3 β-lactamase Origins

Much speculation abounds concerning the origin of β-lactamases. They have been reported to be a part of the bacterial armamentarium for centuries before the introduction of β-lactams into clinical practice (19), with claims of β-lactamase identification in bacterial samples analyzed from soil clinging to plants from the seventeenth century (20). Although most of the newer β-lactamases are plasmid-encoded, many bacteria have β-lactamase genes incorporated into their chromosomes, thus endowing them with a form of permanence as they are passed from one generation to the next. The appearance of β-lactamase genes on plasmids, in fact, appears to be a fairly recent occurrence; studies of culture collections from 1917 to 1954 showed that the same conjugative plasmids existed in the older strains, but β-lactam-inactivating activities were not associated with these plasmids (21, 22). Datta and Hughes concluded that plasmid-encoded resistance determinants were introduced by transposons that accumulated in previously existing plasmids.

If β-lactamases have been a part of the physiology of bacteria for thousands of years, the question remains as to their origin and their reason for existence. Serine β-lactamases most likely evolved from PBPs, as there are many notable similarities between the two sets of enzymes. Not only do they catalyze the same enzymatic reactions using conserved amino acids, but they have also been found to exhibit very similar three-dimensional structures (14). Even the metallo-β-lactamases appear to be folded in a spatial pattern that resembles PBPs and serine β-lactamases.

In the few organisms that do not produce traditional β-lactamases, notably *Streptococcus pneumoniae* (23) and *Helicobacter pylori* (24), resistant PBPs may play that role through a more rapid deacylation reaction than for other PBPs. This has been reported for *S. pneumoniae*, where resistant PBP2x variants demonstrate from 70- to 110-fold increases in deacylation rates, compared to the corresponding PBP from a susceptible strain (25, 26). In amoxicillin-resistant *H. pylori,* several surrogate β-lactam-hydrolyzing enzymes have been identified: a) a mutant form of PBP 1A (24) and b) HpcB, an unusual cysteine-rich protein that may play a role as a PBP from a new structural class (27).

Because β-lactams are prevalent in soil samples that contain β-lactam-producing actinomyces and bacteria (28, 29), it is an obvious suggestion that β-lactamases exist in bacteria to provide an ecological advantage to the β-lactamase-producing cells (30). A soil bacterium that can out-compete its bacterial neighbors by destroying potent β-lactams secreted into the soil would have a distinct evolutionary advantage (31). Notably, many of the first "penicillinases" that were described in the literature in the 1940s were from soil organisms, e.g., *Nocardia* spp., *Streptomyces* spp., and *Bacillus* spp. (32).

However, others argue that β-lactams in the soil would not diffuse far enough to be a threat to surrounding bacteria (33). To ensure survival, bacteria generally conserve resources for only the most critical functions. Thus, when bacteria produce large amounts of β-lactamase in preference to other proteins, there must be a reason other than protection against natural predators. Investigators such as A. Medeiros believe that β-lactamases instead have a major, but poorly understood, role in bacterial physiology (34), possibly by serving to regulate cell growth. Although this latter argument cannot be dismissed lightly, the proliferation of β-lactams in soil isolates suggests that a protection mechanism may have been an important selecting factor in bacterial physiology.

4 Classification Schemes

Classification schemes for β-lactamases have been described since 1970, when eight β-lactamases were separated into categories (35). For the most part, these schemes have focused on differences in enzymes that appear in Gram-negative bacteria, where increased numbers of both chromosomal- and plasmid-encoded enzymes contribute to resistance. There has been less interest in the β-lactamases in Gram-positive bacteria, primarily because the enzymes in Gram-positives that contribute to clinical resistance have been mainly the staphylococcal penicillinases, a rather homogenous set of enzymes that have also appeared

sporadically in enterococci (36, 37), and the β-lactamases in the Gram-positive bacilli that have been studied more as academic curiosities than as contributors to therapeutic failures (38, 39).

When the heterogeneity of β-lactamases was investigated, enzymes were differentiated on the basis of their functional characteristics. Some of the earliest attempts to classify these enzymes were described by Sawai et al. (40), who included the concept of "species specific" β-lactamases, and by Jack and Richmond (35), who evaluated functional characteristics such as hydrolysis profiles of penicillins and cephalosporins, and sensitivity to inhibitors. Others built upon this approach for β-lactamase classification, resulting in the widely accepted schemes of Richmond and Sykes (41) and, later, Bush et al. (42). At the time the first functional schemes were being proposed, no β-lactamases had been fully characterized with respect to amino acid sequence. By 1980, the sequences of four enzymes had been substantially determined after long and tedious processes of protein digestions and sequencing of many small peptide fragments (43). In 1978, the first β-lactamase sequence was reported as the result of nucleotide sequencing of a bla_{TEM} gene, a breakthrough for molecular biologists (44).

Technological advances associated with nucleotide sequencing marked a major change in the approach to the characterization of β-lactamases. Initially, new enzymes had been characterized on the basis of substrate profiles, inhibitor properties, and isoelectric points (41). Only the most important enzymes were analyzed to determine their amino acid sequences (43). However, once it became almost effortless to obtain a nucleotide sequence for a new β-lactamase gene, the in-depth enzymology of β-lactamases was relegated to only a few groups in the world. Thus, today, many β-lactamases have been characterized only on the basis of gene sequences, and frequently, but not always, on the basis of elevated MIC values for selected β-lactam antibiotics. More than 500 unique β-lactamase sequences have now been recorded in the literature, or in compilations of gene bank data (45), but only a small fraction of new β-lactamases are being characterized for their enzymatic properties.

Molecular classifications for Class A and Class B β-lactamases were initially proposed by Ambler, on the basis of the four amino acid sequences available in 1980 (43). There are now four major molecular classes of β-lactamases. Classes A, C, and D include β-lactamases with an active-site serine (46, 47), whereas class B β-lactamases include zinc at their active site. In Table 1, the most commonly used molecular and functional classification schemes are aligned. Although the functional classification schemes were first proposed

Table 1 Alignment of molecular and functional β-lactamase classification schemes (based on (19, 42, 43))

Active-site	Molecular class	Functional class	Typical enzymes	Enzyme characteristics: Typical substrates	Enzyme characteristics: Inhibitors[a]
Serine	A	2a	Staphylococcal penicillinases	Penicillins	CA, TZB
		2b	TEM-1, SHV-1	Penicillins, narrow-spectrum cephalosporins	CA, TZB
		2be	ESBLs (TEM, SHV, CTX-M families)	Penicillins, cephalosporins, monobactams (aztreonam)	CA, TZB
		2br	TEM-IRT enzymes, SHV-10	Penicillins, narrow-spectrum cephalosporins	TZB active Resistant to CA
		2c	PSE-1	Penicillins, including carbenicillin	CA
		2e	*Proteus* and *Bacterioides* cephalosporinases	Cephalosporins	CA
		2f	SME and KPC familes; IMI-1	Penicillins, cephalosporins, carbapenems	CA, TZB
Serine	C	1	AmpC, Chromosomal cephalosporinases	Cephalosporins	Aztreonam, cloxacillin
Serine	D	2d	OXA-1, OXA-10	Penicillins, including cloxacillin/oxacillin	(CA)[c]
		2de	OXA-ESBLs	Penicillins including cloxacillin/oxacillin; cephalosporins except cephamycins	(CA)[c]
		2df	OXA-24, OXA-40	Penicillins, including cloxacillin/oxacillin; carbapenems	CA
Zinc	B	3	L1, CcrA, VIM-and IMP families	Penicillins, cephalosporins, carbapenems, but not aztreonam	EDTA

[a] *CA* clavulanic acid; *TZB* tazobactam; *EDTA* ethylenediaminetetraacetic acid
[b] Extended-spectrum β-lactamase
[c] Dependent upon specific enzyme

in the absence of many sequences, the structure-function relationships predicted in 1988 appear to remain valid (31). Thus, the molecular class C enzymes with larger molecular sizes than the other serine β-lactamases (42), continue to be identified with elevated rates of cephalosporin hydrolysis. All zinc β-lactamases in class B exhibit the ability to hydrolyze carbapenems, but do not hydrolyze monobactams effectively (48). Although class A and class D β-lactamases encompass a broad heterogeneity in their functional properties, they can be readily broken into functional subgroups based on substrate and inhibitor profiles.

5 Historical Development of β-Lactam Antibiotics

Fleming's fortuitous discovery of the antibiotic activity of penicillin heralded a new therapeutic approach to the treatment of infectious disease. Although penicillin was not commercialized until after World War II, the knowledge that natural environmental microbes could produce antibacterial activities led many to examine additional sources for new structural classes of antibiotics. Academic investigators such as Waksman at Rutgers University (49), as well as most of the pharmaceutical companies (50), utilized vast resources to examine natural products for the production of novel compounds capable of killing bacteria. During this period of intense investigation beginning in the 1940s and continuing for at least 50 years, β-lactam structures were among the most prevalent compound class in any antibiotic screening program (K. Bush, personal communication). Most of these programs relied on extracts of soil samples to provide their new antibiotics, and pharmaceutical microbiologists devised clever screening techniques to identify new compounds from these extracts for antibiotic production (51). In these studies, a variety of soil-dwelling microorganisms, including fungi, actinomyces, and bacteria, were shown to produce new β-lactams. In fact, it was possible to identify specific microenvironments that could serve as rich sources of these new molecules, such as leaf litter in New Jersey that provided multiple bacterial sources of monobactams (51).

As a result of the natural occurrence of β-lactams, these natural products in the environment had already served as a natural selection for various families of β-lactamases. It is easy to envision how penicillins in common molds selected for the common penicillinases in the Gram-positive bacilli and cocci (9). Cephalosporins produced by *Cephalosporium* spp. (52) served to apply pressure on the soil-dwelling pseudomonads to maintain their chromosomal AmpC cephalosporinases. Carbapenems and olivanic acids produced by the streptomyces (53) encouraged the production of metallo-β-lactamases by organisms such as bacilli and anaerobes.

However, as shown in Fig. 2, the major driving force for the plethora of β-lactamases described in recent literature has been the introduction and widespread clinical use of β-lactams, both natural and synthetic (or semi-synthetic). In the 1940s, following the introduction of penicillin, the production of penicillinases in staphylococci increased from less than 10% to almost 60% in one British hospital over a 5-year period (9, 10). What makes this even more interesting

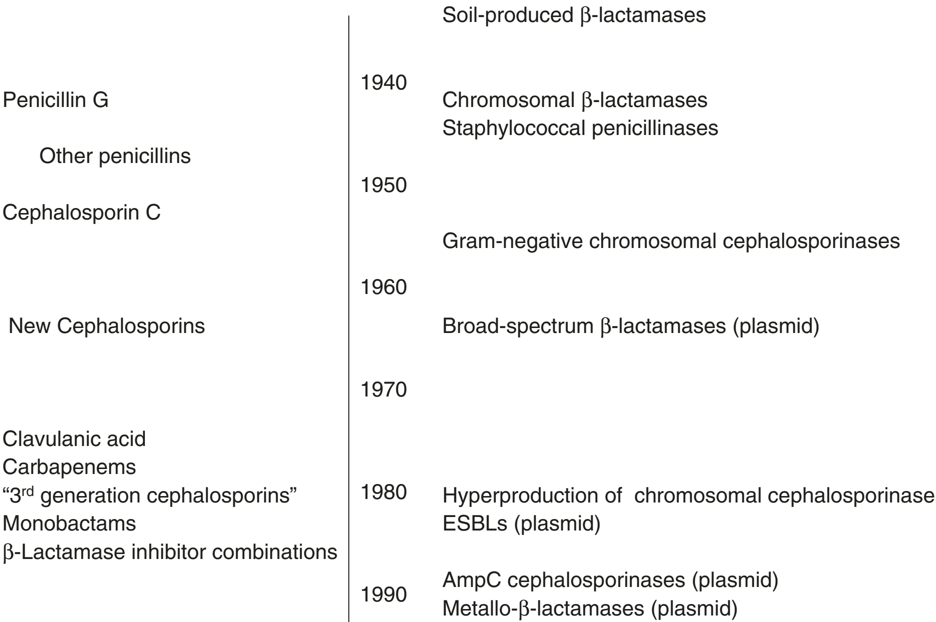

Fig. 2 Correlation of β-lactamase identification with the therapeutic introduction of new β-lactam antibiotics

is that this was the result of collateral damage, as penicillin was used extensively to treat streptococcal infections.

After cephalosporin C was identified as a modifiable chemical entity in the mid-1950s (52), the introduction of penicillinase-stable cephalosporins led to the emergence of Gram-negative bacteria that produced species-specific cephalosporinases capable of hydrolyzing these new molecules. The result was the continued introduction of even more cephalosporins, with chemical substitutions designed to render them stable to β-lactamase hydrolysis. The result was the identification of broad-spectrum β-lactamases such as TEM-1, which appeared in Greece in 1962 (54), in addition to organisms that produced high levels of chromosomal cephalosporinases (55).

The mid-1970s and early 1980s resulted in an explosion of new β-lactams from natural product sources, with the identification of the structurally unique clavulanic acid (56), the carbapenems (57), and monobactams (29), and the introduction of the synthetic β-lactamase inhibitors (58). These classes of β-lactams were developed into new antimicrobial agents that could circumvent the most common β-lactamases that were appearing among clinical isolates. Screening of new β-lactams, whether antimicrobial agents themselves or enzyme inhibitors, frequently included testing against an RTEM β-lactamase and an AmpC cephalosporinase – usually the *Enterobacter cloacae* P99 enzyme, because it was produced in high quantities and could be readily purified for enzymatic studies (56, 59). In addition, the K1 β-lactamase from *Klebsiella oxytoca* (or, as it was known in the 1970s, *Klebsiella pneumoniae*) was a part of many initial screening panels (56, 60), perhaps because this enzyme served as a precursor to the then-unknown ESBLs, with the capability of hydrolyzing some of the oxyimino-substituted cephalosporins and monobactams. With this screening panel, pharmaceutical investigators could discriminate among various cephalosporins and monobactams, on the basis of their potential lability to hydrolysis or vulnerability to inhibition.

By the mid-1980s, it appeared that organisms producing all known β-lactamases of clinical importance could be treated with one of these newer agents, or with a combination product that incorporated a β-lactamase inhibitor with a labile penicillin. However, insidious plasmids bearing resistance determinants for β-lactamases also became loaded with genes conveying resistance to a multiplicity of antibiotic classes. Thus, new β-lactamases did not need to be selected only by β-lactams if their genes were linked to resistance determinants for other drugs.

Following the introduction of the extended-spectrum cephalosporins and the monobactam aztreonam, the emerging resistance mechanism that was anticipated in the β-lactamase community was selection of β-lactamase hyperproduction in the Enterobacteriaceae (61). Class A plasmid-encoded TEM β-lactamases were appearing with strong promoters, leading to high enzyme levels that could not be inhibited by the inhibitor combinations (62). More important, it was predicted that high levels of AmpC cephalosporinases, coupled with porin mutations, would be the major factor leading to cephalosporin resistance (63). Many laboratories weighed the various contributions of β-lactamases induction, selection of derepressed mutants, and decreased permeability, as they affected susceptibility to the new β-lactams (12, 61, 64, 65). Although these Enterobacteriaceae began to be associated with clinical failures of agents such as cefoxitin or cefamandole, an unexpected resistance mechanism rapidly emerged in the late 1980s: the selection of mutant class A β-lactamases, with the ability to hydrolyze the previously stable extended-spectrum cephalosporins and monobactams (66, 67).

The Extended-Spectrum β-Lactamases, ESBLs, were first identified in Europe (66), followed by their appearance in the United States (68–70). These enzymes initially arose as a result of point mutations in the TEM and SHV broad-spectrum penicillinases, with no more than two mutations necessary to confer high-level resistance to cephalosporins such as cefotaxime, ceftazidime, and aztreonam. Some enzymes, such as TEM-3 and TEM-5, exhibited a preference for either cefotaxime or ceftazidime (67, 71), while others, such as TEM-26, were promiscuous and readily hydrolyzed both sets of substrates (72). Although substrate specificities vary among all these enzymes, virtually all class A ESBLs remain susceptible to inhibition by the β-lactamase inhibitors, clavulanic acid and tazobactam (42). In a number of ESBL epidemiological studies, the appearance of these enzymes was directly associated with the use of extended-spectrum cephalosporins, such as cefotaxime or ceftazidime (67, 70–72). As a result, the carbapenem class became an attractive alternative, especially in institutions with major ESBL problems (70, 73, 74).

Concurrently, with the proliferation of plasmid-encoded ESBLs, there appeared plasmid-borne AmpC-type cephalosporinases, such as MIR-1 (73) and ACT-1 (74), presumably selected by the same cephalosporins as the ESBLs. By the mid-1990s, plasmid-encoded class B metallo-β-lactamases (MBLs) began to appear outside Japan, where they had first been described in 1990 (75), probably selected by widespread use of carbapenems. Today, two major families of MBLs have emerged, with the IMP and VIM families appearing in abundance in diverse geographic areas such as southern Europe, South America, and Asia (76–78), but only rarely in North America (79).

As an alternative to synthesizing β-lactams that were stable to hydrolysis, combinations of penicillins were developed with β-lactamase inhibitors to treat infections caused by many class A (serine) β-lactamases, including ESBLs (33, 58). The first of these were clavulanic acid combinations, followed by sulbactam combinations in the early to mid-1980s. In a somewhat delayed response to these combinations, inhibitor-resistant class A β-lactamases were first reported in 1994 (80), when a set of TEM variants was described from clinical isolates that demonstrated unexpected resistance to

clavulanic acid, yet retained antimicrobial activity against common cephalosporins (81). These enzymes have not yet posed a major problem globally and remain generally confined to Europe, with infrequent reporting of their presence in North America (82, 83). It appears that the use of a β-lactam combination rather than a single agent has provided a greater hurdle for resistance selection.

6 Emergence of β-Lactamase Families

6.1 Gram-Positive Bacteria

In Gram-positive bacteria, β-lactamase-mediated resistance is important only among the staphylococci, where penicillinase production became the first emergent resistance mechanism (9). Decades after penicillin entered clinical practice, a second, even more far-reaching β-lactam resistance mechanism evolved, but only after the introduction of the cephalosporins. This latter resistance, due to the introduction of a new penicillin-binding protein, PBP 2a (or PBP 2), is most often combined with penicillinase production, with co-regulation of the two proteins in many strains, indicating that the β-lactamase is still an important commodity (84).

Eventually, β-lactamase production was reported in the enterococci (36), but these strains seem to have become less prevalent (85). Upon close examination, the enterococcal β-lactamase appeared to have been introduced intact from the staphylococci, and probably did not provide a major ecological advantage to the producing organism.

Among the Gram-positive cocci, multiple β-lactamases have been identified, with both zinc and serine β-lactamases appearing as chromosomal enzymes in a single strain. The most studied set of enzymes include the class A penicillinase and class D metallo-enzyme from *Bacillus cereus* (39), with counterparts to these enzymes appearing in *Bacillus anthracis* (86, 87). It is most interesting that these organisms are most frequently found as soil organisms, again supporting an association between soil-produced β-lactams and β-lactamases.

6.2 Gram-Negative Bacteria

In spite of the widespread use of penicillin as an agent to treat Gram-positive infections in the 1940s, the first literature citation referring to β-lactamase production was associated with a penicillinase from *E. coli* (88). As more β-lactamases were identified, investigators assumed that species-specific β-lactamases were the rule (32, 40), hence the naming of enzymes by simple names referring to their producing organism, e.g. K1 or KOXY from *Klebsiella oxytoca* (previously, *K. pneumoniae),* or AER from *Aeromonas* spp. (89). This hypothesis was supported by the identification of what appeared to be species-specific chromosomal cephalosporinases among the Enterobacteriaceae and *P. aeruginosa*. However, as increasing numbers of plasmid-encoded β-lactamases were identified among the Gram-negative spectrum, it became evident that multiple enzymes, both of chromosomal and plasmidic origins, could survive among these organisms. In some recent clinical isolates, as many as five different β-lactam-hydrolyzing enzymes were identified from multidrug-resistant Klebsiellae (74, 82), with multiple enzymes even encoded on the same plasmid (74, 90).

Although the species-specific concept was retained for chromosomally encoded β-lactamases for many years, even this idea was challenged with the identification of MIR-1, the plasmid-encoded cephalosporinase in *K. pneumoniae* that appeared to originate from an *Enterobacter cloacae* AmpC enzyme (73). To date, dozens of plasmid-encoded AmpC-related cephalosporinases have been identified (45, 90), with their sequences clustering in families. Plasmid-encoded AmpC families originating from chromosomal genes from *Citrobacter freundii*, *Enterobacter, M. morganii*, *H. alvei,* or *Aeromonas* have all been shown to share more than 90% homology among the individual members of their respective clusters (90).

For a number of years, the predominant families of ESBLs arose from the TEM and SHV β-lactamases, such that in mid-2006 there were more than 155 TEM variants and more than 90 SHV mutant enzymes (45). However, it was not long before the OXA family of enzymes began to emerge, derived from the third most common family of plasmid-encoded β-lactamases from epidemiological evaluations of Gram-negative bacteria in the late 1970s and early 1980s, prior to the introduction of the later generation of cephalosporins. In fact, seven variants within the OXA family had already been described by 1985 (91). By the early1990s, new families of ESBLs were identified (Table 2), with the most notable being the CTX-M family of enzymes. CTX-M β-lactamases have become the predominant ESBL in South America and many parts of Asia, and have invaded all continents, including Africa (19, 97). The origin of the enzyme is somewhat obscure, but it is thought to be derived from a β-lactamase produced by *Kluyvera* spp. (98).

Some of the more worrisome β-lactamases are the recently identified plasmid-encoded carbapenem-hydrolyzing enzymes in both the serine and the metallo-β-lactamase families. These enzymes not only inactivate carbapenems, but also hydrolyze all other β-lactams, with the exception of aztreonam. Although the serine carbapenemases, such as the KPC enzymes, are inhibited by the classical β-lactamase inhibitors in isolated enzyme assays, commercially available penicillin-inhibitor combinations are generally ineffective when tested in whole cells (82). Most disturbing are recent reports of the increasing numbers of plasmid-encoded

Table 2 Introduction and proliferation of major ESBL families

Enzyme family	First report of parent enzyme	Location	First ESBL report	Location	Number of enzymes in family in 2006	References
TEM	1962	Greece	1987	France	155[a]	(54, 67), Jacoby, personal communication
OXA	1967	Japan	1993	Turkey	97[b]	(92, 93), Jacoby, personal communication
SHV	1972	France	1983	Germany	91	(66, 94), Jacoby, personal communication
CTX-M	1988	Germany	1988	Germany	58	(95), Jacoby, personal communication
PER	1991	France	1991	France	3	(96)

[a] Not all enzymes in the family can be considered to be ESBLs. Included are inhibitor-resistant TEMs (IRTs) and complex-mutant TEMS with characteristics of both ESBLs and IRTs

[b] Not all enzymes in the family can be considered to be ESBLs. Included are oxacillinases with diverse hydrolytic properties, and carbapenemases

ultra-broad-spectrum enzymes, such as the KPC enzymes and the VIM or IMP metallo-β-lactamases, in localized geographical areas where colistin is now being used in empiric therapy for infections caused by panresistant Klebsiellae or *P. aeruginosa* (99, 100).

7 Future Directions

Many β-lactams have been developed for commercial use, based on their abilities to treat infections caused by the most important pathogens known at the time. However, bacteria have consistently demonstrated their survival tactics over time, and have successfully counteracted the multiplicity of attempts by pharmaceutical companies to decimate their populations. Although unremitting attempts have been made by synthetic chemists to circumvent β-lactamase resistance mechanisms, perhaps the most successful to date have been the β-lactamase inhibitor-penicillin combinations. Unfortunately, the current plethora of β-lactamases includes transferable enzymes resistant to all known inhibitors. Therefore, if β-lactams are to remain within our antibacterial armamentarium, it will be essential to devise new agents stable to all known β-lactamases, or to conceive and implement a new approach to β-lactamase inhibition. As has been demonstrated quite convincingly in the past, these measures will only buy us time before the next β-lactamase-related calamity emerges.

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Chapter 13
Penicillin-Binding Proteins and β-Lactam Resistance

André Zapun, Pauline Macheboeuf, and Thierry Vernet

1 What Are PBPs?

Penicillin-binding proteins (PBPs) are the targets of β-lactam antibiotics. These enzymes catalyze the last stages in the polymerization of peptidoglycan, the major constituent of the cell wall. The peptidoglycan, or murein, is a giant molecule, which forms a molecular mesh around the plasma membrane. Chains of tandemly repeated disaccharides form the glycan strands that are linked to each other by short peptide bridges. The discoveries of the PBPs and their cross-bridging mechanism were intimately intertwined. On the basis of studies of the effect of penicillin on peptidoglycan synthesis, it was concluded that cross-linking of the glycan chains resulted from a transpeptidation reaction, which is inhibited by β-lactams (1, 2). The first PBPs were isolated a few years later by covalent affinity chromatography on penicillin-substituted resin (3). Some of these PBPs were DD-carboxypeptidases or endopeptidases rather than transpeptidases. In the intervening three decades, intense research has been carried out on PBPs, particularly on their role in the resistance to β-lactams of some important pathogens such as *Staphylococcus aureus*, *Enterococci* and *Streptococcus pneumoniae*.

PBPs are characterized by the presence of a penicillin-binding domain, which harbors three specific motifs: SXXK, (S/Y)XN and (K/H)(S/T)G. This signature is common to the ASPRE protein family (for active-site serine penicillin-recognizing enzymes), which includes the class A and C β-lactamases. The topology of these β-lactamases is shared with the penicillin-binding domain of the PBPs (4, 5). The penicillin-binding domain is characterized by an active-site cleft between an α-helical sub-domain and an α/β-sub-domain, which consists of a 5-stranded β-sheet covered by a C-terminal α-helix. Following the topological nomenclature for β-lactamases (4, 6), the first motif SXXK is on the N-terminus of helix α2 of the helical sub-domain, on the bottom of the active-site groove, in the standard representation. The third KTG motif on strand β3 of the α/β sub-domain is located on the right side of the active site. Note that this strand is termed β3 as a result of the connectivity of the polypeptide chain, although it forms the margin of the 5-stranded β-sheet. The second SXN motif is on the left side of the active site, on a loop between helix 4 and 5 of the helical sub-domain (Fig. 1).

The serine of the SXXK motif is central to the catalytic mechanism, which is thought to occur in the following manner (Fig. 2). The Oγ of the serine carries out a nucleophilic attack on the carbonyl of the penultimate D-Ala amino acid of the stem peptide, which results in the removal of the last D-Ala amino acid and the formation of a covalent acyl–enzyme complex between the "donor" stem peptide and the protein. The carbonyl of the D-Ala amino acid, now forming an ester linkage with the active-site serine, then undergoes a nucleophilic attack from a primary amine linked in various ways to the third residue of a second "acceptor" stem peptide. This second reaction forms a peptide bond between the two stem peptides and regenerates a free active-site serine. What was just described is the catalysis of transpeptidation (Fig. 2a). In the case of DD-carboxypeptidases, the acyl–enzyme intermediate is hydrolyzed (Fig. 2b).

β-lactams resemble the D-Ala-D-Ala dipeptide in an elongated conformation (Fig. 3). More than the similarity of linked atoms, it is the distribution of three electrostatic negative wells that accounts for the resemblance. With PBPs, β-lactams act as suicide inhibitors. The active-site serine attacks the carbonyl of the β-lactam ring, resulting in the opening of the ring and formation of a covalent acyl–enzyme complex. This complex is hydrolyzed very slowly, thus effectively preventing the active-site serine from engaging in further productive reactions. β-lactamases differ in that they react with β-lactams rather than with D-Ala-D-Ala dipeptides, and that hydrolysis of the acyl–enzyme complex is extremely fast, thus releasing an active enzyme and an inactive compound.

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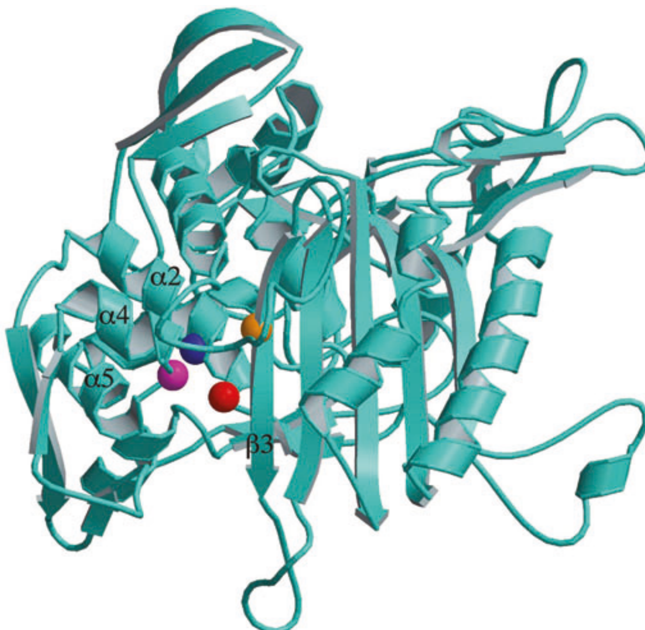


Fig. 1 Topology of the penicillin-binding domain. The example presented is the transpeptidase domain of *S. pneumoniae* PBP2x. The positions of the serine and lysine of the first SXXK motif are shown by red and blue spheres, respectively. The serine of the second SXN motif is indicated by a purple sphere. The lysine of the third KTG motif is shown in yellow. The elements of secondary structure, which bear the catalytic motifs, are indicated with the standard nomenclature (*See Color Plates*)

The reaction of PBPs and serine β-lactamases with β-lactams can be described kinetically as follows (Fig. 4). A noncovalent complex EI is formed between the enzyme E and the inhibitor I, with the dissociation constant K_d, from which acylation proceeds to form the covalent complex EI^* with the rate k_2. EI^* is finally hydrolyzed with the rate k_3 to regenerate the enzyme E and an inactivated product P. The rate described by k_3 is extremely rapid with β-lactamases, whereas it is negligible for PBPs on the time scale of a bacterial generation. The following nomenclature will be used throughout this review. The rate constants k_2 and k_3 describe the acylation and deacylation reactions respectively. The second order rate constant k_2/K_d will be referred to as the efficiency of acylation, which allows calculation of the overall acylation rate at a given concentration of antibiotic. Note that the inhibitory potency of a particular β-lactam for a PBP is given by c_{50}, which is the antibiotic concentration resulting in the inhibition of half the PBP molecules at steady state (i.e., when the acylation and deacylation reactions proceed at the same rate). The value of c_{50} is equal to the ratio $k_3/(k_2/K_d)$. In this review as in the literature in general, PBPs are referred to as being (or having) high or low "affinity" for β-lactams. This "affinity" implicitly refers to c_{50}, and should not be confused with the strength of a noncovalent interaction, which can be described by an association–dissociation equilibrium with a K_d constant, such as the formation of the preacylation complex.

Despite the availability of several crystal structures of PBPs and β-lactamases, and detailed kinetic studies, the enzymatic mechanism is still a matter of debate. Several mechanisms have been proposed that involve various residues of the conserved catalytic motifs and the carboxylate of the antibiotic. It is likely that the precise mechanisms differ between various ASPRE enzymes, and even for a single protein between different β-lactams (7).

2 Classification of PBPs

PBPs are commonly classified into three groups according to their molecular weight and domain structure: high molecular weight PBPs, which fall into two broad families called class A and B, and low molecular weight PBPs. Note that the nomenclature of the PBPs is particularly confusing as it is historically based on the observed electrophoretic pattern exhibited by proteins labeled with radioactive penicillin. Thus there is no necessarily functional or genetic relationship between homonymic PBPs of various organisms.

Class A PBPs comprise a single transmembrane segment, sometimes preceded by a short N-terminal cytoplasmic region, and two extracellular domains. The first extracellular domain carries the glycosyltransferase activity that is responsible for the polymerization of the glycan strands. The glycosyltransferase activity has been demonstrated for various purified recombinant class A PBPs including *Escherichia coli* PBP1b and PBP1a (8–12) and *S. pneumoniae* PBP1b and PBP 2a (13). The glycosyl transferase activity is inhibited by the glycopeptide antibiotic moenomycin (14), which is not used in therapy due to poor pharmacokinetic properties. As the focus of this review is on β-lactam resistance, the glycosyltransferase domain of the class A PBPs will not be discussed further. The C-terminal region of class A PBPs constitutes the penicillin-binding domain that catalyzes transpeptidation, thus bridging adjacent glycan strands. Demonstration of the transpeptidase activity in vitro with a purified recombinant protein has been achieved only recently for *E. coli* PBP1b and PBP1a (8, 11, 12).

Class B PBPs consist of a transmembrane anchor, a domain of unknown function, and a transpeptidase penicillin-binding domain. Although the transpeptidase activity of class B PBPs has never been demonstrated with recombinant proteins, studies of the peptidoglycan composition of *E. coli* cells following treatment with aztreonam, a β-lactam specific to PBP3, indicated that this class B PBP is indeed a transpeptidase (15). The transmembrane segment and non-penicillin-binding domain are certainly involved in proper cellular targeting through probable interactions with other proteins, as demonstrated in the case of *E. coli* PBP3 (16).

Low molecular weight PBPs constitute the third group. These consist mainly of a penicillin-binding domain with a

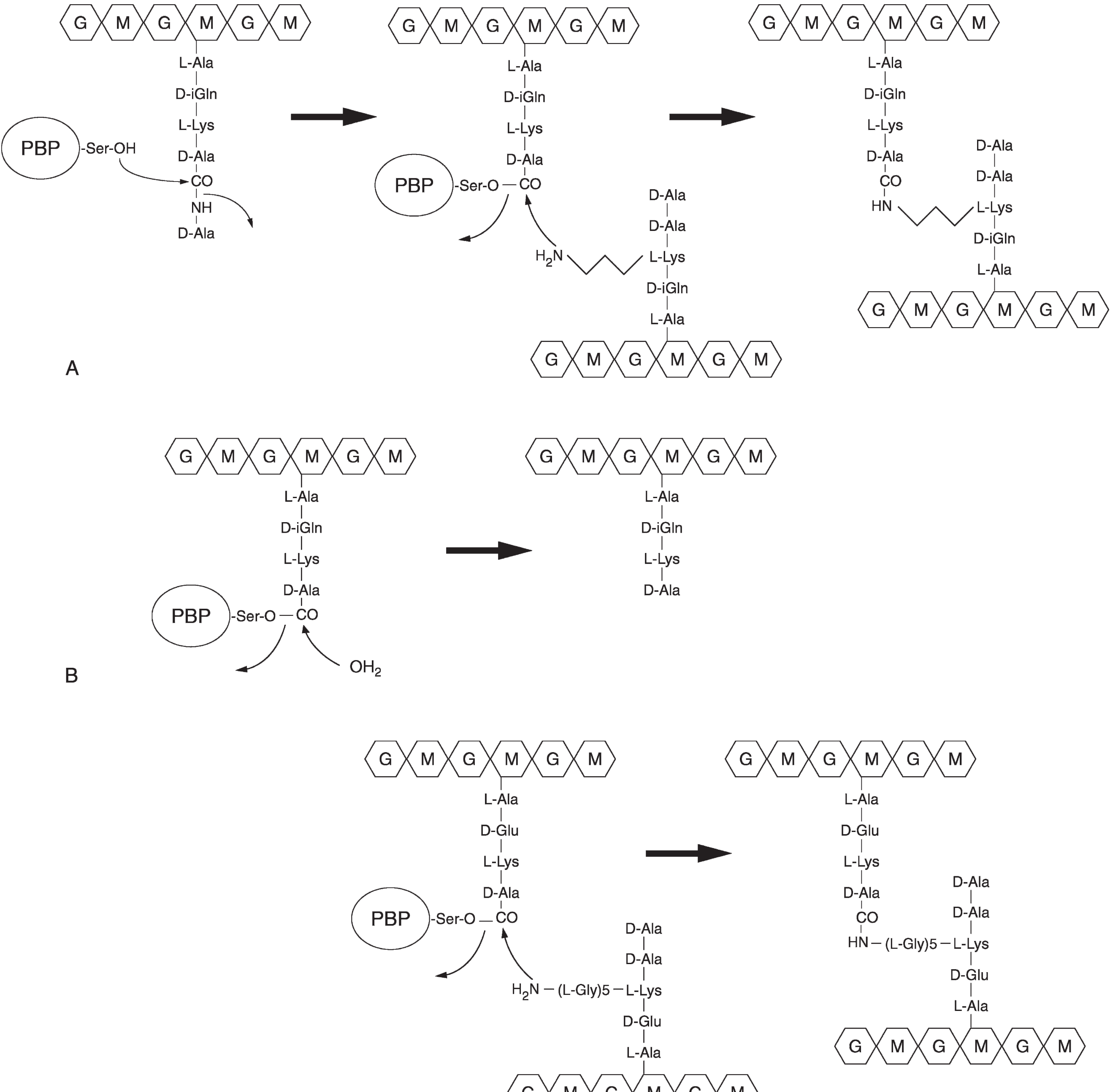


Fig. 2 (**a**) Catalysis of transpeptidation. Fragments of glycan strands are represented by chains of hexagons standing for the hexoses *N*-acetyl glucosamine (G) and *N*-acetyl muramic acid (M). The "donor" pentapeptide is depicted on the upper glycan strand, whereas the "acceptor" is attached here on the lower strand. The peptides shown are those from *Streptococcus pneumoniae*. The second and third amino acids may differ in various species. (**b**) Reaction catalyzed by DD-carboxypeptidase PBPs. With such proteins, the acyl–enzyme inter-mediate is hydrolyzed. (**c**) Transpeptidation reaction scheme in *Staphylococcus aureus*. Note that in many instances, including in *S. pneumoniae*, various intervening amino acids are attached to the third residue of the acceptor peptide, and provide the free amine that attacks the acyl–enzyme intermediate. Such stem peptides are called "branched"

small additional C-terminal domain, which is anchored to the plasma membrane either through a transmembrane segment or an amphipathic helix presumably lying on the lipid bilayer (17). Low molecular PBPs have either demonstrated DD-carboxypeptidase or DD-endopeptidase activities (18–21).

3 Physiological Function of PBPs

The cellular function of some PBPs has been inferred from various lines of evidence, but our knowledge remains sketchy. One type of data is the phenotype of mutant strains, or of cells treated with β-lactams that are specific to particular

Fig. 3 Structural similarity between β-lactams and the natural substrate of the PBPs. (**a**) *N*-acyl-D-alanyl-D-alanine peptide. (**b**) Penicillin backbone. (**c**) Cephalosporin backbone. The regions of negative electrostatic potential are indicated by arcs

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P$$

$$K_d = k_{-1}/k_1$$

Fig. 4 Kinetic scheme of the reaction between a PBP (E) and a β-lactam (I). EI is a preacylation non-covalent complex. EI* is the covalent acyl–enzyme complex. P is the open inactivated product

PBPs. The second type of result is the cellular localization of various PBPs, determined by immunofluorescence or fusion with the green fluorescent protein. Thus, various class A and B PBPs are involved in peptidoglycan synthesis during cell enlargement, cell division, or sporulation. In *E. coli*, for example, the class B PBP3 plays a role in division (22), whereas the class B PBP2 is involved in cell elongation and the onset of the division (23–25). Nothing is known of the specific role of the *E. coli* class A PBPs, as they show some degree of functional redundancy. In *B. subtilis*, the class A PBP1 and PBP4a appear to participate in cell division and elongation, respectively (26, 27), while the class B PBP2b is specific to the division (28) and the class B PBP5, PBP3, and PBP2a take part in the enlargement process (27, 29). In *S. pneumoniae*, and similarly shaped streptococci, enterococci, and lactococci, there certainly are two mechanisms of peptidoglycan synthesis (30, 31), with class B PBP2x and PBP2b participating in septal and peripheral cell wall synthesis, respectively. However, the peripheral localization reported is incorrect (32), and all the high molecular weight PBPs are present at mid cell. The function of the class A PBPs remains therefore unknown in *S. pneumoniae*. *S. aureus* is a spherically shaped coccus, whose division appears to produce entirely the new hemisphere of the daughter cell, in a process that involves its single class A PBP2 (33). The relative role of the two class B PBP1 and PBP3 in *S. aureus* is not clear.

In summary, peptidoglycan synthesis occurs in different phases, sometimes at different locations, depending on the morphology of the organism considered, with different participating PBPs. The class B PBP strictly involved in cell division can be generally identified by sequence comparisons with well-characterized examples and by the localization of its gene in a cluster coding for division proteins (34). The specific cellular function of the other class B and class A PBPs is more difficult to determine without dedicated genetic and localization studies.

4 PBP-Based β-Lactam Resistance

Inhibition of PBPs produces an imbalance in cell wall metabolism resulting in lysis or growth inhibition. The link between PBP inhibition and the biological outcome, lysis or growth arrest, remains poorly understood (e.g., *Escherichia coli* (35), *Staphylococcus aureus* (36), *Enterococcus hirae* (37)). Despite our ignorance of the detailed physiological consequences of β-lactam treatment, various means of resistance have been uncovered and investigated. Resistance to β-lactams arose from decreased permeability of the outer membrane, export of the antibiotics by efflux pumps (these two mechanisms are restricted to Gram-negative bacteria), degradation of the antibiotic by β-lactamases or utilization of PBPs with low affinity for the β-lactams. The following sections will be devoted to the PBPs of organisms that exploit this latter strategy.

4.1 *Staphylococcus aureus*

After the spread of *S. aureus* strains that were resistant to penicillin through the acquisition of a β-lactamase, the semisynthetic β-lactam methicillin was introduced, which was not degraded by β-lactamases. A methicillin-resistant clinical strain was isolated soon afterwards (38). The so-called MRSA (*m*ethicillin *r*esistant *S. aureus*) strains are particularly dangerous in that they exhibit blanket resistance to virtually all β-lactams, often associated with resistance to other classes of antibiotics. MRSA strains were initially found in hospitals causing difficulty in treating nosocomial infections which are increasingly found in the community. The true

origin of community-acquired MRSA strains is debated (39). Vancomycin, a glycopeptide antibiotic, has long been used as a last resort weapon to fight MRSA strains. However, strains exhibiting both high methicillin and vancomycin resistance have appeared recently (40).

The wide spectrum β-lactam resistance of MRSA strains results from the expression, in addition to the four native PBPs, of a fifth PBP termed PBP2a or PBP2' with low affinity for the antibiotics (41, 42). PBP2a is the product of the *mecA* gene whose transcription is controlled by the *mecI* and *mecR1* regulatory elements. MecI is a DNA-binding protein that represses *mecA* transcription (43). By analogy with the homologous BlaI and BlaR1 systems that control the expression of the β-lactamase BlaZ, the Mec system is thought to function in the following manner (44). MecR1 is a signal-transduction protein with an extracellular penicillin-binding domain that senses the presence of β-lactams in the medium, and activates its cytoplasmic domain. The intracellular domain of MecR1 is a protease that undergoes activation through autocatalytic cleavage, which results directly or indirectly in the cleavage of the MecI repressor. The *mecA* gene and its regulatory system are found on a large mobile genetic element called the staphylococcal cassette chromosome *mec* that integrates at a unique site in the chromosome (45). Several variants of the cassette have been found that include in addition to the *mec* genes, several genes encoding resistance to other types of antibacterial agents. A thorough presentation of the current understanding of these genetic elements and their history can be found elsewhere (46, 47).

Interestingly, the intact *mec* system does not confer resistance, as the expression of PBP2a is normally well repressed. Only few β-lactams, not including methicillin, can alleviate this repression. Mutations, for example in *mecI* or in the *mecA* operator region, lead to derepression of *mecA*. Even so, strains with unrestricted expression of PBP2a exhibit methicillin resistance only in a small sub-population (at a frequency of 10^{-4}–10^{-6}), when maintained without β-lactam selective pressure. Following exposure to β-lactams, a homogenous resistant population is selected. When the antibiotic selective pressure is removed, heterogeneity is rapidly restored, with only a small sub-population retaining resistance. These observations indicate that the functioning of PBP2a in cell-wall synthesis bears a cost that is best avoided in the absence of β-lactams. The nature of the genetic determinants of homogenous methicillin resistance in wild strains remains mysterious.

PBP2a is of class B and therefore the glycosyltransferase activity that is also required for peptidoglycan synthesis. Although PBP2a supports all the transpeptidase activity when this activity is inhibited by β-lactams in the four native high molecular weight PBPs, the presence of the class A PBP2 with an active glycosyltransferase domain is nevertheless required (48, 49).

Other genes have been found to be necessary for the full expression of the resistance offered by PBP2a. Over 30 of these auxiliary genes, often termed *fem* (*f*actor *e*ssential for *m*ethicillin resistance, or *aux* for auxiliary) have been identified (50). Several *fem* genes are involved in cell-wall metabolism, other genes take part in regulatory or putative sensory functions. How they cooperate to allow the *mecA*-based resistance is a complex and unresolved issue. The *fem AB* operon, for example, adds the second to fifth glycine residues to the peptidoglycan precursor to form the pentaglycine branch that serves afterwards as the cross-bridge of staphylococcal peptidoglycan (51, 52) (Fig. 2c). A trivial conclusion is that PBP2a has a specific requirement for "acceptor" peptides with a pentaglycine branch. This expectation may be naïve, for PBP2a can confer resistance to *Enterococcus faecalis* and *faecium*, which lack *fem AB* and have the alternative peptidoglycan cross-bridges $(Ala)_2$ and D-Asx, respectively (53).

PBP2a belongs to a sub-group of class B PBPs characterized by the presence of an insertion of about 100 residues following the transmembrane anchor (Fig. 5). This group also includes chromosomally encoded PBP5 from *Enterococcus faecium*, *hirae,* and *faecalis*, and plasmid-encoded PBP3 conferring *Enterococcus hirae*, which are all low-affinity PBPs conferring β-lactam resistance (see below). There are other members of this sub-group of PBPs in *Bacillus subtillis* and related species, in *Listeria monocytogenes* and *L. innocua* and in *Clostridium acetobutylicum*, although these do not appear to confer reduced susceptibility to β-lactams. The origin of PBP2a remains mysterious. A close *mecA* homologue has been found both in susceptible and resistant *Staphylococcus sciuri* strains (54, 55). The *mec* system may thus have spread from a hitherto unidentified staphylococcal species, not only to *S. aureus*, but also to *S. epidermitis*, *S. haemolyticus*, *S. hominis,* and *S. simulans* (56).

The reaction of PBP2a with β-lactams is extremely slow. The acylation efficiency of PBP2a by penicillin G, characterized by the second order rate constant k_2/K_d of approximately $15\,M^{-1}s^{-1}$, is roughly 500-, 800-, 900- and 20-fold smaller than that of the native PBP1, PBP2, PBP3, and PBP4 from *S. aureus*, respectively (57–59). When compared to PBP2x from the susceptible *S. pneumoniae* strain R6, PBP2a is acylated three to four orders of magnitude more slowly (57, 60, 61). With such a poor acylation efficiency (57, 58), the acylation rate of PBP2a at therapeutic concentrations of β-lactams is negligible compared to the bacterial generation time ($t_{1/2}$ for acylation greater than 1 h with 10 µM of penicillin).

The low efficiency of acylation results both from a poor "true" affinity of PBP2a for the β-lactams, with dissociation constants (K_d) of the preacylation complex in the millimolar range, and extremely slow acylation rates (k_2) ranging from 0.2 to 0.001 s^{-1} (57, 62). Although published values differ for various β-lactams and means of measurement, the acylation rate k_2 of PBP2a by penicillin G, for example, is three orders

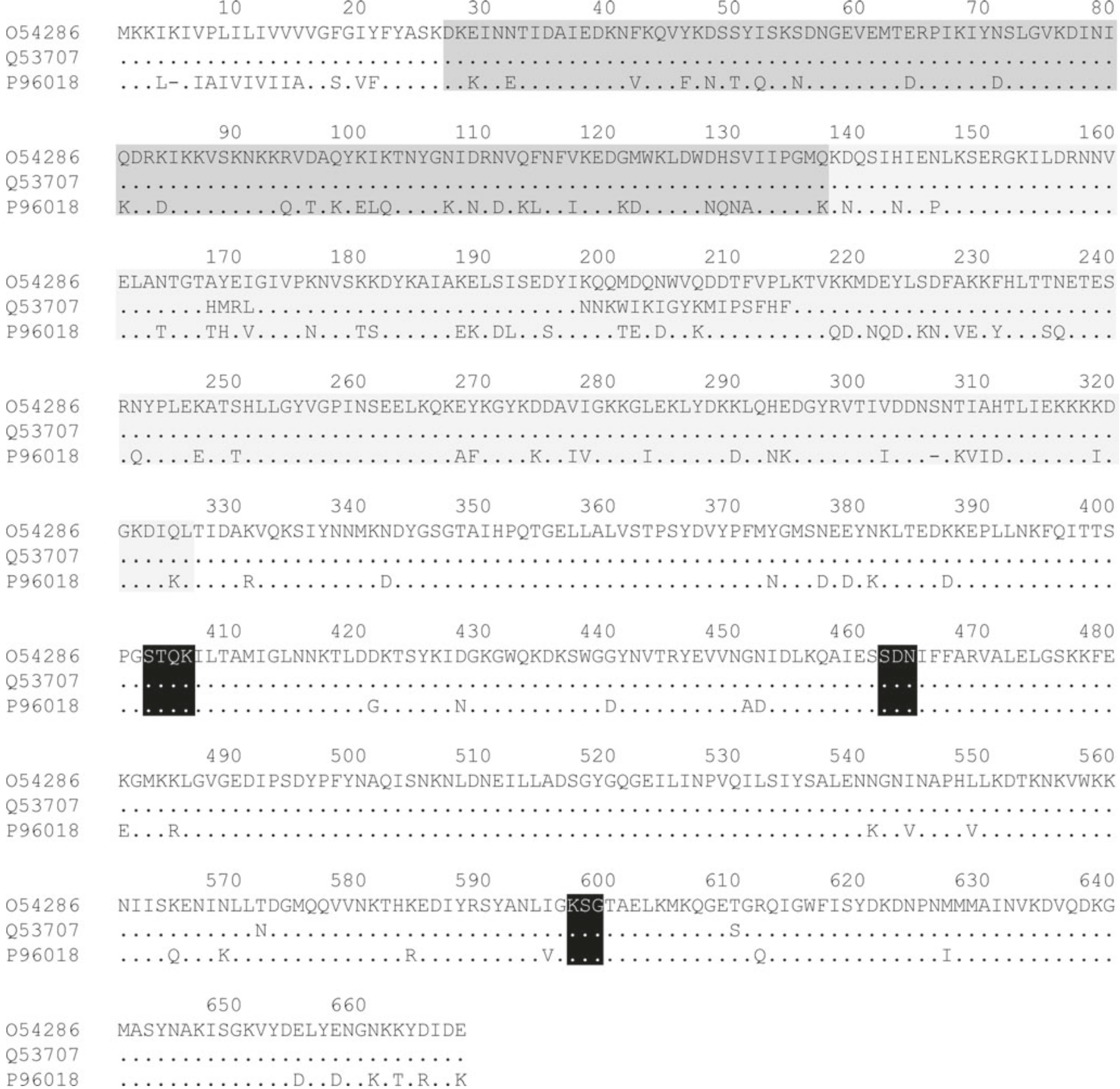


Fig. 5 Sequence alignment of staphylococcal PBP2a (designated by their Uniprot accession numbers). Three out of 16 available sequences are shown. There are three additional sequences that closely resemble # O54286, differing at one or six positions. One additional sequence is very similar to # Q53707, with one substitution and the insertion of two residues. Finally two other sequences resemble # P96018, differing each at 15 positions. The # P96018 sequence and close variants are from *S. sciuri*. The N-terminal domain is shaded. *Dark shading* indicates the extension specific to the sub-group of class B PBPs that includes PBP2a. The catalytic motifs are in *black boxes*

of magnitude slower than that of the susceptible PBP2x from *S. pneumoniae* (57, 60).

The structure of a soluble form of PBP2a without its transmembrane anchor has been solved to a resolution of 1.8 Å (63). The N-terminal non-penicillin-binding domain (residues 27–328) is bilobal, with the first lobe (27–138) formed by the sub-group specific extension. The transpeptidase domain shares its overall fold with other PBPs. The N-terminal domain confers a rather elongated shape to the whole molecule with the active site reaching approximately 100 Å from the membrane anchor.

In the absence of bound antibiotic, the active site of PBP2a appears to be rather closed with the active site S403 poorly positioned for a nucleophilic attack and a twisting of strand β3 that is required to accommodate the N-terminus of helix α2 and the active site S403. The structures of PBP2a with covalently bound nitrocefin, methicillin, and penicillin G revealed a tilt of the whole helical subdomain with respect to the

α/β-subdomain (2.3° with nitrocefin, O. Dideberg, personal communication). This rotation opens the active site and is accompanied by a substantial local rearrangement of the active site (Fig. 6). The Oγ of S403 is displaced by 1.8 Å (with nitrocefin), whereas the strand β3 is straightened. It has been argued that this conformational rearrangement is costly and impedes acylation. The 20-fold slower acylation by methicillin ($k_2 = 0.008\,s^{-1}$) compared to penicillin G ($k_2 = 0.2\,s^{-1}$), has likewise been rationalized on the basis that bound methicillin is translated along the active-site cleft relative to bound penicillin G. This relative displacement increases the distance between the putative proton donor (S462) of the second catalytic motif and the nitrogen group of the opening β-lactam ring. Although possible, these explanations rely on the assumption that the conformations of the acyl–enzyme intermediates are relevant to the transition states of the acylation reaction. However, it must be remembered that there is a complete absence of correlation between the efficiency of acylation (k_2/K_d) and the strength of the noncovalent interaction between the covalently bound antibiotic and the PBP, as demonstrated with *E. coli* PBP5 (64). Therefore, analysis of the complementarity of bound open antibiotics may bear little relevance to the understanding of the acylation process.

Mildly β-lactam resistant strains of *S. aureus* that lack both *mecA* and β-lactamases have also been isolated. There are good indications that the resistance of these strains is due to modified native PBPs. Alterations of penicillin binding by PBP1, PBP2 and elevated amount of PBP4 were observed in such strains (65, 66). The acylation rates of PBP1 and PBP2 were decreased, and the deacylation rates increased (59). The kinetic modifications result from point mutations, as demonstrated for PBP2 (67). A tenfold decrease of the acylation efficiency results from the double substitution S569A and A576S. Another variant with the A450D and A462V substitution surrounding the SXN motif and the Q629D mutation has a k_2/K_d lowered 20-fold (67). A laboratory mutant selected with ceftizoxime has the single substitution P458L close to the SXN catalytic motif (68).

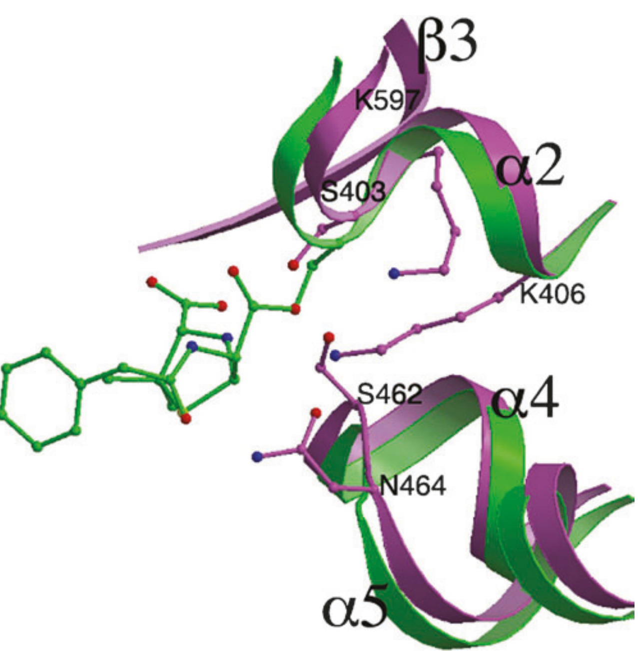


Fig. 6 Superposition of the active site of *S. aureus* PBP2a without (purple) and with (green) bound penicillin (shown in balls and sticks). The first motif on helix α2 and the second motif between α4 and α5 are moved away from strand β3, which bears the third catalytic motif (*See Color Plates*)

Thus, *Staphylococcus aureus* has been found to resist β-lactams in three ways, by using β-lactamases to degrade the antibiotic, by lowering the affinity of its endogenous PBPs for β-lactams, and most dangerously through the recruitment of an additional PBP that is unaffected by β-lactams.

4.2 Enterococci

The intrinsic resistance to β-lactams is a characteristic of enterococci. Isolates of *Enterococcus faecalis* typically exhibit MICs for penicillin of 2–8 mg/L (e.g., (69)), and *E. faecium* of 16–32 mg/L (e.g., (70)). These two species, which cause important human health problems, particularly nosocomial infections, have been the subject of intense molecular studies over the past two decades, together with *E. hirae*, which is more of a concern in veterinary medicine.

Enterococci morphologically resemble streptococci, which may be related to the fact that they share the same set of three class A and two class B high molecular weight PBPs (71). However, the intrinsic moderate resistance to β-lactams results from the presence of an additional sixth high molecular weight PBP, which takes over the transpeptidase function of the other PBPs when these are inhibited by the antibiotics (72, 73). This was concluded from three lines of evidence in early studies of an *E. hirae* strain and several derivatives (initially identified as *Streptococcus faecium* ATCC 9790). Firstly, it was found that one of the high molecular weight PBPs (PBP5) had a much lower affinity for penicillin, and spontaneous mutants with greater resistance had elevated amounts of this PBP (73). Secondly, a mutant hypersensitive to penicillin was found to lack PBP5 expression (72). Finally, saturation of PBP5 with β-lactams led to bacterial death (74).

Subsequent and parallel studies uncovered the same mechanism underlying intrinsic β-lactam resistance in *E. faecium* (75, 76) and *E. faecalis* (77). The wide range of elevated levels of resistance exhibited by clinical isolates of *E. faecium* was found to arise from two mechanisms: increased expression of PBP5 and mutations of PBP5 that further decrease its affinity for β-lactams (78, 79). Strains with intermediate level of resistance (MIC for ampicillin of 8 mg/mL) appear to rely mainly on the first mechanism, while extremely resistant strains (MIC for ampicillin of up to 512 mg/L) appear to combine both overexpression and reduced affinity (78, 79) or use

only the latter mechanism (80, 81). Note that the exclusive use of the PBP5 transpeptidase, when the others are inhibited by β-lactams, does not modify the composition of the peptidoglycan cross-bridges (75). A peculiar strain of *E. hirae* (S185) was found to express, in addition to its chromosomally encoded PBP5, a second PBP with low β-lactam affinity. This related but plasmid-encoded PBP is termed PBP3r (82, 83).

When the genes encoding PBP5 from various *E. faecium* clinical isolates were sequenced (Fig. 7, Table 1), several point mutations were found to be correlated with a low affinity for β-lactams and high resistance (80, 81, 84). However, as clinical isolates are not isogenic, assessment of the effect of various PBP5 sequences awaited their introduction in a single strain. When three PBP5 sequences originating from strains with MICs for ampicillin of 2, 24, and 20 mg/L were introduced in a strain with no PBP5 expression (MIC ampicillin of 0.03 mg/L), the resulting strains had MICs of 6, 12, and 512 mg/L, respectively (75). These results demonstrate that variants of PBP5 indeed confer different MICs, but that this effect is strongly modulated by other unknown factors (75, 85). The particular mutation M485A was hypothesized to have a very important effect as it was found in two highly resistant strains and is located close to the second catalytic motif SXN482 (80). When introduced individually, this mutation caused only a modest increase of resistance, when compared to the resistance of the clinical strains that harbor this substitution (75, 80). However, in an isogenic background, the M485A substitution accounted for most of the difference

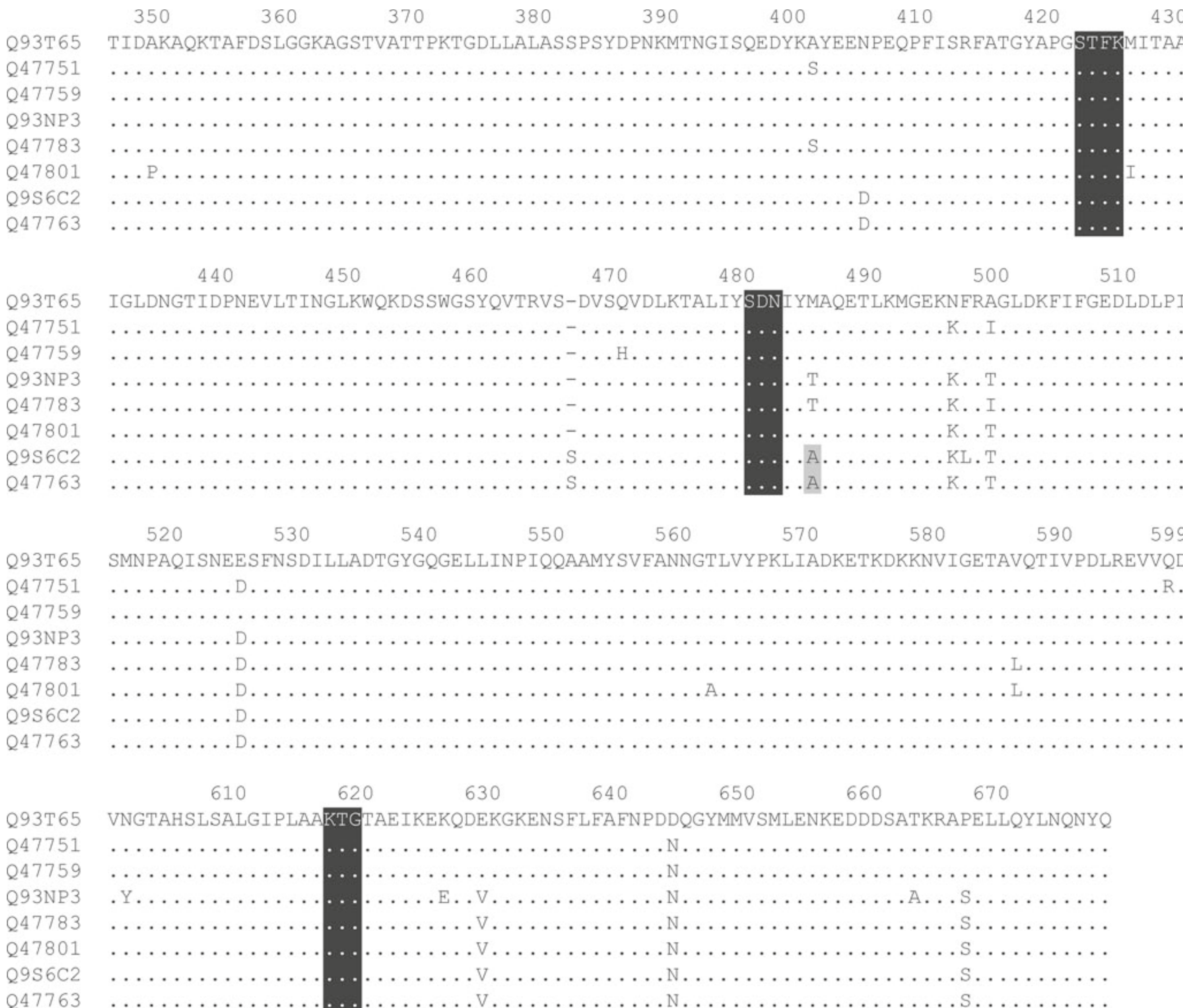


Fig. 7 Alignment of publicly available sequences of *E. faecium* PBP5 transpeptidase domain. Sequences are ordered according to the MIC of their originating strain (see Table 1). Catalytic motifs are blackened. The M485S substitution that was investigated and shown to increase resistance is *highlighted in gray*

Table 1 Characteristics of *E. faecium* strains and their PBP5, for which sequences are publicly available

Uniprot #	Strain	MIC (mg/L)	MIC (mg/L) in isogenic strains[b]	Expression level	k_2/K_d ($s^{-1}M^{-1}$)[a]
Q93T65	BM4107	2 (Amp)[b]	6	→[b]	
Q47751	D366	16 (Pen)[a]		↗[a]	17
Q47759	D63	5 (Pen)[a]		→[a]	24
	D63r	70 (Pen)[a]		↗↗[a]	20
Q93NP3	D344	24 (Amp)[b] 64 (Pen)[a]	12	↗↗[a]	17
Q47783	EFM-1	90 (Pen)[a]		→[a]	1.5
Q47801	9439	128 (Amp)[d]		↗[c]	
Q9S6C2	C68	256 (Amp)[e]			
Q47763	H80721	512 (Amp)[b] 512 (Pen)[a]	20	→[a]	<1.3

[a] (80); [b] (75); [c] (78); [d] (84); [e] (89)

of resistance conferred by two PBP5 variants that otherwise differed at seven positions in the transpeptidase domain (75).

Enterococcal PBP5 belongs to the same sub-group of class B PBPs as the acquired *S. aureus* PBP2a, with an insertion of about 120 residues following the transmembrane helix. The crystal structure of *E. faecium* PBP5 bound to penicillin was solved to a resolution of 2.4 Å (86). The originating strain (D63r) had an MIC for penicillin of 70 mg/L that appears to result solely from overproduction of the same PBP5 found in the parental strain (D63), which has the basal MIC of 5 mg/L (80). Therefore, the structure is that of a "wild-type" PBP5, without substitutions that further decrease the affinity for β-lactams. The efficiency of acylation of D63r *E. faecium* PBP5 defined by the second order rate constant $k_2/K_d = 20\,M^{-1}s^{-1}$ is similar to that of *S. aureus* PBP2a, that is 2–3 orders of magnitude slower than that of a "regular" high-affinity PBP (80).

As no structure was obtained in the absence of antibiotic, no comment could be made regarding a possible rearrangement upon acylation, although the authors speculate that some loop residues, which are conserved in this sub-group of PBPs (residues 461–465), may have been pushed aside to allow antibiotic binding (86). Another proposal is that S480 of the second catalytic motif may not be appropriately positioned to act as the proton donor for the nitrogen of the opening β-lactam ring (86), much as proposed in the case of *S. aureus* PBP2a and methicillin (63). The important role of the substitution of M485 by Ala or Thr in the expression of high resistance (75, 80, 81) was rationalized as follows. The side chain of M485 lies behind K425 of the first catalytic site, which may be involved in the proton abstraction of the catalytic S422. Smaller residues in position 485 may result in greater conformational freedom of K425 and thus hinder acylation. The same argument might apply to the M426I substitution found in a highly resistant strain (84). The addition of a second serine after S466 that is found in a PBP5 with an extremely low efficiency of acylation (80) was tentatively explained by a reinforcement of the steric hindrance due to the rigid loop 451–466 (86).

PBP5, as a class B PBP, does not support the necessary glycosyltransferase activity for peptidoglycan synthesis, although it can take over all the required transpeptidase activity. Deletion studies in *E. faecalis* have demonstrated that the glycosyltransferase activity must be provided by at least one of the two class A PBPs encoded by *ponA* or *pbpF* (71). The third class A PBP encoded by *pbpZ* is not required.

Although the high resistance of many enterococcal clinical strains results from their greater amount of PBP5, the reasons underlying this overexpression are still unclear. An open-reading frame upstream of the gene encoding PBP5 is truncated in an *E. hirae* strain overproducing PBP5. This finding suggested that this gene might be a PBP5 synthesis repressor (*psr*) (87). However, subsequent tests of this hypothesis in *E. hirae* using isogenic strains have ruled out a role of *psr* in the regulation of PBP5 expression (88). Similarly, no role for *psr* was found in PBP5 expression in *E. faecium* (89) or *E. faecalis* (77).

Four isolates of *E. faecalis* were found to exhibit high resistance to ampicillin and imipenem without overexpression of PBP5. Instead, the resistance is due to two substitutions, P520S and Y605H, in PBP4 (the orthologue of streptococcal PBP2x) (90).

In addition to the modes of resistance presented above, the plasmid-borne expression of β-lactamases has been documented in some clinical strains of *E. faecalis*, and less frequently in *E. faecium* (91). Although not found in clinical isolates (yet?), an intriguing mechanism of β-lactam resistance was selected in the laboratory strains of *E. faecium* (92–94). These mutants appear to by-pass altogether the need for PBPs. A β-lactam insensitive L,D-transpeptidase activity appears to be responsible for cross-linking of the peptidoglycan, generating L-Lys-D-Asx-L-Lys instead of D-Ala-D-Asx-L-Lys bridges. However, increased resistance does not result from higher L,D-transpeptidase activity, but from a greater amount of precursor that lacks the terminal D-Ala. This elevated amount of truncated precursor is due to the cytoplasmic overexpression of a β-lactam insensitive DD-carboxypeptidase (93). This precursor cannot be a

"donor" substrate for the PBPs but is adequate for the L,D-transpeptidase activity. If ever found in clinical isolates, this mechanism would spell the end of β-lactam-based therapy for enterococci, as it completely obviates the transpeptidase function of the PBPs.

4.3 Streptococcus pneumoniae

Expression of a β-lactamase or an additional low-affinity PBP has never been reported in pneumococcus. Instead, β-lactam-resistant strains of *S. pneumoniae* always harbor modified versions of their own PBPs that are inefficiently acylated by β-lactams (95, 96).

Once electrophoretic techniques were good enough to resolve the six PBPs from *S. pneumoniae*, it became apparent that PBP1a, PBP2b, PBP2x, and sometimes PBP2a were altered in resistant clinical isolates. These modified PBPs bound less radio-labeled antibiotic, whereas the affinity of PBP1b and PBP3 was unchanged (97). Sequencing revealed that mosaic genes encode PBP2b (98), PBP2x (97), and PBP1a (99) in resistant clinical strains. Mosaicity is the product of recombination events between different alleles within a species or between homologous genes of related species. *S. pneumoniae* as a naturally competent organism is particularly apt to this type of genomic plasticity (100).

Mosaic sequences of *pbp* genes are very difficult to classify and organize. Comparison of nucleotide sequences originating from susceptible strains show that they exhibit the same level of polymorphism as other loci, with less than 1% of difference leading to one or two amino acid substitutions over the protein length (97, 98). In contrast, mosaic *pbp* genes show blocks of sequences that differ from non-mosaic alleles by about 14–23% (PBP2b (98, 101); PBP1a (99); PBP2x (97)). The diverging blocks span various lengths of the regional coding for the transpeptidase domain or even most of the extracellular domain. The degree of difference compared to the normal level of intraspecies polymorphism suggested that the diverging sequence blocks originate from other streptococcal species (97, 98). Parallel examination of various mosaic *pbp* genes showed that multiple sources of homologous DNA had been tapped by pneumococcal strains to survive antibiotic selection (97, 98, 102). Evidence of multiple recombinational events in the history of individual *pbp* alleles further complicates the analysis, although favored sites of recombination can be identified (102).

The origin of the sequence blocks found in mosaic *pbp* genes remains largely mysterious with the possible following exceptions for *pbp2x*. Fragments of the *pbp2x* sequences of two penicillin-susceptible strains of the commensal *Streptococcus mitis* and *Streptococcus oralis* could be identified in many alleles encoding PBP2x from resistant pneumococci (102). Although large fragments of these *S. oralis* and *S. mitis pbp2x* sequences can be recognized in resistant strains of *S. pneumoniae*, the identity in these blocks is not perfect. Differences are found in some codons that are important for the resistance including positions 338 and 339 of the first catalytic motif. This observation supports the following scenario for the emergence of pneumococcal resistance. Commensal streptococci sharing the same niche, such as *S. oralis* and *S. mitis*, have acquired resistance through point mutations selected by repeated exposure to β-lactam treatment for various ailments. Fragments of genes encoding PBPs with reduced affinity were subsequently exchanged between closely related streptococcal species, including *S. pneumoniae*, and selected by antibiotherapy (103). The recognition of these multiple horizontal gene transfers in commensal *Streptococci* and *Pneumoccus* has led to the concept of global gene pool of altered *pbp* sequences for β-lactam resistance (104). Since *S. pneumoniae* can easily exchange genetic material, closely related strains can differ in capsular biosynthetic genes (hence serotype) and *pbp* genes. Conversely, identical *pbp* alleles or capsular biosynthetic genes can be found in unrelated strains (105, 106). Nevertheless, despite the complications that horizontal gene transfers bring to the definition of pneumococcal lineage, it appears from numerous studies that the worldwide spread of pneumococcal β-lactam resistance results from the dispersion of a limited number of successful clones (107).

Besides mosaicity resulting from inter- and intra-species homologous recombination, point mutations in *pbp* genes have certainly contributed to the resistance phenomenon. A case in point is the T550A substitution in PBP2x that confers resistance to cephalosporins but susceptibility to penicillin. This substitution was found in the laboratory upon selection with cefpodoxime or cefotaxime (108–110), as well as in PBP2x from clinical isolates where it was caused by a mutation either within a mosaic (111) or a "virgin" *pbp2x* gene (112).

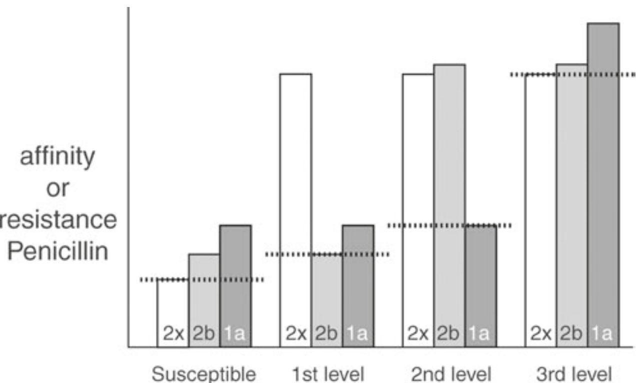
Selection in the laboratory has demonstrated that PBP2x and PBP2b are the primary resistance determinants for cefotaxime (a cephalosporin) and piperacillin (a penicillin), respectively (110, 113). This can be interpreted as PBP2x and PBP2b being the essential PBPs most reactive towards cefotaxime and piperacillin, respectively. Indeed, cefotaxime does not react with PBP2b (114). Surprisingly, the amino acid substitutions selected in the laboratory do not match those found in clinical isolates, with the exception of the aforementioned T550A in PBP2x (108–110) and T446A in PBP2b (110). This discrepancy may simply reflect the limited sampling. Alternatively, the most useful substitutions may be different in the molecular context of the native PBPs from *S. pneumoniae*, as selected in the laboratory, or in the PBPs from the commensal streptococcal species where they were probably originally selected by their host.

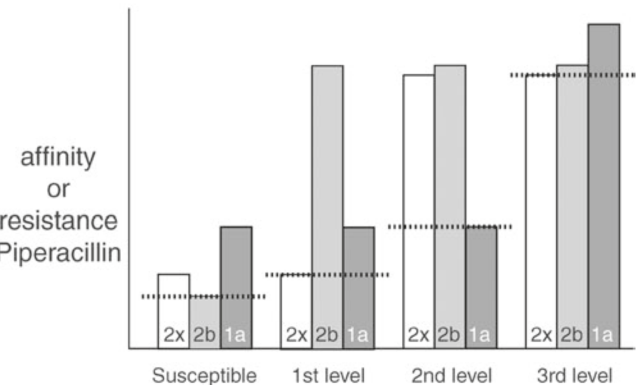
Like the laboratory point mutants, transfer of *pbp2x* genes from clinical resistant isolates to a susceptible strain can confer a moderate level of resistance to both cephalosporins and most penicillins (97, 112, 115–120). Introduction of mosaic *pbp2b* genes can be selected by a modest reduction of the susceptibility to piperacillin (121). Increased resistance to penicillins is achieved upon transfer of both mosaic *pbp2x* and *pbp2b* genes (115–118). Higher level of resistance to cephalosporins and penicillins results from the additional introduction of a mosaic *pbp1a* gene (116–119, 122). A high level of resistance restricted to the cephalosporins is obtained following transformation of a susceptible strain with mosaic *pbp2x* and *pbp1a* (111, 116, 117, 119, 123). The above observation can be rationalized by invoking a threshold effect, as depicted in Fig. 8.

These experimental findings are mirrored in clinical strains (124, 125). Most resistant clinical isolates harbor three mosaic *pbp* genes encoding PBP1a, PBP2b, and PBP2x (e.g., (118, 126–129)). However, some weakly resistant strains have mosaic alleles only of *pbp2x* and *pbp2b* (e.g., (127, 128)). At least one example was found of a clinical strain with barely reduced susceptibility to penicillin that has only *pbp2x* modified (128). Some isolates with cephalosporin resistance, yet susceptible to penicillin, were found to have mosaic *pbp2x* and *pbp1a* while retaining a "virgin" *pbp2b* (130, 131).

The identification of amino acid substitutions that are relevant to the reduction of affinity of a particular PBP is a difficult task. Due to the process of recombination, superfluous substitutions have probably been imported together with the ones that provide antibiotic resistance (the "hitchhiking" effect). Indeed, even genes neighboring *pbp2b* or *pbp1a* have been incidentally modified through recombination of large DNA fragments (132, 133). Nevertheless, a number of probable important substitutions were proposed based on their absence in susceptible strains, presence in many resistant strains, and proximity to the catalytic motifs. The role of some of these substitutions was probed by detailed genetic, enzymatic, and structural studies.

PBP2x has been the subject of the most detailed investigations. The transpeptidation reaction with substrates mimicking the physiological reaction (such D-Ala-D-Ala-L-Lys containing peptides) has never been achieved in vitro with PBP2x or other pneumococcal PBPs (134). In contrast, PBPs were shown to catalyze the hydrolysis of thiol–ester substrate analogues. With such a substrate called S2d, a benzyl-D-alanyl–enzyme intermediate is formed transiently and hydrolyzed (135). With PBP2x, some D-amino acids could provide their free primary amine to attack such acyl–enzyme intermediates, thus completing a transpeptidation reaction (134). However, the significance of these in vitro reactions is unclear as L-amino acids were ineffective, although the physiological primary amine is provided by the side-chain of an L-lysine (134).





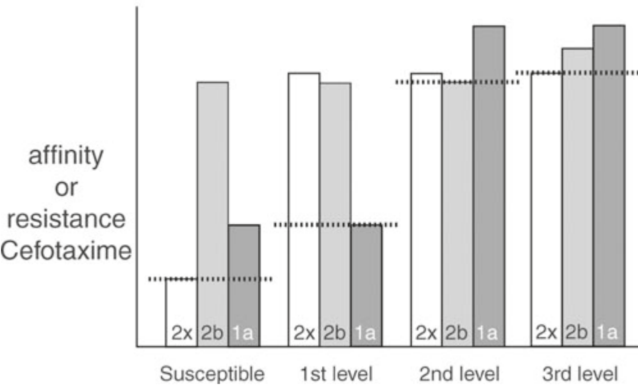


Fig. 8 Schematic representation of the threshold effects that may account for the relationship between which PBPs are modified and the level of pneumococcal resistance. Of PBP2x, PBP2b, and PBP1a, the one with the highest affinity for the β-lactam considered sets the susceptibility threshold of the recipient strain (*dashed line*). The sequence of introduction of altered PBPs, which produces an incremental increase of resistance, depends on the relative affinities of the PBPs of the susceptible strain for a particular β-lactam

In contrast to transpeptidation, the reaction of PBPs with β-lactams occurs readily in vitro. By measuring the decrease in intrinsic fluorescence of a recombinant soluble form of PBP2x upon antibiotic binding, the overall acylation efficiency defined by the second order rate constant k_2/K_d was

determined to be between 60,000 and 110,000 $M^{-1}s^{-1}$ for penicillin and about twice as fast for cefotaxime (61, 134, 136, 137). The deacylation rate k_3 measured in different ways (recovery of enzymatic activity, loss of bound radiolabeled penicillin, mass spectrometry) is between 0.8 and 5 s^{-1} for penicillin and somewhat slower for cefotaxime (60, 134, 136, 137). The very fast acylation and slow deacylation reactions result in a concentration of antibiotic at which half the enzyme is acylated at the steady state (c_{50}) that lies in the micromolar range. This value of c_{50} is consistent with MIC of susceptible strains (60, 119). Attempts have been made to delineate the dissociation constant of the noncovalent preacylation complex K_d and the rate of acylation k_2 with penicillin. One study found a K_d of 0.9 mM and a k_2 of 180 s^{-1} (60), whereas a second study reported a K_d of 20 mM and a k_2 of 1,600 s^{-1} (138). The published data lend more credence to the latter higher numbers. Thus penicillin has a very poor "true" affinity for PBP2x, and this finding presumably applies to β-lactams and PBPs in general. The efficacy of β-lactams against susceptible bacteria does not result from a particularly good fit of the antibiotic to its target (K_d), but rather from the extremely high rate of acylation (k_2).

The crystal structure of PBP2x from the susceptible strain R6, truncated of its cytoplasmic and transmembrane regions, was solved to a resolution of 2.4 Å (5, 139). The extracellular part of PBP2x consists of a transpeptidase domain within the common fold of the ASPRE proteins (residues 266–616), flanked by an elongated N-terminal domain (residues 49–265) and a small globular C-terminal domain (residues 617–750). The N-terminal domain is shaped like a pair of sugar tongs with a hole of about 10 Å in diameter (5). The function of this domain remains unknown although it was proposed to interact with other protein partners. Alternatively, this domain may recognize some chemical motif of the peptidoglycan. When all the amino acid substitutions found in different mosaic sequences of PBP2x are mapped onto the crystal structure (i.e., 30 of the 217 positions of the N-terminal domain), they are all distributed onto the outer surface of the domain and none is found within the hole. The conservation of the residues forming the inner surface of the hole supports the idea that the sugar tong serves to grasp an unknown partner (140). The function of the C-terminal domain is completely unknown, although it is found only in the class B PBPs involved in the division of some Gram-positive bacteria.

The main feature of the transpeptidase domain, with respect to other known structures of the ASPRE family, is the presence of a very long groove, at the center of which is found the active site. Modeling showed that this cleft can accommodate two molecules (NAG-NAM)-L-Ala-D-Glu-L-Lys-D-Ala, one of which is covalently bound to the active-site serine, and the other providing the Nζ of its L-Lys ready to complete the transpeptidation. Both disaccharide moieties can sit in the larger valleys at both ends of the groove (5).

Regarding the precise mechanism of acylation by antibiotics, the crystal structure of PBP2x and a number of theoretical studies have left the question open (e.g., (7)). The conservation of the hydrogen-bonding pattern involving residues of the three catalytic motifs SXXK, SXN, and KTG in PBP2x and the TEM-1 β-lactamase, suggests that the acylation mechanisms are similar (7, 139). The pH dependence of the acylation rate is consistent with a model where a residue with a pK_a of 4.9 functions as a base to help deprotonate the active-site serine, a group with a pK_a of 7.6 triggers upon deprotonation a rearrangement to a less reactive conformation, and a residue with a pK_a of 9.9 is hydrogen bonded in its protonated form to the free carboxylate of the substrate (138). The base was proposed to be K340 of the first motif with the unusual pKa of 4.9. T550, which binds the carboxylate of the antibiotic (139), would have the pKa of 9.9. Investigation of solvent isotope effects on the rate of acylation suggested a complex process partially rate-limited by the chemistry (the proton exchanges) and by solvation and/or conformational rearrangement (138).

Based upon sequence comparisons and the proximity to the catalytic motifs, the substitutions most likely to impart some resistance include T338A, T338G, T338P, and M339F found within the SXXK motif (61, 111, 112, 116), H394Y and M400T that surround the SXN motif (111, 113, 126, 128), and L546V, T550A, and Q552E, which are close to the KTG motif (111, 112, 127, 141). The effect of some of these substitutions has been characterized in detail as discussed below. These mutations do not appear randomly in sequences, but some families can be recognized.

Examination of approximately 100 publicly available sequences of the transpeptidase domain of PBP2x reveals three broad families (Fig. 9). One family contains non-mosaic sequences that are very similar to the PBP2x from the reference susceptible strain R6. The mosaicity complicates the picture of the two other families and the grouping would differ for various sequence blocks. Nevertheless, the emerging pattern suggests that two main mechanisms have been selected that reduce the affinity of PBP2x for the antibiotics (120). Figure 10 shows the distribution of the substitutions in the structure of the transpeptidase domain of PBP2x from two resistant isolates, representing two modes of reducing the affinity for β-lactams.

One family of sequences is characterized by the T338A substitution. About 30 other substitutions in the transpeptidase domain accompany this defining mutation, although no mutation is consistently found together with T338A, and never found in the absence of the T338A mutation. The side chain of T338 is pointing away from the active-site cavity and is hydrogen-bonded to a buried water molecule. It has been proposed that suppression of the hydrogen bonding by

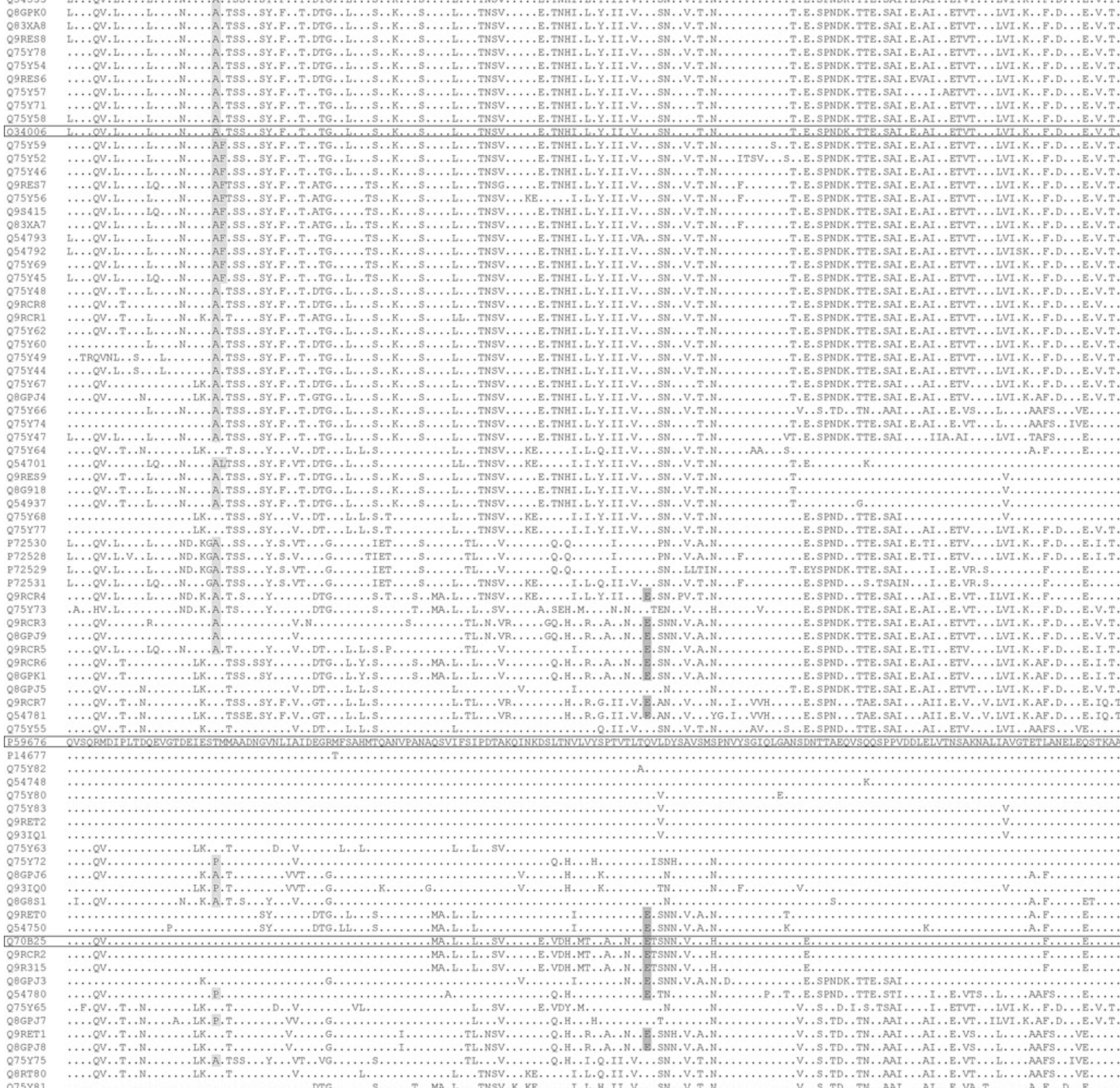


Fig. 9 Alignment of PBP2x publicly available sequences (aligned and clustered with CLUSTALW). Only positions where at least one sequence differs from the R6 reference (Uniprot accession number # P59676) are shown. Substitutions at position 338, 339, and 552 are highlighted. Although the mosaicity confounds effort to classify unambiguously these sequences, this representation allows to visualize that sequences characterized by a mutation in position 338 differ substantially from sequences with the Q552E substitution, although a few sequences harbor both mutations. The crystal structure of the high affinity PBP2x from strains R6 (# P59676), as well as the two low affinity protein from strains Sp328 (# O34006) and 5259 (# Q70B25) have been solved, revealing two modes of reducing the affinity for β-lactams

replacement of T338 can lead to destabilization of the active site due to the loss of the water molecule (61). Introduction of the sole T338A mutation in PBP2x from the susceptible strain R6 reduces its efficiency of acylation by penicillin by a factor of two (61), which is not enough to be selected following transformation into a susceptible strain (119). Reversion of the substitution in the related PBP2x from resistant strains Sp328 and 4,790 increases the acylation efficiency sixfold (61, 119).

A subset of sequences that contain the T338A mutation also have the adjacent M339F substitution. These sequences are from strains with particularly high levels of resistance

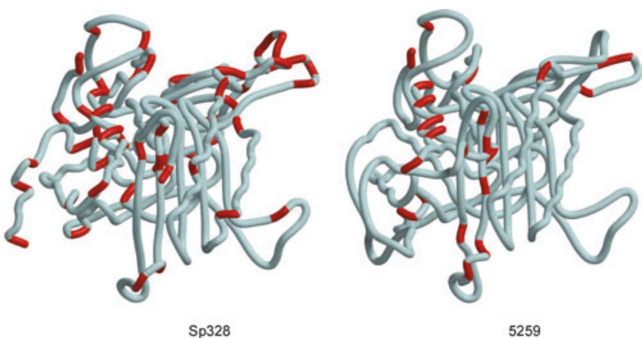


Fig. 10 Distribution of the amino acid substitutions (red) in the PBP2x transpeptidase domain from *S. pneumoniae* strains Sp328 (sequence # O34006) and 5259 (# Q70B25), with respect to PBP2x from strain R6 (*See Color Plates*)

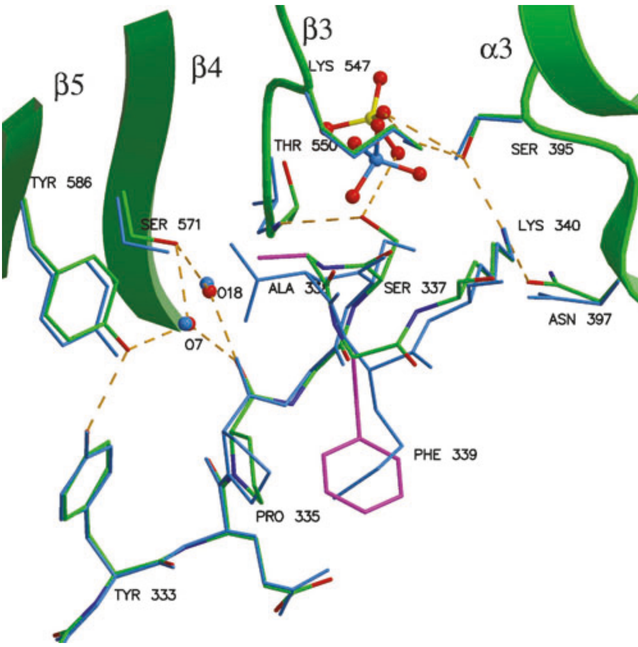


Fig. 11 Superposition of the active site of wild type R6 PBP2x (blue) and of the double mutant T338A/M339F (green carbon atoms and side chains of the mutated residues in purple). Note that the hydroxyl of the catalytic S337 is pointing in opposite directions (*See Color Plates*)

(111, 112, 119, 125–127). PBP2x molecules from such isolates have an efficiency of acylation by penicillin reduced more than 1,000-fold (60, 119). Most of this reduction is due to a slower rate of acylation (k_2 decreased 300-fold), although a weaker preacylation binding (K_d fourfold higher) also contributes to the overall extremely poor affinity of the PBP2x with the double T338A/M339F (60, 119). In addition, these PBP2x variants have significantly faster deacylation kinetics (k_3 increased 40- to 70-fold), an effect mostly due to the M339F substitution (119, 137). The slow acylation and fast deacylation combine to elevate the c_{50} (concentration of antibiotic resulting in the steady-state acylation of half the enzyme) by four to five orders of magnitude (60, 119).

The M339F mutation alone, introduced in the reference R6 PBP2x, reduces the efficiency of acylation by penicillin by sixfold and is sufficient to confer a measurable level of resistance (119). Combination of the M339F and T338A mutations produces a greater effect. The structure of the latter double mutant has been solved to a resolution of 2.4 Å. The salient feature of the mutated active site is the reorientation of the hydroxyl of the catalytic S337 that is now pointing away from the active site center and is hydrogen-bonded to the main chain nitrogen of T550 instead of to K340 (119) (Fig. 11). The active-site serine 337 may exist in an equilibrium between two rotamers, only one of which can be acylated. Mutations such as M339F, by subtly altering the active site, may shift the equilibrium towards the unproductive rotamer. Note that this effect could be restricted to the reaction with β-lactams if binding of the physiological substrates favors a conformation that offsets the effect of the mutations.

The detailed studies of a few mutations fell short of explaining the reduction of affinity measured for PBP2x from clinical resistant isolates. The individual reversions of the 41 mutations of the PBP2x transpeptidase domain from a highly resistant strain, revealed by in vitro kinetic and in vivo phenotypic characterization the importance of four substitutions, in positions 371, 384, 400 and 605, in addition to those in position 338 and 339 (142). The combined reversion of the six substitutions nearly restored the normal rapid rate of acylation by β-lactams. Conversely, introduction of five combined mutations diminished the reactivity towards β-lactams almost to the level of the original PBP2x with 41 substitutions. A conceptually similar study in vivo with a different PBP2x, also identified the I371T and R384G substitutions as central for the reduced acylation rate (143).

Resolution of the structure of PBP2x from the resistant strain Sp328, which belongs to the family defined by the T338A substitution, has confirmed the absence of the buried water molecule (140). The most striking feature of Sp328 PBP2x is the great flexibility of the loop-spanning residues 365–394. This instability extends in part to the SXN motif in positions 395 to 397, with S395 being somewhat displaced. Thus, the 60-fold reduction of the acylation efficiency by cefotaxime, for example, is due to a distortion of the active site (61, 119). The 365–394 segment forms one side of the groove leading to the active site. The flexibility of this region generates a more accessible "open" active site that may better accommodate alternative physiological substrates with branched stem peptides (140). The destabilization of the 365–394 region was shown to result from the I371T and R384G mutations (142).

A second family of PBP2x molecules from resistant strains can be defined by the presence of the Q552E substitution. Introduction of this single substitution in PBP2x reduces about fourfold the efficiency of acylation and confers a modest level of resistance to the recipient R6 strain

(120, 141). The structure of a PBP2x from a clinical strain that possess the Q552E substitution has been solved to a resolution of 3 Å. This PBP2x has an efficiency of acylation reduced more than 15-fold (120). The only significant difference found in comparison to the structure of R6 PBP2x is the displacement of strand β3, which carries the KTG motif (120) (Fig. 12). This displacement of 0.5 Å narrows the active site, and is reminiscent of the closed conformation of PBP2a from *S. aureus*, which is thought to cause the low efficiency of acylation of this enzyme by coupling the reaction to a major structural rearrangement (63). In addition to this conformational effect, the introduction of a negative charge in position 552 greatly affects the entry of the active site and does not favor binding of β-lactams, which are negatively charged (120).

Consequently, it appears that two distinct mechanisms have been selected that reduce the reactivity of PBP2x towards β-lactams. One mechanism primarily affects the chemistry of the active site S337, whereas the second mechanism hinders acylation by requiring an opening of the active site. These two mechanisms may be a reflection of two major sources of exogenous genetic material that have been incorporated in strains of *S. pneumoniae*. Note that a few sequences of PBP2x have both T338A and Q552E substitutions and may thus combine the effect of both mechanisms.

Another significant substitution is T550A, which confers resistance to cephalosporins only, both in laboratory and clinical strains (108, 110–112). When the T550A point mutation occurs within a mosaic PBP2x, which contains the T338A/M339F double mutation, it further increases the resistance to cephalosporins, while it almost abolishes resistance to penicillin (111). This effect is mirrored in the acylation efficiency of a T550A point mutant of R6 PBP2x, which is decreased 20-fold towards cefotaxime and unaffected towards penicillin (141). This effect has been rationalized by the abolition of the hydrogen bond between T550 and the carboxylate that is attached to the six-member ring of second- and third-generation cephalosporins (139).

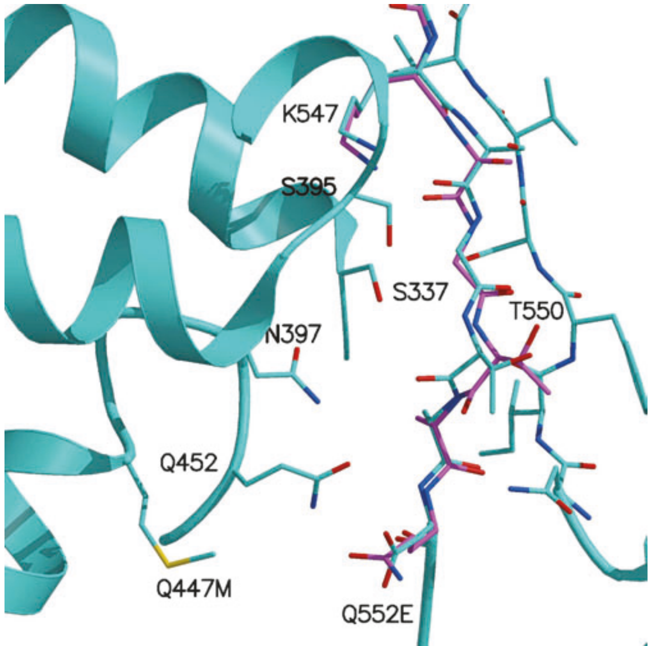


Fig. 12 Structure of the PBP2x active site from strain 5259 (cyan). The position of strand β3 from R6 PBP2x is shown in purple. Note the slight closure of the active site from 5259 PBP2x (*See Color Plates*)

PBP2b, the other class B PBP from *S. pneumoniae*, has not been subjected to such thorough investigations, presumably because of the absence of high-resolution structure. Over 90 sequences are available and two substitutions, T446A or T446S and E476G are always found in PBP2b from clinical resistant strains. The probable importance of these two substitutions was pointed out in numerous studies (101, 115, 125, 127). The T446A mutation, which is immediately adjacent to the SXN motif, is also selected by piperacillin in the laboratory (110). T446A is the only substitution that has been characterized biochemically (121) and it reduces the affinity for penicillin by 60%. The affinity of various PBP2b molecules from clinical isolates with 6 to 43 mutations in addition to T446A is reduced by 90 to 99%.

In addition to the mutations in positions 446 and 476, some PBP2b sequences are distinguished by other salient features such as the substitution of six to seven adjacent residues at position 426/427–432 (98, 101). Three related PBP2b sequences from Japanese isolates are noteworthy by the insertion of three residues (SWY) after position 422 (144). This is one of two occurrences of a change in the number of residues in a mosaic PBP. The other case was found in PBP1a (see below). In all other cases, the total length of the proteins and the position of the catalytic motifs are fully conserved, despite extensive sequence remodeling. Seven related sequences from Korean clinical strains show a substitution within the third catalytic motif KTG, which is changed to KSG (145). In contrast to PBP2x and PBP1a where mutations within the first catalytic motif are commonplace, a single case was reported of a V388A substitution within the SVVK motif (146). The recent emergence of strains that show a particularly high resistance to amoxicillin, relative to other β-lactams, appears to result from a set of ten substitutions in the region 591–640 surrounding the third catalytic motif KTG (118, 147). The relative importance of these and other mutations for the resistance awaits investigation, and mechanistic insight will require the resolution of the crystal structure of PBP2b from susceptible and resistant strains.

PBP1a may be considered clinically as the most important and troublesome PBP. Indeed, the resistance potentially provided by mosaic PBP2x and PBP2b is capped by the presence of a "virgin" PBP1a, which still warrants some efficacy to β-lactam therapy. High level of resistance depends on a

modified PBP1a. Despite its clinical importance, PBP1a is the least studied of the three PBPs clearly involved in resistance. Biochemical studies have been limited, although the crystal structure of the transpeptidase domain is now available at the resolution of 2.6 Å (148). The acylation efficiency of PBP1a from the susceptible strain R6 was measured to be about 70,000 $M^{-1}s^{-1}$ for penicillin and the deacylation rate constant to be about $10^{-5}\,s^{-1}$ (149). These values are of the same magnitude as those reported for PBP2x. No biochemical data have been published for a mosaic PBP1a. About 50 PBP1a sequences are publicly available. The T471A substitution within the first catalytic motif, analogous to the T338A mutation in PBP2x, is commonly found in PBP1a sequences from resistant strains (117, 126–128, 150). Reversion of this substitution reduced but did not abrogate the resistance that PBP1a confers in addition to PBP2x and PBP2b (117). Some mosaic sequences lack the T471A mutation, including PBP1a from a highly resistant Hungarian isolate (MIC for penicillin of 16 mg/L) (151). Another remarkable feature is the mutation of a stretch of four residues (TSQF to NTGY) at position 574–577, which is observed in all the mosaic sequences. Amino acids at positions 574–577 belong to a loop between strands β3 and β4, which form the side of a tunnel at the entrance of the catalytic cleft. This wall has a hydrophobic character conferred by Phe577, which is certainly changed in the mutant (148). Reversion of this set of substitutions decreased the additional resistance conferred by PBP1a (151). A similar effect of the reversion was found for the L539W substitution, although the sequence in which the experiment was performed is the only one that presents this particular mutation (151). Much remains to be learnt about the detailed mechanism by which the reactivity of PBP1a is reduced.

Although PBP2x, PBP2b, and PBP1a are the major PBPs responsible for the resistance of *S. pneumoniae*, a number of studies have hinted at the possible involvement of various other PBPs. Transfer of a high level of resistance from a strain of *S. mitis* to a laboratory strain of *S. pneumoniae* was shown to require transfer of the genes encoding the five high molecular weight PBPs (152). A point mutation in the low molecular weight PBP3 was found to contribute to the resistance of a strain selected on cefotaxime in the laboratory (109). In contrast to these laboratory experiments, examination of the PBPs from clinical isolates failed to reveal significant modification of PBP1b or PBP3 (125, 153). Early studies, which examined various strains through the labeling of PBPs with radioactive penicillin, found several instances where binding to PBP2a was diminished in resistant strains (97, 104). Also, transfer in the laboratory of resistance from a *S. mitis* strain to *S. pneumoniae* involved modification of PBP2x, PBP2b, PBP1a, and PBP2a, but not of PBP1b and PBP3 (116). Various combinations of point mutations, including silent ones, were observed in some PBP2a sequences, suggesting events of intraspecies recombination (154). The role of PBP2a in β-lactam resistance is now firmly established in at least one instance (155). A strain isolated from an AIDS patient was found to harbor a mosaic PBP2a in addition to mosaic PBP2x, PBP2b, and PBP1a. Transformation experiments demonstrated that this PBP2a variant is indeed responsible for an elevated resistance to various β-lactams. The sequence shows 25 substitutions including 12 within the transpeptidase domain. The absence of crystal structure precludes a detailed analysis, but it is noteworthy that the threonine following the catalytic serine is replaced by an alanine, like in numerous variants of PBP2x and PBP1a.

Both class B PBPs, PBP2x and PBP2b, are essential in *S. pneumoniae*, which is consistent with the selection of variants of these proteins by β-lactams (146). PBP1b and PBP3 are not essential (156, 157), which again is consistent with the fact that these proteins are not involved in the resistance process. PBP1a and PBP2a are not essential individually, but one of them must be present and functional (156, 158). The fact that PBP1a, rather than PBP2a, is the main target of antibiotic selective pressure may be due to PBP2a having a low intrinsic affinity for β-lactams (159).

A puzzling discovery was made, which is directly related to PBP-based β-lactam resistance. Clinical resistant isolates have an abnormal peptidoglycan structure with an elevated proportion of cross-bridges that involve branched stem-peptides (160). Instead of having the L-Lys of the "acceptor" peptide cross-linked directly to the D-Ala of the "donor" peptide, there are intervening L-Ala-L-Ala or L-Ala-L-Ser dipeptides. The genetic determinants of this cell wall abnormality could nevertheless be separated from the resistance determinants (the mosaic *pbp* genes) (161). The genes responsible for the synthesis of branched precursors were found to constitute the *murMN* operon (162), also known as the *fibAB* operon (163). Mosaic *murM* genes often increase the resistance level conferred by a set of mosaic *pbp* genes (130, 162). A naïve explanation is that mosaic PBPs prefer branched substrates. However, deletion of *murM* abolishes the resistance but does not have impact on the growth rate in the absence of antibiotic challenge (162), demonstrating that mosaic PBPs can efficiently use linear precursors. The situation is reminiscent of the role of *femAB* operon in *S. aureus*, which is required for expression of *mecA*-based resistance, while the *mecA*-encoded PBP2a can nevertheless function with alternative substrates produced in the absence of *femAB* (52, 53). It has been proposed that branched stem-peptides may be superior competitors against β-lactams for the active site of some PBPs of resistant strains, or that they may be involved in some signaling function of cell wall metabolism, or that they play a particular role in the integrity of the peptidoglycan, a role that becomes critical when some PBPs are inhibited by antibiotics (162).

Besides MurM, other unknown factors modulate β-lactam resistance. Indeed, five clinical isolates with significantly

different levels of resistance were found to have the same MurM allele and strictly identical sequence of their penicillin-binding domains, for the six PBPs (154).

Although much is known about the biochemistry of the PBPs, the MurM complication highlights our limited understanding of the physiological function of the PBPs in cell wall metabolism, both in the absence and presence of antibiotics.

4.4 Neisseria

Neisseria meningitidis and *Neisseria gonorrhoeae* are pathogens that have acquired reduced susceptibility to penicillin via two routes. The modification of at least one chromosomally encoded PBP will be discussed below. Alternatively, production of a plasmid-encoded β-lactamase is common in *N. gonorrhoeae* (e.g., (164)), while it is rare in *N. meningitidis* (165).

Neisseria species contain only three PBPs called PBP1, PBP2, and PBP3, which are respectively class A, class B, and a low molecular weight carboxypeptidase. Gonococcal strains with reduced susceptibility to β-lactams that do not express a β-lactamase were found to exhibit reduced labeling of PBP2 and PBP1 with radiolabeled penicillin (166). Reduced labeling of PBP2 was observed in meningococci (167). PBP2 is encoded by the *penA* gene, which is mosaic in resistant strains of *N. gonorrhoeae* (168) and *N. meningitidis* (169). The mechanism of acquisition of non-plasmidic resistance in *Neisseria* is therefore similar to that of *S. pneumoniae*.

Like pneumococcus, *Neisseria* species are naturally competent organisms and horizontal gene transfers are common (170). The origin of the foreign sequence fragments that are found in the *penA* gene of resistant gonococci and meningococci has been investigated in some depth. Several commensal species, such as *Neisseria flavescens*, *Neisseria cinerea*, or *Neisseria perflava*, appear to have each contributed sequence blocks to *penA* genes from resistant strains (169, 171–173). *N. flavescens* isolates recovered from the preantibiotic era have relatively high penicillin MICs and a PBP2 with an intrinsic low affinity for penicillin (171). Transfer in the laboratory of the *penA* gene from such *N. flavescens* isolates could indeed confer some resistance to *N. meningitidis* (171). In contrast, *N. cinerea* is not naturally resistant, and accordingly, no resistance was achieved in *N. meningitidis* upon transfer of the *penA* gene from this species (171). It was found that the PBP2 sequences of *N. cinerea* origin found in resistant meningococci have an additional aspartic acid following D345, which is not present in the susceptible *N. cinerea* strains (171). This insertion was also found in mosaic PBP2 sequences from most resistant gonococcal strains. Site-directed mutagenesis has demonstrated that this insertion is sufficient to decrease the reactivity of PBP2 for β-lactams and to confer some resistance to *N. gonorrhoeae* (174). A clinical resistant strain was later discovered that only has this additional aspartic acid (175). The sequence identity between *Neisseria* PBP2 and PBPs of known structures is too low to obtain reliable alignment. Nevertheless, by simply aligning the SXXK and SXN motifs, it appears that the insertion following position 345 is in a region close in space to the SXN motif. This region was found to be destabilized in a low-affinity PBP2x of *S. pneumoniae* (140).

Thus, it appears that *penA* alleles that confer penicillin resistance have arisen both from the recruitment of sequence blocks from naturally resistant species, such as *N. flavescens*, and new mutations such as a codon insertion. When, how often, and in which species these recombination and mutation events have occurred are difficult questions. As commensal *Neisseria* species readily exchange genetic material, the *penA* alleles conferring resistance may be considered as forming a common gene pool, which is shared by several species (176, 177).

The cell wall of strains with altered penA alleles has a greater amount of unprocessed pentapeptides, suggesting that the transpeptidase and/or carboxypeptidase activity of low-affinity PBP2 is modified (178).

Early studies hinted at the possibility that PBP1, the class A PBP, also had decreased reactivity for penicillin in gonococci (166), but subsequent studies failed to uncover mosaicity in the *ponA* gene encoding PBP1. Recently, an allele of *ponA* encoding PBP1 with the single substitution L421P was found to contribute to the high resistance of some *N. gonorrhoeae* strains (179). This substitution is 40 residues N-terminal to the catalytic S461. The sequence identity with the only PBP of class A of known struture (*S. pneumoniae* PBP1b, (180)) is too low to determine the location of the L421P substitution. Nevertheless, the L421P substitution was shown in vitro to diminish about fourfold the acylation efficiency of PBP1 by various β-lactams (179).

Note that three non-*pbp* loci have been found to contribute to β-lactam resistance in *Neisseria* species. The *mtr* locus encodes an efflux pump (181), while *penB* codes for a porin (182). The nature of the third locus *penC*, which is required to allow phenotypic expression of the *ponA* mutation, remains undetermined (179).

4.5 Other Pathogens

Modified PBPs as a means to resist β-lactams has been documented in a few other pathogens, including the species where the most frequently encountered mode of resistance is the production of a β-lactamase. Some examples will be briefly presented below.

Most resistant clinical isolates of *Haemophilus influenza* evade the action of β-lactams by producing a β-lactamase. However, the number of β-lactamase-negative ampicillin-resistant (BLNAR) strains is rising, particularly in Japan (183). First documented in 1980 (184), BLNAR strains were found to express PBPs with a reduced reactivity towards penicillin (185). Early studies that monitored the PBPs by reaction with radio-labeled penicillin found modifications in PBP2, PBP3, PBP4, PBP5, and PBP6, depending on the resistant strain (186, 187). Further scrutiny and gene sequencing confirmed only the role of modifications in PBP3, the division specific class B PBP (188–190). Truncation of PBP4, a low molecular weight PBP, was found in some BLNAR strains, but this anomaly was not correlated with resistance (191). Another study failed to find significant substitutions in the high molecular weight PBPs: PBP1a, PBP1b, and PBP2 (192).

Sequencing of the gene fragment encoding the transpeptidase domain of PBP3 revealed in excess of twenty mutation patterns, with a number of mutations per sequence ranging from one to nine, affecting 21 different positions (191–193). These PBP3 sequences are not mosaic but show an accumulation of point mutations. Various classification schemes have been proposed (191, 193, 194). Some sequences are characterized by the presence of an R517H substitution (group I), while others have the N526K mutation (groups II and III). Both substitutions are relatively close to the third KTG514 catalytic motif. Position 517 with respect to the KTG motif is analogous to the position 552, which is also mutated in a group of PBP2x sequences from *S. pneumoniae* (120). Sequences that contain the N526K substitution can also possess the three additional mutations M377I, S385T, and L389F surrounding the second SSN381 catalytic motif (group III). Site-directed mutagenesis and transformation experiments have shown that S385T and L389F increase the resistance conferred by N526K. M377I does not and may be a neutral mutation linked to the S385T substitution (194). Modeling of the structure of *H. influenza* PBP3 on that of *S. pneumoniae* PBP2x showed that residues 517, 526, 377, 385/ and 389 are probably lining the active-site cavity (191). It has been noted that the PBP3 sequence of group III, found only in Japan, is association with a high resistance to cefotaxime, whereas group I and group II sequences confer only weak resistance to this cephalosporin (193).

The affinity for penicillin of a few *H. influenza* PBP3 variants has been measured in vitro (192). PBP3 of group II, including one variant that has only the N526K mutation had lower affinity than a PBP3 of group I, in agreement with the resistance level of the originating strains. Surprisingly a PBP3 with only the R517H substitution, the mutation defining group I sequences, had the same high affinity as a wild-type PBP3. This substitution in isolation therefore cannot confer resistance.

BLNAR strains with high level of resistance can combine mechanisms that involve alteration of PBP3 and an efflux pump (192), must be added. In addition, it is now evident that a low-affinity PBP3 can also be found in strains expressing a β-lactamase, and that both mechanisms can cooperate to increase the resistance sense combinations of β-lactams and β-lactamase inhibitors such as the widely used amoxicillin/clavulanate formulations (193, 195).

The genome of *Helicobacter pylori* encodes three recognizable PBPs. These are the homologues of the class B PBP2 and PBP3, and of the class A PBP1a from *E. coli*. Using a fluorescein-labeled penicillin, a fourth low molecular weight penicillin-binding protein was identified (196). Its sequence shows no homology with proteins of the ASPRE family and the catalytic motifs cannot be recognized in their usual positions. The status of this protein with respect to the subject of this review is therefore uncertain.

Clinical amoxicillin-resistant *H. pylori* strains have been isolated that lose their resistance following storage as frozen samples (197). This type of unstable resistance may be related to the transient loss of expression of the fourth mysterious penicillin-binding protein (198).

The isolation of a few stable amoxicillin-resistant strains was also reported (199–202). In one strain, the resistance was shown to result entirely from the single point mutation S414R in PBP1a, although another substitution was also present (202). Two other stable resistant strains were found to have the three substitutions T556S, N562Y, and T593A as well as the insertion of a Glu after residue 464 (201). One strain had ten substitutions, all of them in the second half of the transpeptidase domain, including the T556S and N562Y mutations (203). It may be noteworthy that the T556S is within the third catalytic motif KTG. In vitro selection on amoxicillin also yielded strains with modified PBP1a (204). The PBP1a of one such strain had four substitutions, including the S414R mutation (205).

To our knowledge, no clinical isolates of *Escherichia coli* were found to resist through the expression of modified PBPs. However, as a laboratory workhorse, *E. coli* was used to demonstrate that β-lactam pressure can select altered PBPs (206). Several point mutations in PBP3 were found to confer resistance to cephalexin and other cephalosporins. Note that *E. coli* PBP3 is the class B PBP dedicated to division. Interestingly, the substitution T308A, next to the active site S307, is analogous to the PBP2x T338A and PBP1a T471A that confer resistance to *S. pneumoniae* (207, 208). Another mutation was found in the second catalytic motif, changing SSN361 into SSS361 (207).

A few reports must be added to complete this overview of pathogens with modified PBPs. PBP alteration has also been found in imipenem-resistant clinical isolates of *Proteus mirabilis (*209) and *Pseudomonas aeruginosa* (210). A cefsulodin-resistant clinical isolate of *P. aeruginosa* also had one PBP with reduced affinity, although not the same as the imipenem-resistant isolate (211). Overexpression of

PBP3, in addition to decreased outer-membrane permeability, was found in a highly resistant strain of *Salmonella muenchen* (212). The various levels of resistance of several strains of *Acinetobacter calcoaceticus* could be correlated with the production of PBPs with altered expression or affinity for β-lactams (213). In the laboratory, imipenem could select a resistant clone of *Acinetobacter baumanii* with an altered PBP (214). Alterations of PBP3 or PBP2 were selected in laboratory mutants of *Listeria monocytogenes* (215, 216). Altered PBPs were also found in laboratory resistant mutants of the *Bacteroides fragilis* group (217) and of *Rhodococcus equi* (218).

Pathogens have been submitted to severe antibiotic pressure over the past five decades, leading to the emergence of resistant strains. In a natural setting, β-lactam-producing bacteria need to be protected against drugs of their own making. Two examples have been documented, which involve low-affinity PBPs. Expression of a particular PBP is responsible in part for the resistance of β-lactam-producing *Streptomyces clavuligenus* (219). None of the eight PBPs of cephamycin C-producing *Nocardia lactamdurans* bind the β-lactam secreted by this bacteria, although it also expresses a β-lactamase (220).

5 Are the PBPs Sustainable Targets?

The PBPs involved in the β-lactam resistance of the major pathogens are summarized in Table 2. The use of β-lactams to treat staphylococcal, enterococcal, and pneumococcal infections is already largely compromised. The isolation of strains with modified PBPs from species that usually resist by producing β-lactamases is worrying. The long-term efficacy of β-lactams may thus be compromised even in the advent of efficient β-lactamase inhibitors. It is therefore reasonable to ask whether PBPs are still valid targets for future antimicrobial therapies.

Half a century of β-lactam therapy has largely validated the targeting of PBPs. The uniquely eubacterial synthesis of peptidoglycan is a good predictor of the near absence of negative secondary effects in vertebrates. These two reasons justify the continued effort to target the PBPs. In which direction should the research effort be headed?

The main lesson from detailed kinetic studies of the reaction between PBPs and β-lactams is that these antibiotics are a poor fit to the enzyme-active site. The high dissociation constant of the noncovalent complex guarantees the broad specificity of the β-lactams, but also hints that attempts to improve their affinities may be misguided. Moreover, crystal structures of PBPs complexed covalently to various antibiotics can only suggest what might be the interactions taking place in the preacylation complexes. The structure of a preacylation complex would help to understand both the noncovalent affinity (K_d) and the acylation rate (k_2), the latter being most affected in altered PBPs.

Instead of focusing on the reaction between PBPs and β-lactams, research should be directed towards what may be PBPs' Achilles' heel: their physiological reaction of transpeptidation. Indeed, the remarkable feature of the low-affinity PBPs is their retained capacity to catalyze peptidoglycan cross-linking, even though the acylation chemistry is expected

Table 2 High molecular weight PBPs of organisms that resist β-lactams by expressing low-affinity PBPs

	Class A (bifunctional)			Class B (monofunctional)		
Staphylococcus aureus	PBP2			PBP1	PBP3	PBP2a ↗
						mecA
Enterococci	PBP1a	PBP1b	PBP2a	PBPC(B)	PBP2b	PBP5 ↗
	ponA	pbpZ	pbpF	pbpB	pbpA	
Streptococcus pneumoniae	PBP1a	PBP1b	PBP2a	PBP2x	PBP2b	
Neisseria	PBP1			PBP2		
	ponA			penA		
Haemophilus influenzae	PBP1a	PBP1b		PBP3	PBP2	
				ftsI		
Helicobacter pylori	PBP1a			PBP3	PBP2	

Low-affinity PBPs are boxed. *Hatched borders* indicate an intrinsic low affinity. An *arrow* indicate overexpression. *No shading* indicates point mutations, *light shading* indicates mosaicity, and *dark shading* indicates acquisition of exogenous origin. Alternative gene names are given below their respective product

to be similar to β-lactams and D-Ala-D-Ala-containing substrates. Understanding how the natural PBP substrates maintain the reactivity of the catalytic serine even in PBPs from resistant bacteria should help the design of novel compounds. Such new drugs could react with all PBPs, regardless of their reactivity with β-lactams (221). Alternatively new molecules might serve as adjuvant to restore or maintain the reactivity of all PBPs towards traditional β-lactams.

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Chapter 14
Aminoglycosides: Mechanisms of Action and Resistance

Maria L. Magalhães and John S. Blanchard

1 Antimicrobial Mechanism of Action

Aminoglycosides are amongst the most important compounds used to treat serious nosocomial infections caused by aerobic, Gram-negative bacteria (1, 2). They are pseudo-polysaccharides containing amino sugars and can therefore be considered polycationic species for the purpose of understanding their biological interactions. Since they are highly positively charged at physiological pH values, they show high binding affinity for nucleic acids, especially for certain portions of the prokaryotic ribosomal RNA (rRNA). Different classes of aminoglycosides bind to different sites on rRNA, as will be discussed.

Aminoglycoside uptake by bacterial cells has been shown to occur in three phases (3). The initial step involves electrostatic interactions between the antibiotic and the negatively charged lipopolysacharide (LPS) of the Gram-negative outer membrane (4). The polycationic antibiotic competitively displaces essential divalent cations (magnesium) that cross-bridge and stabilizes adjacent LPS molecules. Disruption of the outer membrane by this mechanism has been proposed to enhance permeability and initiate aminoglycoside uptake (4–6). Aminoglycoside transport across the cytoplasmic membrane involves an initial lag phase followed by a second phase in which the drug is rapidly taken up. Transport across the cytoplasmic membrane requires energy from the electron transport system in an oxygen-dependent process (3, 7–9). The intrinsic resistance of anaerobic bacteria to aminoglycosides can then be explained by the failure to transport the drug inside the cell. Once inside the cell, the drug binds to the 30S ribosomal subunit, at the *A*minoacyl-tRNA (aa-tRNA) acceptor site (*A*) on the 16S ribosomal RNA (rRNA), affecting protein synthesis by induction of codon misreading and inhibition of translocation (10, 11).

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Some aminoglycosides, like spectinomycin and kasugamycin, were found to have no effect on chain elongation (codon misreading) but block initiating ribosomes completely. Streptomycin and other aminoglycosides similarly block the initiation complex, but act later, decreasing the accuracy of translation (12).

It is believed that fidelity of translation depends on two steps—an initial recognition between the codon on mRNA and the anticodon of the charged aa-tRNA, and a subsequent proofreading step. During the initial selection, the cognate codon is recognized, inducing GTP hydrolysis and the release of elongation factors from aa-tRNA (13). The aminoacyl end of aa-tRNA is free to move into the peptidyl transferase center on the 50S subunit, where peptide bond formation occurs (14). A similar sequence of events happen when a non-cognate codon is recognized. However, in such a case, following GTP hydrolysis and release of additional factors, non-cognate aa-tRNAs dissociate from the ribosome rather than enter the peptidyl transferase center, due to the lower stability of the codon-anticodon complex (13).

Although the precise mechanism of aminoglycoside-induced miscoding is not completely understood, it has been shown that aminoglycosides enhance the binding stability of cognate aa-tRNAs to the small ribosomal subunit (15). It has been proposed that such stability enhancement would allow non-cognate tRNAs to enter the peptidyl transferase site, being incorporated into the nascent polypeptide chain.

Recently, high-resolution crystal structures of the 30S ribosomal subunit (16, 17) as well as nuclear magnetic resonance (NMR) derived structures (18) of ribosomal constituents bound to aminoglycoside molecules have provided valuable information about the molecular mechanisms of aminoglycoside binding and action.

The NMR structure of the complex between an A site-mimicking RNA molecule and the aminoglycoside paromomycin revealed how this aminoglycoside binds to the ribosome (18). Critical nucleotides for binding include A^{1492}, U^{1495}, as well as the C^{1407}–G^{1494} and A^{1408}–A^{1493} base pairs. These studies showed that the antibiotic binds in the major groove of the A-site in an L-shaped conformation. The

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2-deoxystreptamine and 2,6-dideoxy-2,6-diamino-glucose rings contribute the most important intermolecular contacts. The N1 and N3 amino groups of the central deoxystreptamine ring, found in all typical aminoglycosides are required for specific binding to the 16S rRNA.

A high-resolution crystal structure of the 30S subunit from *Thermus thermophilus* complexed with different antibiotics was reported in 2000, providing important insights into the molecular mechanisms of translation as well as the mode of action of aminoglycosides (17). In this work, Carter et al. proposed a model to address how typical aminoglycoside molecules increase the affinity of the aa-tRNA for the A-site. During translation, the selection of aa-tRNA occurs by formation of a mini-helix between the codon of mRNA and the anticodon of the cognate tRNA. They propose that when this tRNA-mRNA complex is formed, two adenines (A^{1492} and A^{1493}) from 16S rRNA flip out from their intrahelical positions and form a hydrogen bonding-network with the 2′-OH groups on both sides of the codon-anticodon helix. The two adenines would sense the width of the minor groove, allowing for discrimination of distortions arising from mispairing. In the absence of any aminoglycoside molecule, some energy would be required to flip out these two adenine bases, but presumably this energy cost would be compensated by the formation of favorable interactions with the cognate aa-tRNA. By binding to the A site, the aminoglycoside stabilizes the flipped-out structure, thus reducing the energy cost of both cognate and non-cognate aa-tRNA binding and increasing aa-tRNA affinity for the A-site (17, 19). Therefore, typical aminoglycosides like paromomycin induce miscoding by mimicking the conformational change in the 16S rRNA induced by a correct codon-anticodon pair.

Indeed, it has been reported that aminoglycosides stabilize aa-tRNA binding about sixfold (15). In contrast, the rate of aminoglycoside-induced misreading ranges from 20- to 200-fold (20, 21) (depending on the codon and the antibiotic), suggesting the existence of additional mechanisms by which binding of aminoglycosides induces codon misreading (Fig. 1).

The structure of the atypical aminoglycoside streptomycin bound to the 30S subunit has also been reported (17). The data revealed that the drug makes interactions with residues from four different domains of the 16S rRNA, including U^{14} in helix 1, C^{526} and G^{527} from helix 18, A^{913} and A^{914} from helix 27 and 28, respectively, and C^{1490} and G^{1491} from helix 44. It also makes contacts with K45 from protein S12.

The reported data offers a structural rationale for the observed properties of streptomycin. It had previously been reported by Lodmell and Dahlberg (22) that there are two alternative base pairing schemes in *E. coli* rRNA during translation—one which leads to a ribosomal ambiguity (*ram*) conformation, with high affinity for tRNA which results in increased miscoding, and a second that leads to a restrictive state with low tRNA affinity—and the balance of these two states could be involved in the proofreading process (22, 23). The structural data from the streptomycin complex indicate that this aminoglycoside preferentially stabilizes the *ram* state (17), providing an explanation for the error-prone translation induced by this drug. By stabilizing the *ram* state, streptomycin would increase initial binding of non-cognate

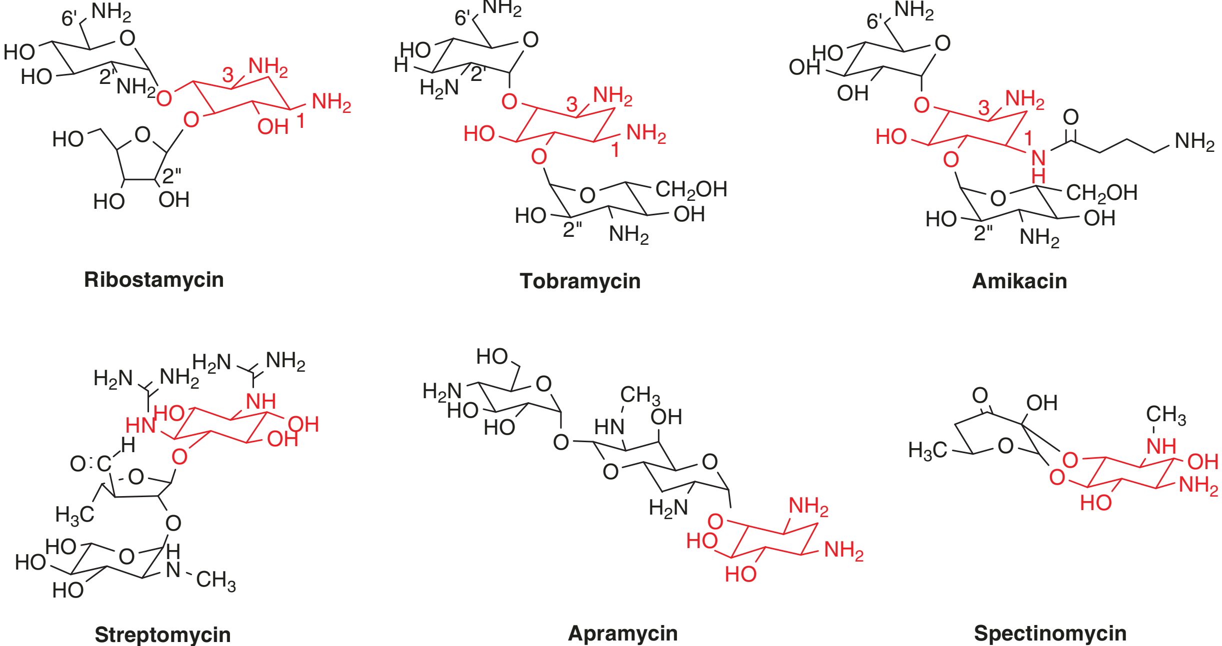

Fig. 1 Structures of typical (upper) and atypical (lower) Aminoglycosides

tRNAs as well as make the transition to the restrictive state more difficult, thereby affecting the proposed balance of such states and hence, proofreading.

Although the mechanism of action at the translational level of aminoglycosides has been extensively clarified by the data above, the connection between protein misreading and bactericidal activity remains unclear. Most antibiotics that target ribosomes are bacteriostatic, while aminoglycosides are unique inhibitors of translation that cause "cidal" activity (24). In addition to codon misreading, early studies on streptomycin have revealed an additional effect: membrane damage. Several studies showed that the treatment of *E. coli* cells with streptomycin led to the loss of intracellular nucleotides (25), amino acids (26) and K^+ (27). Later studies (12, 28, 29) have proposed that misreading would play an indirect, but essential and determinant role in the bactericidal action of aminoglycosides. The following model has been widely accepted: (1) small amounts of the antibiotic penetrate the cell, by a mechanism that is not completely understood, and binding to the A site in ribosomes that are actively elongating proteins cause a small degree of misreading; (2) the misread proteins are misfolded and are incorporated into the membrane, where it creates channels that permit a larger influx of antibiotic; (3) the intracellular antibiotic concentration rises and the drug is trapped inside the cell (30), resulting in the complete inhibition of protein synthesis, which causes bacterial death.

The only difference in the sequence of the 16S rRNA between prokaryotes and eukaryotes is at position 1408, which is adenosine in all prokaryotic and eukaryotic mitochondrial sequences, but guanosine in cytoplasmatic eukaryotic sequences. The A^{1408}–A^{1493} base pair in the bacterial ribosome creates a binding pocket for the primed ring that doesn't occur in the eukaryotic structure, explaining the specificity of the drug for the bacterial target (18, 31).

2 Mechanism of Drug Resistance

2.1 Ribosomal Mutations

Although target modification is a very common mechanism of bacterial drug resistance, clinical aminoglycoside resistance is generally not manifested by mutations of the ribosome. Most bacteria have multiple copies of the genes encoding rRNA, and thus to generate resistance, every copy of such gene would have to be mutated, and the probability of such occurrence is virtually nonexistent. *Mycobacterium* is the only genus that contains a single copy of the ribosomal operon (32) and, accordingly, is the only case in which clinical resistance due to ribosomal mutations is relevant. High-level resistance to streptomycin in *Mycobacterium tuberculosis* has been reported to result from mutations in the genes encoding two components of the ribosome, the 16S rRNA (33–35) and the S12 protein (35, 36).

The most frequently occurring mutation associated with streptomycin resistance in *M. tuberculosis* consists of point mutations in the ribosomal S12 protein encoded by gene *rpsL*. Mapping of these mutations revealed that all mutations occurred in highly conserved regions of the gene encoding one of the two critical lysines (K43 and K88) (35, 37, 38). Although structural studies have revealed that streptomycin makes direct contacts with S12 (17), mutations in this protein appear to affect streptomycin binding by perturbing the overall structure of 16S rRNA (39). In addition to streptomycin resistance, *rpsL* mutations can also lead to a different phenotype: streptomycin dependence. These mutations are associated with a hyper accurate phenotype, having extremely low translational error rates (38, 40).

The mutations in the *M. tuberculosis rrs* gene, encoding the 16S rRNA, affect two highly conserved regions, the loop 530 and the region around nucleotide 912 resulting in decreased affinity for streptomycin (1, 2, 34, 36). Recently, it has also been observed that certain mutations in the conserved 530 stem-loop of 16S rRNA also results in streptomycin dependence phenotype (33).

2.2 16s rRNA Methylation

Aminoglycoside-producing organisms have a range of defensive options available to avoid self-inactivation, including target modification and enzymatic inactivation of the drug. Members of the Actinomycetes produce inactive aminoglycosides, which are acetylated or phosphorylated molecules that are occasionally cleaved to produce the final active molecules (2, 41, 42). However, to further resist the secreted active compounds, many aminoglycoside-producing organisms also express rRNA methylases capable of modifying the 16S rRNA molecule at specific positions, thus preventing further binding of the drug (42, 43). A number of genes encoding S-adenosylmethionine (SAM)-dependent methylases have been identified from several aminoglycoside producers (44–49), and such enzymes are members of the Agr family (for aminoglycoside resistance). In aminoglycoside-producing actinomycetes, methylation of residue G^{1405} has shown to result in high-level resistance to kanamycin and gentamicin (43, 45) while methylation of residue A^{1408} gave resistance to kanamycin, tobramycin, sisomycin and apramycin, but not gentamicin (43, 44, 50). Methylation of these nucleotides abolishes important intermolecular contacts between rRNA and the aminoglycoside molecule.

Until recently, ribosomal protection by methylation of 16S rRNA had been restricted to aminoglycoside-producing actinomycetes. Recently, however, several plasmid-encoded 16S rRNA methylases, encoded by the genes *armA*, *rmtA*, *rmtB*, and *rmtC*, have emerged in clinical isolates that exhibit a high level of resistance to numerous aminoglycosides (51–55).

Analysis of the genetic environment of *armA, rmtA*, and *rmtB* genes imply that these resistance genes are present on mobile genetic elements carried by transferable large plasmids (54). Since these methylases can modify all copies of 16S rRNA, conferring a very high level of resistance against most clinically important aminoglycosides, the emergence of this type of resistance among clinically important microbial pathogens is of special concern for the future, as these genes can be easily disseminated.

2.3 Efflux-Mediated Resistance

Aminoglycosides are vital components in the treatment of infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* in cystic fibrosis patients. Such infections are difficult to treat because of intrinsic and acquired resistance caused by the expression of multidrug efflux systems in these organisms. Efflux systems are able to export an impressive variety of structurally unrelated molecules reducing the intracellular accumulation of antibiotics necessary for target inhibition. Intrinsic resistance is characterized by the constitutive expression of efflux pumps causing a natural low-level resistance to various antibiotics (56, 57). Mutations in the regulatory genes of the pumps, or induction of expression in the presence of a substrate, can lead to the overexpression of the formerly constitutive genes, causing high-level antibiotic resistance (2, 57).

From a clinical perspective, the most relevant multidrug efflux systems are members of the resistance-nodulation-division (RND) family (56). Several RND proteins have been shown to be involved in intrinsic aminoglycoside resistance in various Gram-negative pathogens (58–63). RND transporters use membrane proton motive force as energy source and are localized in the cytoplasmic membrane of Gram-negative bacteria. A membrane fusion protein (MFP) localized in the periplasmic space connects the RND transporter to the outer membrane pore (OMP) forming a continuous tripartite channel able to export substrates efficiently out of the cell (64, 65).

Early studies have shown that intrinsic low resistance to aminoglycosides, tetracycline and erythromycin, in *P. aeruginosa* is mediated by the expression of the Mex (for *m*ultiple *e*ffl*ux*) pumps, which are composed of a transmembrane protein (MexY), an outer membrane channel (OprM), and a periplasmic membrane fusion lipoprotein (MexX) (59, 60). The identity of the outer membrane channel of this tripartite system remains under debate, but auxiliary OprM-like proteins such as OpmG and OpmI may also interact with MexX and MexY to form a tripartite functional pump (66).

Later studies have proposed that the upregulation of *amrAB* genes, encoding a multidrug efflux system belonging to the NDR family in *P. aeruginosa,* also plays an important role in clinical resistance to aminoglycosides (61).

Burkholderia pseudomallei is the causative agent of melioidosis, a disease that can be rapidly fatal if manifested in acute form. This Gram-negative bacillus is intrinsically resistant to a wide range of antimicrobial agents caused by the expression of efflux systems. Studies have also identified the presence of AmrAB-OprA multidrug efflux system specific for aminoglycoside and macrolide antibiotics (67).

The *E. coli* genome contains several genes coding for RND transporters. The AcrD transporter was first identified based on the sequence similarity with the MexY sequence, and further shown to participate in the active effux of aminoglycoside molecules (63). Interestingly, recent studies have shown that AcrD not only captures aminoglycoside molecules from the cytoplasm, but also from the periplasmic space, followed by the active efflux of the drug out of the cell (68).

In mycobacteria the majority of drug efflux pumps identified so far belong to the major facilitator superfamily (MFS). Efflux-mediated resistance to aminoglycoside and tetracycline have been recently described in *Mycobacterium fortuitum* by the expression of the *tap* gene (69), as well as in *Mycobacterium bovis* and *Mycobacterium tuberculosis* by the expression of the *P55* gene (69, 70). Sequence analysis revealed 16 open reading frames encoding putative drug efflux pumps belonging to MFS class in *M. tuberculosis* (71). Such putative efflux pumps could account for streptomycin-resistant clinical isolates of *M. tuberculosis* that cannot be assigned to any other mechanism to date.

2.4 Enzymatic Drug Modification

The most common mechanism of aminoglycoside clinical resistance is the structural modification of the aminoglycoside molecule resulting from the action of intracellular bacterial enzymes that catalyze the covalent modification of specific amino or hydroxyl functions (Fig. 2). The chemically modified drug exhibits diminished binding to the A site of bacterial 16S rRNA, causing loss of antibacterial activity in resistant organisms that harbor these enzymes (72). Structural studies of aminoglycosides complexed to the 16S rRNA have highlighted the importance of several amino and hydroxyl groups for the proper binding of aminoglycoside molecules (17, 18). The N1 and N3

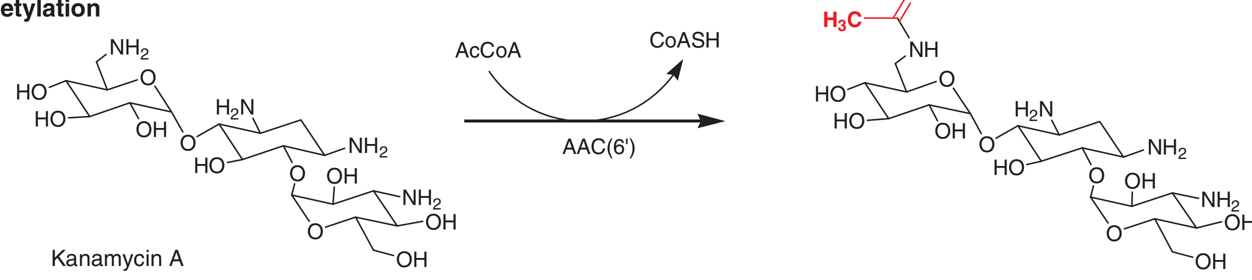

Fig. 2 Mechanisms of Enzyme-catalyzed Covalent Modification of Aminoglycosides

amino groups of the deoxystreptamine ring hydrogen-bond to nucleotides U^{1495} and G^{1494}; the 3′ and 4′-hydroxyl groups of the primed ring contact A^{1493} and A^{1492} phosphates, respectively; the 2′-amino position forms an internal hydrogen bond with the doubly primed ring that is important for the correct positioning of the primed ring; and the amino and hydroxyl groups of the triply primed ring make electrostatic interactions with the phosphate backbone of several rRNA residues. Therefore, it is clear that modifications of these conserved or semi-conserved positions would lead to deleterious effects on the binding properties and thus the antibacterial activity of the drug.

There are three classes of aminoglycoside modifying enzymes: aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside phosphotransferases (APHs), and aminoglycoside acetyltransferases (AACs). They are divided into subtypes according to which position on the drug the modification occurs. For instance, the APH(3′) modifies the 3′-hydroxyl of susceptible aminoglycosides. The enzymes are further classified on the basis of the pattern of resistance designated by a Roman numeral and, in some cases, a letter designating a specific gene. Aminoglycoside-modifying enzymes can be either plasmid or chromosomally encoded, the former being associated with transposable elements, facilitating the rapid spread of the resistance phenotype not only within a given species but also among a large variety of bacterial species.

2.4.1 Aminoglycoside Adenylyltransferases

Aminoglycoside adenylyltransferases catalyze the reaction between Mg-ATP and aminoglycoside molecules to form the *O*-adenylated aminoglycoside and the magnesium chelate of inorganic pyrophosphate. These enzymes adenylate hydroxyl groups on the positions 2″, 3″, 4′, 6, and 9 where the most relevant reactions, from a clinical perspective, are catalyzed by ANT(2″) and ANT(4′) (1, 2).

ANT(3″) is characterized by resistance to the atypical aminoglycosides streptomycin and spectinomycin, modifying the 3″-hydroxyl position of streptomycin and 9-hydroxyl group of spectinomycin (73). ANT(6) and ANT(9) adenylate 6-hydroxyl and 9-hydroxyl groups of streptomycin and spectinomycin, respectively, in Gram-positive organisms (74, 75).

ANT(2″) was first identified in a clinical isolate of *Klebsiella pneumoniea* in 1971 (76), catalyzing the *O*-adenylylation of the 2″-hydroxyl group of 4,6-substituted aminoglycoside molecules. This enzyme causes resistance to multiple aminoglycosides, since it adenylylates a broad range of substrate molecules (77). Mechanistic studies have shown that the enzyme follows a Theorell-Chance kinetic mechanism in which the nucleotide binds first followed by the aminoglycoside, pyrophosphate is released prior to the nucleotidylated aminoglycoside, and turnover is controlled by the rate-limiting release of the final product (78). Further studies by Van Pelt et al. (79) have indicated that the nucleoside monophosphate is transferred directly to the hydroxyl group of the antibiotic in one step and that the reaction proceeds through inversion of the stereochemistry about the α-phosphorous. Recent substrate specificity studies have confirmed the importance of the 2′-substitution on 2″-*O*-adenylation, where molecules containing 2′-amino groups, instead of a 2′-hydroxyl, favor adenylation to occur (77, 80).

The ANT(4′) kanamycin nucleotidyltransferase was originally isolated from clinical isolates of *Staphylococcus aureus* in 1976, adenylylating the 4″-hydroxyl group of kanamycin. The enzyme can utilize ATP, GTP, or UTP as the nucleotide substrate and can inactivate a wide range of aminoglycosides including kanamycins A, B, and C, gentamicin A, amikacin, tobramycin, and neomycins B and C (81).

Recently, a chromosomally encoded 4′-*O*-nucleotidyltransferase from *Bacillus clausii* has been reported to cause resistance to kanamycin, tobramycin, and amikacin. The *aadD2* gene was chromosomally located in all strains and was not transferable by conjugation, suggesting that *aadD2* is specific to *B. clausii* (82).

2.4.2 Aminoglycoside Phosphotransferases

The APH class of enzymes is the second-largest group of aminoglycoside-modifying enzymes. These enzymes catalyze the transfer of the γ-phosphoryl group from ATP to hydroxyl groups on aminoglycoside molecules. As a consequence, favorable electrostatic interactions that formerly existed between the hydroxyl group and specific residues on rRNA are abolished, resulting in the poor binding of the drug to its target—the ribosome. The majority of these enzymes belong to the APH(3′) subfamily, which is also the most widespread among pathogenic organisms (83).

The *aph(3′)-IIIa* gene is found primarily in Gram-positive cocci and confer resistance to a wide range of aminoglycoside antibiotics, including kanamycin, amikacin, neomycin, and butirosin (83). The three-dimensional structure of the corresponding APH(3′)-IIIa has been solved to 2.2 Å and has been shown to have significant structural similarity to eukaryotic serine/threonine and tyrosine protein kinases (EPK) (84). In addition to structural similarities, APH(3)′-IIIa is inhibited by specific EPK inhibitors (85), and is able to phosphorylate several EPK substrates (86). Recent evidence has shown that Ser/Thr kinases are not exclusive to eukaryotes and many aminoglycoside-producing organisms have been shown to encode eukaryotic-like kinases (87). Based on such evidence an attractive possible origin for APHs is that an ancestral bacterial protein kinase also provided a means of protection against the toxic effects of aminoglycosides in producing organisms, and then diverged, during evolution, for detoxification purposes.

APH(3′)-IIIa operates by a Theorell-Chance mechanism, where ATP binds prior to the aminoglycoside; the modified drug is the first product to leave, followed by the rate-limiting dissociation of ADP (88).

In Gram-positive organisms, the expression of a bifunctional enzyme 6′-*N*-acetyltransferase and 2″-*O*-phosphotransferase is responsible for high-level resistance to most aminoglycosides currently used in clinical practice (89). Both activities can be separately expressed and the kinetic properties of the bifunctional enzyme do not differ from its monofunctional counterparts (90).

Streptomycin resistance due to aminoglycoside phosphotransferases is the result of two classes of enzymes, the APH(3″) and the APH(6) (91). Both enzymes are found in the streptomycin producer *Streptomyces griseus* and the *aph (6)*-encoding gene is clustered with streptomycin biosynthetic genes. The reason for such redundancy in aminoglycoside self-defense is not known at the present.

APH(4) and APH(9) are responsible for resistance to hygromycin and spectinomycin, respectively, by phosphorylation of the 4- and 9-hydroxyl positions on the respective aminoglycoside molecules (87).

2.4.3 Aminoglycoside Acetyltransferases

Aminoglycoside acetyltransferases are the largest group of aminoglycoside-modifying enzymes and catalyze the acetyl-CoA-dependent *N*-acetylation of amino groups of typical

aminoglycoside molecules. This class of enzymes include four major subclasses, which modify the amino groups of positions 1 and 3 of the central deoxystreptamine ring as well as the 2′ and 6′ amino groups of the 2,6-dideoxy-2,6-diamino-glucose ring (2, 50, 92, 93).

The first aminoglycoside-modifying enzyme reported in bacteria was kanamycin 6′-*N*-acetyltransferase IV, first identified in 1965 by Okamoto and Suzuki (94). This enzyme was the second example (after the discovery of penicillinase) of a bacterial enzyme causing antibiotic resistance by drug inactivation or modification. AAC(6′)-IV was the subject of the development of new kinetic diagnostics of enzymatic mechanisms by Radika and Northrop (95) who used these methods to establish that AAC(6′)-IV follows a rapid equilibrium random kinetic mechanism (96).

A chromosomally encoded aminoglycoside 6′-*N*-acetyltransferase (AAC(6′)-Iy) has been identified in clinical isolates of aminoglycoside-resistant *Salmonella enterica* (97). The *aac*(6′)-Iy gene was located at the end of a long operon in sensitive strains, however, a massive 60 kbp deletion placed the constitutive *nmp* promoter directly upstream of the gene, resulting in the observed resistance phenotype. The deduced AAC(6′)-Iy sequence of 145 amino acids showed significant primary sequence homology with the Gcn5-related *N*-acetyltransferases (GNAT) superfamily. This is an enormous superfamily of enzymes (>10,000 identified to date from published sequenced genomes), whose members show sequence homology to the histone acetyltransferases (HAT) (98). To date, over three dozen members of the GNAT family have been structurally characterized, revealing a structurally conserved fold. The kinetic characterization of AAC(6′)-Iy has shown that the enzyme presents narrow acyl-donor specificity, but very broad specificity with respect to aminoglycosides containing a 6′-amino functionality. Both substrates must bind to the enzyme before catalysis occurs, and the order of substrate binding was proposed to be random (99).The structural characterization of this enzyme in 2004, confirmed that AAC(6′)-Iy is a member of the GNAT superfamily and revealed strong structural similarities with the *Sacharomyces cerevisiae Hpa*2-encoded histone acetyltransferase (100). The authors also demonstrated that AAC(6′)-Iy catalyzes acetylation of eukaryotic histone proteins. Such structural and catalytic similarities suggest that bacterial aminoglycoside acetyltransferases and eukaryotic histone acetyltransferases may be evolutionarily linked.

The *aacA29b* gene was identified from a multi-drug resistant clinical isolate of *Pseudomonas aeruginosa*, exhibiting high-level resistance to various aminoglycosides. On the basis of amino acid sequence homology, it was proposed that this gene encoded a 6′-*N*-acetyltransferase. Surprisingly, this enzyme was found to confer aminoglycoside resistance not by acetylating the drug, but by sequestering aminoglycoside molecules as a result of tight binding, thus preventing the molecule from reaching its target: the ribosome (101).

As previously discussed, high-level aminoglycoside resistance, in *E. faecalis*, is often due to the plasmid-mediated expression of the bifunctional AAC(6′)-APH(2″) (89). In *E. faecium*, intrinsic resistance is mediated by the expression of the chromosomally encoded *aac*(6′)-Ii gene, conferring low-level resistance to aminoglycosides (102).

Kinetic studies have shown that AAC(6′)-Ii follows an ordered Bi-Bi mechanism, in which acetyl-CoA binds first to the enzyme followed by the aminoglycoside (103). Chemistry is not rate-limiting, as evidenced by very small solvent isotope effects and large dependence of the maximum velocity on the solvent micro viscosity, arguing that a physical step, probably product dissociation governs the overall rate of catalysis (103). The molecular mechanism of this enzyme was investigated by mutagenesis studies and the role of several potential catalytic residues on the active site of the Enterococcal AAC(6′)-Ii were explored (104). These studies indicate that Glu72 is critical for the proper positioning and orientation of aminoglycoside substrates in the active site. In addition, the amide NH group of Leu 76 is implicated in important interactions with acetyl-CoA and transition state stabilization. The three-dimensional structure of the *E. faecium* AAC(6′)-Ii was solved at 2.7 Å resolution, revealing a compact GNAT fold (105).

In a very recent report, Robicsek et al. identified a variant of gene *aac*(6′)-Ib in clinical isolates of Gram-negative bacteria that has acquired the ability to modify fluoroquinolones (106). This enzyme was shown to reduce the activity of ciprofloxacin by *N*-acetylation of the secondary amino nitrogen of its piperazinyl substituent. The acquisition of this additional substrate activity by an aminoglycoside acetyltransferase represents a notable adaptation that justifies considerable future concern.

AAC(2′) is a class of aminoglycoside acetyltransferases with significantly more restricted occurrence in bacteria. All *aac*(2′) genes reported so far are chromosomally encoded and universally present in mycobacteria, where the physiological role is not understood (107). The *aac*(2′)-Ic gene of *M. tuberculosis* was cloned and expressed in *E. coli* and the purified enzyme acetylated all aminoglycoside substrates tested in vitro. Dead-end inhibition studies as well as alternative substrate diagnostic studies supported an ordered sequential mechanism with a degree of randomness, where binding of acyl-CoA is preferred followed by the aminoglycoside. The enzyme is able to perform both *N*-acetyl as well as *O*-acetyl transfer (108). The *aac*(2′) genes are not responsible for clinical resistance in mycobacteria.

The AAC(3) family of aminoglycoside acetyltransferases regioselectively modify the 3-amino group of the deoxy-streptamine ring, and at present this family includes five major types: I–V, based on the pattern of aminoglycoside resistance that they confer. As previously discussed, the 3-amino group is found in all aminoglycosides and is required for specific binding of these molecules to the A site of the rRNA. Acetylation at this position would disrupt crucial interactions required for specific binding, resulting in poor binding to the ribosome.

The AAC(3) I and II isoenzymes preferentially modify the gentamicin group of aminoglycosides (109, 110). Initial velocity, product, dead-end, and substrate inhibition studies have reveled that this enzyme follows a random Bi-Bi kinetic mechanism where both substrates must bind to the enzyme active site before catalysis can occur (111).

AAC(3)-III enzymes catalyze the covalent acetylation of a wide variety of aminoglycosides including gentamycin, tobramycin and neomycin (112).

The AAC(3)-IV enzyme was the first aminoglycoside-modifying enzyme identified as capable of modifying the novel aminoglycoside used for veterinary use, apramycin (113). This enzyme was originally found in *E. coli* and *S. typhimurium* animal isolates (113, 114) but was quickly identified in human clinical isolates from hospitalized patients (115), representing a serious concern given the activity of this enzyme with essentially all therapeutically useful aminoglycosides (116). The enzyme from *E. coli* has been recently kinetically characterized, revealing the broadest aminoglycoside specificity range of all AAC(3) enzymes (116). Dead-end inhibition and isothermal titration calorimetry (ITC) experiments reveled that the enzyme follows a sequential, random, Bi-Bi kinetic mechanism. Substrate specificity studies showed that acylation at the 1-*N* position sterically interfere with 3-*N* acetylation. Similar results have been observed with other AAC(3) enzymes, including AAC(3)-III and AAC(3)-I. Sequence alignment studies indicate that this enzyme is not a member of the GNAT superfamily, but currently no structural data have been reported to confirm such findings.

The last member of the AAC(3) class of enzymes identified to date, was AAC(3)-V, isolated from a clinical isolate of *Pseudomonas aeruginosa* resistant to kanamycin, gentamicin, tobramycin, and sisomycin (117).

The only member of this class of enzymes to be structurally characterized to date is the *Serratia marascens* AAC(3)-I (118). The monomer fold was typical of the GNAT superfamily, with the characteristic central antiparallel β-sheet containing two amino-terminal helices on one side of the sheet and the two carboxy-terminal helices on the other.

3 Mechanism of the Spread of Resistance

In general, the process by which bacteria become resistant to antibiotics occurs either by mutations or by horizontal gene transfer; in which one bacterium transfers genetic material to another, either of the same or different genus. The rapid spread of drug resistance among pathogenic bacteria is usually attributed to horizontal gene transfer since the development of antimicrobial resistance by mutational changes is a relatively slow process (119, 120).

The natural history of the emergence of bacterial resistance has been proposed to involve gene transfer from antibiotic-producing soil organisms to Gram-positive bacteria, and then to Gram-negative bacteria (120). Many of the genes that mediate resistance are found on transferable plasmids or on transposons that can be disseminated among various bacteria. Transposons are mobile pieces of DNA that can insert themselves into various locations on the bacterial chromosome, as well as move into plasmids or bacteriophage DNA (121).

Three mechanisms of gene transfer in bacteria have been identified: transformation, which involves the uptake and incorporation of naked DNA; conjugation, which depends on cell–cell contact to transfer DNA elements; and transduction whereby the host DNA is encapsulated into a bacteriophage that acts as the vector for its injection into a recipient cell (122).

The rapid dissemination of aminoglycoside resistance among pathogenic organisms has been largely attributed to conjugation of plasmids and non-replicative transposons among bacteria (119, 120, 123, 124). A clinical example of the ongoing importance of conjugative plasmid transfer on resistance to aminoglycosides is the shocking case of untreatable and fatal neonatal septicemia mediated by *Klebsiella pneumonia* EK105, which carries a mobile plasmid encoding resistance to amikacin, ampicillin chloramphenicol, kanamycin, streptomycin, tobramycin, netilmicin, oxacillin, gentamicin, and mezlocillin (125).

Although aminoglycosides are not first-line therapy for staphylococcal infections, the recent increase in nosocomial infections caused by aminoglycoside-resistant strains is worrisome because it is often associated with resistance to drugs commonly used to treat staphylococcal infections (126). In addition, aminoglycoside resistance plasmids can reside in avirulent *Staphylococcus epidermides* strains present in skin flora of ill patients, being a reservoir that can be further transferred to virulent strains via conjugative transfer (127, 128). Recent studies have shown that 80% of methicillin-resistant *S. aureus* (MRSA) infections showed resistance to multiple aminoglycosides including gentamycin, tobramycin, kanamycin, amikacin, astromicin, and arbekacin, where 56% of such cases carried a transferable plasmid encoding a bifunctional

aminoglycoside-modifying enzyme AAC(6′)-APH(2″) (129). The gene *aac*(6′)-*aph*(2″) is present in the Tn 4100-like transposon which is inserted in both the R plasmid and the chromosome of aminoglycoside-resistant isolates (89).

The worldwide-disseminated *armA* gene confers high-level resistance to essentially all clinically important aminoglycosides by methylation of the 16S rRNA. Recent studies have shown that *armA* gene is part of the functional transposon Tn-1548 together with an *ant*(3″)(9) gene (130, 131). The reported data suggest that *armA* gene is spread by conjugation followed by transposition. This combination accounts for the worldwide dissemination of aminoglycoside resistance by 16S rRNA methylases in pathogenic organisms.

The fact that bacteria produce a remarkable array of tools to overcome the toxic effects of antimicrobials is already alarming. But the fact that such genetic information is located in mobile DNA elements, which can be easily and rapidly disseminated between most diverse bacteria, is particularly worrisome. Increased incidence of multi-drug resistant bacteria and rising evidence of resistance transfer from one organism to another may lead to increasing emergence of nosocomial pathogens for which there is no antibiotic solution.

4 Cross-Resistance

Aminoglycosides are often combined with a β-lactam drug in the treatment of infections caused by staphylococcal, enterococcal, and streptococcal strains (132). Resistance to β-lactams is usually caused by expression of β-lactamases, which are enzymes capable of hydrolyzing the β-lactam ring of penicillins, cephalosporins and related antimicrobial drugs, rendering them inactive. Since the report of the first β-lactamase-producing organism in 1983, β-lactam resistance is often associated with high-level resistance to aminoglycosides. In fact, genes encoding β-lactamases are usually carried on transferable plasmids that often also contain aminoglycoside resistance genes (119, 121, 133, 134). The resulting cross-resistance can make serious enteroccocal infections, such as endocarditis, extremely difficult to treat. Alarmingly, strains of *E. faecium* resistant to all known antibiotics have emerged as lethal pathogens in intensive care units in hospitals across the United States (24).

5 Alternative Agents

Amikacin is a semi-synthetic derivative prepared from kanamycin A by acylation of the 1-amino group of the 2-deoxystreptamine ring with 2-hydroxy-4-aminobutyric acid. Because of this structural modification, amikacin is less susceptible to the action of many aminoglycoside-modifying enzymes, and therefore is especially effective in the treatment of bacteria resistant to other aminoglycosides (24, 50, 135).

After the success of amikacin in circumventing drug inactivation by modifying enzymes, other 1-*N* substituted derivatives, like isepamicin and arbekacin, were synthesized. In these derivatives, the 1-amino substitution protects against modification at 2″-hydroxyl and 3-amino positions, most likely by steric hindrance. This valuable feature explains the broad success and utility of the 1-amino substituted derivatives in situations of resistance to kanamycin, gentamicin, or tobramycin. These compounds, however, are still largely susceptible to ANT(4′) enzymes (81).

Dibekacin (a 3′,4′-dideoxykanaymcin B derivative) was rationally designed to circumvent inactivation by the APH(3′) and ANT(4′) enzymes. Further modification of this drug by addition of a 4-amino-2-hydroxybutyryl group on the 1-amino group produced arbekacin. Arbekacin is particularly successful against MRSA, and has been used in Japan since 1990 (135, 136). However, strains of *S. aureus* resistant to arbekacin were recently isolated, where a mutation in the *aac*(6′)-*aph*(2″) gene permits arbekacin acetylation at the 4″ position (137).

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Chapter 15
Tetracycline and Chloramphenicol Resistance Mechanisms

Marilyn C. Roberts and Stefan Schwarz

1 Introduction

1.1 Tetracycline Resistance

Tetracycline resistance (Tc^r) is most often due to the acquisition of new genes, which code for energy-dependent efflux of tetracyclines, or for a protein that protects bacterial ribosomes from the action of tetracyclines. Many of these genes are associated with mobile plasmids or transposons and can be distinguished from each other using molecular methods including DNA-DNA hybridization with oligonucleotide probes and DNA sequencing. A limited number of bacteria acquire Tc^r by mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, change regulation of innate efflux systems, or alter the 16S rRNA (1). Currently, two genes are considered related, i.e., of the same class, and given the same gene designation if their gene products share ≥80% of the amino acid sequences in common with each other. Two genes are considered different from each other if their gene products share ≤79% amino acid sequence identity (2). This comparison can now be done using the GenBank sequence information.

Thirty-five different tetracycline resistance (*tet*) genes and three oxytetracycline resistant (*otr*) genes have been characterized (Table 1). The oxytetracycline genes were first identified in oxytetracycline producing organisms, and thus the nomenclature reflers to the organisms first shown to carry the particular gene. Currently there are 23 efflux genes, 11 ribosomal protection genes, three enzymatic genes, which modify and inactivate the tetracycline molecule, and one gene, the *tet*(U) gene, has an unknown resistance mechanism (Table 1). The *tet*(P) is unusual because it has a functional efflux protein coded by the *tetA*(P) gene linked to a *tetB*(P) gene, which codes for a ribosomal protection type protein. The *tetA*(P) has been found without the *tetB*(P) gene, but the *tetB*(P) gene has not been found alone (5). Recently the *otr*(C) has been sequenced and it codes for an efflux protein (GenBank AY509111).

1.2 Chloramphenicol Resistance

Chloramphenicol resistance (Cm^r) is most often due to the presence of chloramphenicol acetyltransferases (CATs) which inactivate chloramphenicol (6). There are two different types of CAT enzymes which are genetically unrelated (Table 2). Cm^r may also be due to the efflux of chloramphenicol via specific membrane-associated transporters (11). Both, the genes coding for CATs and specific exporters, are often associated with plasmids, transposons or gene cassettes. Some chromosomal multidrug transporters have also been identified which export chloramphenicol (12). Cm^r may also occur from mutations which reduce the expression of outer membrane proteins (13), mutations in the 23S rRNA (14), inactivation of chloramphenicol by 3-*O*-phosphotransferases (15) or target site modification by a 23S rRNA methylase (4, 8).

In contrast to the tetracycline resistance genes, there is no internationally accepted nomenclature for chloramphenicol resistance genes currently available. However, when using the same criteria as for the classification of *tet* genes, 22 groups of classical chloramphenicol acetyltransferase genes (*cat*), at least five groups of *cat* genes of the second type, which occasionally are referred to as *xat* genes (6), and 11 different groups of genes coding for specific exporters can be distinguished (Table 2). In addition, a single gene, *cfr*, is known to code for the aforementioned rRNA methylase and to mediate resistance by target site modification (4, 8). During whole genome sequencing, cat-like genes have been annotated in the genomes of several bacteria, e.g.,

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Table 1 Mechanism of resistance for characterized *tet* and *otr* genes

Efflux (23)	Ribosomal protection (11)	Enzymatic (3)	Unknown[a]
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E)	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32),	*tet*(X)	*tet*(U)
tet(G), *tet*(H), *tet* (J), *tet*(V), *tet*(Y)	*tet*(Q), *tet* (T), *tet*(36)	*tet*(34)	
tet(Z), *tet*(30), *tet*(31), *tet*(33)	*otr*(A), *tetB*(P)[b], *tet*	*tet*(37)	
tet(35)[d]			
tet(39)			
tet(K), *tet*(L), *tet*(38)			
tetA(P)			
otr(B), *otr*(C)			
tcr3			

[a]*tet* (U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins
[b]*tetB*(P) is not found alone and *tetA*(P) and *tetB*(P) are counted as one operon
[c]*tet*(X) and *tet*(37) are unrelated but both are NADP-requiring oxidoreductases: *tet*(34) similar to the xanthine-guanine phosphoribosyl transferase genes of *V. cholerae*
[d]Not related to other *tet* efflux genes
Ref. (3) In 2008 3 new efflux genes were characterized, *tet*(40), *tet*(41), *tet*(42)

Brucella melitensis (GenBank NC_003317) and *Bacillus cereus* (GenBank NC_004722). However, comparisons on both nucleotide and amino acid level revealed little, if no homology with the known chloramphenicol resistance determinants and it has not been demonstrated whether these *cat*-like genes actually confer chloramphenicol resistance. More information on phenicol resistance can be found in two recent reviews (9, 10).

2 Mechanisms of Tetracycline Resistance

2.1 *Tetracycline Resistance Due to Efflux Proteins*

The tetracycline resistance efflux proteins are part of the Major Facilitator Superfamily (MFS) and share amino acid and protein structure similarities with other efflux proteins involved in multiple-drug resistance, quaternary ammonium resistance and chloramphenicol and quinolone resistance (12). All the *tet* and *otr* efflux genes code for membrane-associated proteins which export tetracycline from the cell which reduces the intracellular drug concentration and thereby protects the ribosomes within the cell. Efflux genes are found in both Gram-positive and Gram-negative species (Table 3). The Gram-negative *tet*(B) gene codes for an efflux protein, which confers resistance to both tetracycline and minocycline, but not to the new glycylcyclines (1). All the other efflux proteins confer resistance to tetracycline, but not to minocycline or glycylcyclines.

Each of the *tet* efflux genes codes for an approximately 46 kDa membrane-bound efflux protein. The efflux proteins exchange a proton for a tetracycline–cation complex against a concentration gradient (21). The Gram-negative efflux genes have two functional domains, α and β which correspond to the N- and C-terminal halves of the protein respectively, and data suggests that residues dispersed across the protein are important for function (22). Mutations affecting energy coupling have been located in cytoplasmic loops 2–3 and 10–11 of the efflux protein (23).

The Tet(A), (B), (C), (D), (E), (G), (J), (H), (Z), (24), and (25) proteins share 41–78% amino acid homology. All have a repressor protein upstream of the structural gene (16, 26). These tetracycline repressor genes are read in the opposite direction from the structural gene. The repressor proteins share 37–88% amino acid identity. All these genes are of Gram-negative origin except for the *tet*(Z) and *tet*(33) genes which are found in Gram-positive bacteria (Table 3). The *tet*(Z) and *tet*(33) genes have repressor genes upstream from the structural gene, like the Gram-negative efflux genes, and are the first Gram-positive efflux proteins to be controlled by a repressor protein (16, 17, 26).

In contrast, the Gram-positive *tet*(K) and *tet*(L) efflux genes are not regulated by repressors, share 58–59% amino acid identity and confer resistance to tetracyclines, but not to minocycline. The *tet*(K) and *tet*(L) genes are generally found on small transmissible plasmids, which on occasions become integrated into the chromosome. They have primarily been found in Gram-positive species, but both of these genes have been found in a few facultative and/or anaerobic Gram-negative genera where they appear to confer tetracycline resistance (Table 3) (3).

A small number of plasmid borne *tet*(L) genes have been sequenced and share between 98 and 99% sequence identity with each other, while the chromosomal *tet*(L) gene from *B. subtilis* has only 81% amino acid sequence identity with the other *tet*(L) genes (27). The genes are transcribed from a single promoter. The *tet*P operon from *Clostridium perfringens* consists of two overlapping genes. The *tetA*(P) gene codes for a efflux protein, but does not have the conserved motifs that are common in the other tetracycline efflux proteins (28) and the *tetB*(P) gene which codes for a protein with amino acid identity of 37–39% to the Tet(M) ribosomal protection protein. Less work has been done with the efflux proteins found in *Streptomyces* sp. [Otr(B), Otr(C) and Tcr3], and *Mycobacterium* sp. [Tet(V)] (3).

Table 2 Distribution of chloramphenicol resistance genes

Mechanism	Gene designation(s)	Number of genera	Genera
Enzymatic inactivation CAT (classical)	*cat*, *catI*, *catA1*, *pp-cat*	12	*Achromobacter*, *Acinetobacter*, *Aeromonas*, *Corynebacterium*, *Escherichia*, *Klebsiella*, *Photobacterium*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Yersinia*
	cat, *catII*, *catA2*	8	*Aeromonas*, *Agrobacterium*, *Escherichia*, *Haemophilus*, *Klebsiella*, *Photobacterium*, *Shigella*, *Vibrio*
	cat, *catIII*, *catA3*	5	*Eubacterium*, *Mannheimia*, *Pasteurella*, *Shigella*, uncultured bacterium
	cat, *cat(pC221)*, *catC*	4	*Bacillus*, *Enterococcus*, *Staphylococcus*, *Streptococcus*
	cat, *cat(pC223)*	4	*Enterococcus*, *Lactococcus*, *Listeria*, *Staphylococcus*
	cat, *cat(pC194)*, *cat-TC*	3	*Enterococcus*, *Lactococcus*, *Staphylococcus*, *Streptococcus*
	catP, *catD*	2	*Clostridium*, *Neisseria*
	cat	1	*Proteus*
	cat	1	*Streptomyces*
	cat	1	*Deinococcus*
	cat	1	*Zymomonas*
	cat	1	*Bacillus*
	catS	1	*Streptococcus*
	cat	1	*Campylobacter*
	cat	1	*Listonella*
	cat	1	*Bacillus*
	catB	1	*Clostridium*
	catQ	1	*Clostridium*
	cat86	1	*Bacillus*
	cat	1	*Bacteroides*
	cat	1	*Clostridium*
	cat	1	*Clostridium*
CAT (second type)[a]	*cat*, *catB1*	1	*Agrobacterium*
	cat, *catB2*, *catB3*, *catB4*, *catB5*, *catB6*, *catB8*, *catB10*	15	*Acinetobacter*, *Aeromonas*, *Bordetella*, *Enterobacter*, *Escherichia*, *Eubacterium*, *Klebsiella*, *Marinomonas*, *Morganella*, *Pasteurella*, *Pseudomonas*, *Riemerella*, *Shewanella*, *Salmonella*, uncultured bacterium
	catB7	1	*Pseudomonas*
	catB9	1	*Vibrio*
	cat	1	*Vibrio*
Efflux			
Specific exporters	*cmlA*, *cmlA1*, *cmlA2*, *cmlA4*, *cmlA5*, *cmlA6*, *cmlA7*, *cmlB*	8	*Acinetobacter*, *Aeromonas*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella*, uncultured bacterium
	cmlB1	1	*Bordetella*
	cmlA-like, *floR*, *flo*, *pp-flo*	9	*Acinetobacter*, *Escherichia*, *Klebsiella*, *Pasteurella*, *Photobacterium*, *Salmonella*, *Stenotrophomonas*, *Vibrio*
	cml	1	*Escherichia*
	fexA	1	*Staphylococcus*
	cmr, *cmx*	1	*Corynebacterium*
	cmr, *cmrA*	1	*Rhodococcus*
	cml	1	*Streptomyces*
	cmlv	1	*Streptomyces*
	nd[a]	1	*Mycobacterium*
	nd	1	*Nocardia*
Target site modification			
Methylase	*cfr*	1	*Staphylococcus*

[a]No designation
Refs. (4, 7–10)

Table 3 Distribution of *tet* and *otr* genes

Gene	Number of genera	Genera
Efflux		
tet(A)	17	*Aeromonas*, *Actinobacillus*, *Acinetobacter, Bordetella Citrobacter*, *Edwardsiella*, *Escherichia*, *Haemophilus*, *Klebsiella*, *Plesiomonas*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Veillonella*, *Vibrio*
tet(B)	25	*Actinobacillus*, *Acinetobacter*, *Aeromonas*, *Citrobacter*, *Brevundimonsa*, *Erwinia*, *Enterobacter*, *Escherichia*, *Haemophilus*, *Klebsiella*, *Mannheimia*, *Moraxella*, *Neisseria*, *Pantoea*, *Pasteurella*, *Photobacterium*, *Plesiomonas*, *Proteus*, *Providencia*, *Pseudomonas*, *Treponema*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*
tet(C)	14	*Aeromonas, Bordetella, Citrobacter*, *Chlamydia*, *Enterobacter*, *Escherichia*, *Francisella*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*
tet(D)	14	*Aeromonas*, *Alteromonas*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pasteurella*, *Photobacterium*, *Plesiomonas*, *Salmonella*, *Shigella*, *Yersinia*, *Vibrio*
tet(E)	7	*Aeromonas*, *Alcaligenes*, *Escherichia*, *Providencia*, *Pseudomonas*, *Serratia*, *Vibrio*
tet(G)	8	*Escherichia*, *Mannheimia*, *Pasteurella*, *Providencia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Vibrio*
tet(H)	5	*Actinobacillus*, *Acinetobacter*, *Mannheimia*, *Moraxella*, *Pasteurella*
tet(J)	1	*Proteus*
tet(K)	12	*Bacillus*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Haemophilus*, *Listeria*, *Mycobacterium*, *Norcardia*, *Peptostreptococcus*, *Staphylococcus*, *Streptococcus*, *Streptomyces*
tet(L)	21	*Actinobacillus, Acinetobacter*, *Actinomyes*, *Bacillus*, *Clostridium*, *Enterococcus*, *Fusobacterium*, *Geobacillus*, *Listeria, Mannheimia, Morganella, Mycobacterium, Norcardia, Pasteurella, Pedicoccous, Peptostreptococcus*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Veillonella*
tetA(P)	1	*Clostridium*
tet(V)	1	*Mycobacterium*
tet(Y)	1	*Escherichia*
tet(Z)	1	*Corynebacterium*
tet(30)	1	*Agrobacterium*
tet(31)	1	*Aeromonas*
tet(33)	1	*Corynebacterium*
tet(34)	4	*Aeromonas*, *Pseudomonas*, *Serratia*, *Vibrio*
tet(35)	2	*Stentrophomonas*, *Vibrio*
tet(38)	1	*Staphylococcus*
tet(39)	1	*Acinetobacter*
tcr3	1	*Streptomyces*
otr(B)	2	*Mycobacterium*, *Streptomyces*
otr(C)	1	*Streptomyces*
Ribosomal protection		
tet(M)	45	*Abiotrophia*, *Acinetobacter*, *Actinomyces, Aerococcus*, *Afipia*, *Bacteriodes*, *Bacillus*, *Bacterionema*, *Bifidobacterium*, *Catenibacterium*, *Clostridium*, *Corynebacterium*, *Eikenella*, *Enterococcus*, *Enterobacter*, *Erysipelothrix*, *Escherichia*, *Eubacterium*, *Fusobacterium*, *Gardnerella*, *Gemella*, *Granulicatella*, *Haemophilus*, *Kingella*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Listeria*, *Microbacterium*, *Mycoplasma*, *Mycobacterium*, *Neisseria*, *Pantoea*, *Pasteurella*, *Peptostreptococcus*, *Photobacterium*, *Prevotella*, *Ralstonia*, *Selenomonas*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Ureaplasma*, *Veillonella*, *Vibrio*
tet(O)	15	*Anaerovibrio*, *Aerococcus*, *Butyrivibrio*, *Campylobacter*, C*lostridium Enterococcus*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, *Megasphaera, Mobiluncus*, *Neisseria*, *Peptostreptococcus*, *Staphylococcus*, *Streptococcus*
tet(S)	5	*Enterococcus*, *Lactococcus*, *Lactobacillus*, *Listeria*, *Veillonella*
tetB(P)	1	*Clostridium*
tet(Q)	18	*Anaerovibrio*, *Bacteriodes*, *Capnocytophaga*, *Clostridium*, *Eubacterium*, *Gardnerella*, *Lactobacillus*, *Mitsuokella*, *Mobiluncus*, *Neisseria*, *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Ruminococcus*, *Selenomonas*, *Streptococcus*, *Subdoligranulum*, *Veillonella*
tet(T)	1	*Streptococcus*
tet(W)	22	*Acidaminococcus*, *Actinomyces, Arcanobacterium*, *Bacillus*, *Bacteriodes*, *Bifidobacterium*, *Butyrivibrio*, *Clostridium*, *Fusobacterium*, *Lactobacillus*, *Mitsuokella*, *Megasphaera*, *Neisseria*, *Prevotella*, *Porphyromonas*, *Roseburia*, *Selenomonas*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Subdoligranulum*, *Veillonella*
tet(32)	2	*Clostridium*, *Eubacterium*
tet(36)	2	*Bacteroides*, *Clostridium*
tet	1	*Streptomyces*
otr(A)	1	*Mycobacterium, Streptomyces*
Enzymatic		
tet(X)	1	*Bacteroides*
tet(34)	4	*Aeromonas, Pseudomonas, Serratia, Vibrio*
tet(37)	1	Unknown
Unknown		
tet(U)	2	*Staphylococcus*

Refs. (1, 3, 16–20)

2.2 Tetracycline Resistance due to Ribosomal Protection Proteins

Eleven ribosomal protection proteins have been described (Tables 1 & 3). These genes code for cytoplasmic proteins that protect the ribosomes from the action of tetracycline in vitro and in vivo and confer resistance to tetracycline, doxycycline, and minocycline. Most studies have used the Tet(M) and/or Tet(O) proteins, though it is thought that all the 11 proteins have the same mechanism of resistance (1, 29). These proteins have sequence similarity to the ribosomal elongation factors, EF-G and EF-Tu, and are grouped into the translation factor superfamily of GTPases. The ribosomal protection proteins can bind and hydrolyze GTP in a ribosome-dependent manner, although they cannot be substituted for the elongation factors in vivo or in vitro. This differs from the efflux proteins, which require intact membranes to function. Taylor et al. 2003 (29) suggest that they may be evolutionarily derived from the elongation factors. The ribosomal protection proteins interact with the base of h3g. A recent detailed review can be found in this paper (29), but briefly the ribosomal protection proteins bind to the ribosome at the base of h4, which causes an allosteric disruption of the primary tetracycline binding site. This results in the release of the tetracycline molecules from the ribosome. This action allows the ribosome to return to its normal post-translocational conformational state, which was altered by the binding of tetracycline. Once the normal conformation occurs, protein synthesis begins (29).

The Tet(M), Tet(O), Tet(S), Tet(T), Tet(W), Tet(32) share between 78 and 68% amino acid identity (30, 31) but the G+C content of these genes varies from a low of 33% for the *tet*(S) gene to a high of 53% in the *tet*(W) gene and form one homology group. Recently, a recombination event between two 2 tetracycline-resistance genes [*tet*(O) and *tet*(W)] has been identified in tetracycline resistant *Megasphaera elsdenii* (32). Mosaic genes have been found in this species which include parts of *tet*(O), *tet*(W) and/or *tet*(32) genes (18).

The Tet(Q) and Tet(T) proteins form a second homology group, while the TetB(P) and Otr(A) form a third homology group. The *tet*(M) gene is usually associated with conjugative transposons like Tn*916* (33) Within this family of transposons large elements carrying 2–4 different antibiotic resistance genes have also been identified including an *erm*(B) gene coding for an rRNA methylase which confers macrolide, lincosamide and streptogramin B resistance, a gene for a classical *cat* and an aminoglycoside phosphotransferase, *aphA-3*, encoding kanamycin resistance (Km^r) (1). The combination of *tet*(M) and *erm*(B) genes is often found in Gram-positive streptococci, staphylococci, and enterococci (1). The presence of the classical *cat* and *aphA-3* genes within common transposons may explain why Cm^r and/or Km^r *Streptococcus pneumoniae* strains continue to be isolated in areas where the use of these antibiotics has been stopped (33).

The *tet*(Q) gene is associated with large (65–>150 kb) conjugative transposons. Most of these elements carry both *tet*(Q) and *erm*(F) and have been found in a number of aerobic and aerobic genera (24). A region of the transposon is required and is sufficient for conjugal transfer of the element, and for mobilization of both co-resident plasmids, and mediates excision and circularization of discrete nonadjacent segments of chromosomal DNA in *Bacteroides* (34).

2.3 Tetracycline Resistance due to Enzymatic Inactivation

The *tet*(X) gene encodes an enzyme, which modifies and inactivates the tetracycline molecule. However, it does not seem to have much clinical relevance since it requires oxygen to function and is found only in a strict anaerobe, *Bacteroides*, where oxygen is excluded (35). Thus, it is unlikely that the *tet*(X) gene functions in its natural host (*Bacteroides*). The Tet(X) is a cytoplasmic protein that chemically modifies tetracycline in the presence of both oxygen and NADPH, and semi-synthetic drug tigecycline (35a). Sequence analysis indicates that this protein shares amino acid homology with other NADPH-requiring oxidoreductases and degrades tetracyclines, including tigecycline. The tet(X) gene has now been found in an aerobic Gram-negative Tcr Sphingobacterium sp., isolated from agricultural soil. Recently a *tet*(X) positive anaerobic Gram-negative Tc^r *Sphin-gobacterium* sp., isolated from agricultural soil, was identified which degraded tetracycline (35b). More recently, a second gene, *tet*(37), has been identified from the oral cavity of man (25). This also requires oxygen to function. Unfortunately, no attempt to determine the host(s) of this gene has been done, though it will be of great interest to determine if this gene is also associated with anaerobes. Tet(36) is similar to the xanthine-guanine phosphoribosyl transferase genes of *V. cholerae* (3).

2.4 Other/Unknown Mechanisms of Resistance

The *tet*(U) gene confers low-level tetracycline resistance (1). This gene produces a small protein (105 amino acids) which is smaller than the efflux and the ribosomal proteins. There is 21% similarity over the 105 amino acids between the Tet(U) and Tet(M) proteins beginning close to the carboxy terminus of the latter. These similarities do not include the consensus GTP-binding sequences, important for resistance in the ribosomal protection proteins. Thus it is unclear what the mechanism of resistance is.

2.5 Tetracycline Resistance due to Mutations

Laboratory-derived mutations in the *tet*(A) or *tet(*B) gene have led to glycylcycline resistance, suggesting that bacterial resistance may develop over time and with clinical use of glycylcycline (1). Mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, can also affect the bacterial host's resistance to tetracycline. Tc^r *Helicobacter pylori* and *Mycobacterium avium* complex also have mutations. Mutations that up-regulate innate efflux pumps can alter the host's susceptibility profile. One example is *Neisseria gonorrhoeae* which has an innate *mtrCDE*-encoded efflux pump. A 1 bp deletion of an A within the 13 bp inverted repeat sequence of the *mtrR* promoter region leads to a four-fold increased resistance to tetracycline, penicillin, and erythromycin (36). In *N. gonorrhoeae* the chromosomally mediated resistance is often more common than plasmid-mediated antibiotic resistance (37). Mutations in efflux pumps have been documented in a variety of other Gram-negative species (1).

3 Mechanisms of Chloramphenicol Resistance

3.1 Chloramphenicol Resistance due to Chloramphenicol O-Acetyltransferases

Both types of CAT enzymes have a trimeric structure normally composed of three identical monomers which is encoded by the *cat* gene (6). The classical CAT monomers vary between 207 and 238 amino acids, whereas those of the second type of CATs are smaller with 209 to 219 amino acids. All CATs transfer an acetyl group from a donor molecule (usually acetyl-CoA) to the C3 position of the chloramphenicol. This acetyl group is then shifted from C3 to C1 and the C3 position is again available for a second acetylation step. Neither the mono- or di-acetylated chloramphenicol molecules have antimicrobial activity (6). None of the CAT enzymes are able to inactivate florfenicol, a chloramphenicol derivative that is exclusively licensed for use in animals (38), because the C3 position is fluorinated in the florfenicol molecule. As a result, the C3 position of florfenicol cannot act as an acceptor site for the acetyl groups making florfenicol resistant to inactivation by these enzymes.

The classical CATs represent a highly diverse group of enzymes which show an overall identity of 44%. These enzymes have been detected in Gram-positive and Gram-negative, aerobic and anaerobic bacteria (Table 2). They can be placed into 22 genetic groups using ≥80% amino acid identity to define a group. However, fifteen of the groups have a single gene from a single species (Table 2). The CATI, CATII and CATIII, which represent members of the first three genetic groups in Table 2, are exclusively found in Gram-negative genera and are expressed constitutively. The genes coding for these enzymes have been completely sequenced and the biochemical and enzymatic characteristics of the proteins studied in detail (39). The CATIII enzyme was the first to be crystallized and provided insight into the folding of the CAT monomers and helped to identify the amino acids that were important for the structure and the function of the CAT enzyme (40).

The next three genetic groups of classical CAT were named according to the plasmids (pC221, pC223/pSCS7, and pC194), on which they were first detected. These have been identified in a variety of Gram-positive genera (Table 2). The K_M values for chloramphenicol and acetyl-CoA, the isoelectric point, pH optimum, and thermostability for the CATs associated with the Gram-positive plasmids have been determined (39, 41). These *cat* genes are induced by chloramphenicol and have translational attenuators located immediately upstream of the respective *cat* genes which resemble those located upstream of the tetracycline resistance genes *tet*(K) and *tet*(L) (42).

The closely related CATP and CATD proteins were first identified in the Gram-positive anaerobe *Clostridium* sp. where they are located on transposons (43). These genes have also been identified in Cm^r Gram-negative *Neisseria meningitidis* (44, 45). This group is unusual because members are found in both Gram-positive and Gram-negative genera (Table 2). Both genes are expressed constitutively.

The second type of CAT enzymes is only distantly related to the classical CATs and they are structurally similar to acetyltransferases involved in streptogramin A resistance (6). At least five different genetic groups can be distinguished, though all enzymes have approximately 77% identity with each other. These *cat* genes are often associated with gene cassettes and integrons in Gram-negative bacteria (46). Some of these *cat* genes have also been identified in transposons. The CAT protein from *Agrobacterium tumefaciens* has different acetylation kinetics when compared to the classical CATIII enzyme (6). This difference might explain the distinctly lower chloramphenicol MIC mediated by this CAT protein. Because of this lower resistance level, it was speculated that members of this second type of CATs might have a physiological role, other than chloramphenicol resistance (Cm^r), in their host bacteria, though little else is known about these enzymes (6).

3.2 Chloramphenicol Resistance due to Specific Exporters

The specific exporters involved in the export of either chloramphenicol or chloramphenicol and florfenicol are members of the Major Facilitator Superfamily of efflux proteins (12) and commonly exhibit 10–14 transmembrane

segments (TMS) (11). There are eleven genetic groups, though nine are found in a single genus including six from soil and environmental bacteria (Table 2). The *cmr* and *cmx* genes are found on plasmids, while the *cmx* gene is associated with a transposon (11). The *Rhodococcus* genes are associated with plasmids, while the *cmrA* is located on transposon Tn*5561*. The *Streptomyces venezuelae* Cmlv protein is thought to play a role in self-defense of the antibiotic producer from its own products. Several closely related *cmlA* genes have been identified on gene cassettes in Gram-negative bacteria and unlike other cassette-borne genes, *cmlA*, is inducibly expressed by a translational attenuator, similar to that of staphylococcal *cat* genes (47, 48). Recently, a second type of chloramphenicol exporter, CmlB1, which shares 74–77% identity with the known CmlA proteins, was identified on a plasmid from *Bordetella bronchiseptica* (49). The *cmlB1* gene is also preceded by a translational attenuator and inducibly expressed. Both, the CmlA and CmlB1 proteins cannot efficiently export florfenicol from the bacterial cell and bacteria with these genes are florfenicol susceptible.

Resistance to both chloramphenicol and florfenicol is characteristic for the group comprising *floR* and *pp-flo* genes. The *pp-flo* gene was detected in the fish pathogen *Photobacterium damselae* subsp. *piscicida* (50). These genes can be found in the chromosome of multi-resistant *Salmonella enterica* serovars including Typhimurium DT104, *Vibrio cholerae*, *E. coli*, *B. bronchiseptica*, and *Acinetobacter baumannii*, or on plasmids of *E. coli*, *Salmonella* Newport, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Pasteurella trehalosi* renamed *Bibersteinia trehalosi*, and *Stenothrophomonas maltophila*. A recent review of these genes can be found in Butaye et al. (11).

The gene *fexA* is located on a *Staphylococcus lentus* plasmid. Expression of *fexA* is inducible with either chloramphenicol or florfenicol. A translational attenuator similar to those of *cat* genes from *Staphylococcus* sp. and *Bacillus pumilus* was identified immediately upstream of the *fexA* gene (51).

3.3 Chloramphenicol Resistance due to Multidrug Transporters, Permeability Barriers, Mutations, Phosphorylation, or Target Site Methylation

Multidrug transporter systems assigned to the Resistance/Nodulation/Cell Division family have been reported to export phenicols from the bacterial cell and to include the AcrAB-TolC system in *Escherichia coli* (52) and the MexAB-OprM and MexCD-OprJ systems in *Pseudomonas aeruginosa* (12). In Gram-positive bacteria, several 12-TMS multidrug transporters of the Major Facilitator Superfamily, such as Blt and Bmr proteins from *Bacillus subtilis* and NorA from *Staphylococcus aureus*, have been reported to have a substrate spectrum that includes chloramphenicol (12). Another two closely related 12-TMS multidrug efflux proteins, MdfA and Cmr, have been identified in *Escherichia coli* (53, 54).

Cm^r Gram-negative bacteria may be due to the loss, or a distinct decrease in the expression of outer membrane proteins which serve as the entry for chloramphenicol into the bacterial cell. Examples have been reported in *Haemophilus influenzae* (55), and *Salmonella enterica* serovar Typhi (13). Activation of the *mar* locus in *Enterobacteriaceae* may also play a role in the decreased expression of the outer membrane protein OmpF by producing an antisense RNA that interferes with the translation of *ompF* transcripts.

Several mutations in the 23S rRNA of *E. coli* (14) are known to confer Cm^r. Deletions of 6 bp in the gene coding for the ribosomal protein L4 in *Streptococcus pneumoniae* have been reported to confer simultaneous resistance to chloramphenicol, oxazolidinones, and macrolides (56). Inactivation of chloramphenicol by *O*-phosphorylation has only been observed in the chloramphenicol producer *Streptomyces venezuelae* and is believed to contribute to the self-defense of the host (57).

Recently, the plasmid-borne gene *cfr* has been identified in staphylococci and shown to code for a methylase. The Cfr methylase modifies A2503 in 23S rRNA and thereby mediates not only resistance to the chloramphenicol and florfenicol, but also to other unrelated antimicrobial agents, such as lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics, all of which bind in close proximity to A2503 at the ribosome (4, 7–10).

4 Distribution of Resistance Genes

4.1 Distribution of Tetracycline Genes

The 14 Gram-negative *tet* efflux genes have been found in facultative and aerobic Gram-negative species. The most widespread Gram-negative efflux gene is the *tet*(B) gene which has been identified naturally in 25 different genera (Table 3). The *tet*(A) gene has been found in 17 genera while both *tet*(C) and *tet*(D) genes have been found in 14 different genera (Table 3). In contrast, the *tet*(J), *tet*(Y), *tet*(30), *tet*(31), and *tet*(39) genes have been identified in a single genus each. Seven different *tet* efflux genes have been found in *Aeromonas*, *Escherichia, and Pseudomonas* isolates, while eight different *tet* efflux genes have been identified in *Vibrio* sp. (Table 3). One study found a correlation between the plasmid incompatibility group and the particular *tet* genes the plasmid carried (58). These authors suggested that *tet* genes may become genetically linked to

specific incompatibility and/or replication genes which could influence the distribution to specific genera and/or even species (58). This relationship was not shown in an earlier study by Mendez et al. (59).

Very recently, the *tet*(C) gene has been found in the obligate intracellular bacteria *Chlamydia suis* chromosome (60). This marks the first description of any acquired antibiotic resistance gene identified in obligate intercellular bacteria. Associated with the *tet*(C) gene was an ISCS605 element similar to those found in *Helicobacter*. In addition, a 10.1 kb fragment shared 99% identity between the *C. suis* genetic island and an *Aeromonas salmonicida* plasmid pRAS3.2. One can only speculate how the *tet*(C) gene came to be linked to a genetic island that has components from two distantly unrelated genera that inhabit distinctive ecological niches. However, this data suggests that even obligate intercellular bacteria like *Chlamydia* sp. are able to exchange and acquire tetracycline resistance genes commonly found in enteric Gram-negative genera (60).

Nine efflux *tet* genes are found in Gram-positive genera, *Mycobacterium*, and *Streptomyces* (Table 3). Of these the *tet*(K) and *tet*(L) genes are the most widely distributed with 12 and 21 genera respectively. Both of these genes have occasionally been found in Gram-negative genera. However, the prevalence of the *tet*(K) and *tet*(L) genes in the Gram-negative population may be underestimated in Table 3 because Gram-negative isolates are rarely screened for the presence of these two genes. The other seven Gram-positive *tet* efflux genes are found in one to four genera (Table 3).

There have been eleven ribosomal protection genes identified. All appear to be of Gram-positive origin, though these have been identified in Gram-positive, Gram-negative, aerobic, anaerobic, and cell-wall free genera from nature (Table 3). The *tet*(M) and *tet*(Q) genes are generally associated with conjugative chromosomal elements, which code for their own transfer (34). Conjugative transposons appear to have less host specificity then do plasmids, which may explain why the *tet*(M) gene is found naturally in 30 different genera, the *tet*(Q) gene found in 14 genera, and the recently identified *tet*(W) gene found in 13 genera (Table 3). The *tet*(O) gene has been found in ten genera, while the *tet*(S) gene has been found in five genera (Table 3). The *tet*(S) and *tet*(O) genes can be associated with conjugative plasmids, or in the chromosome where they have not been mobile (1). The *tet*(O) gene has been associated with a conjugative transposon which also carries a *mef*(A) gene, which codes for a macrolide efflux protein, in *Streptococcus pyogenes* isolates from Italy (61). This new location has allowed us to move the *tet*(O) gene between unrelated genera in the laboratory and may in time increase its spread to a broader group of bacteria in nature. The remaining four ribosomal protection genes have been found in one genus (Table 3).

The *tet*(X) gene is found in anaerobic *Bacteroides* sp. though it is unlikely to have much clinical relevance, as well as Gram-positive aerobic Gram-negative Tc^r *Sphingobacterium* in this host. It was considered an oddity until the recent identification of a second gene *tet*(39) with the same mechanism of action though genetically unrelated (25). Unfortunately, the host(s) of the *tet*(37) gene is not known. More work needs to be done to understand the role that these two genes may have in nature.

4.2 Distribution of Chloramphenicol Resistance Genes

A wide distribution of the classical *cat* genes has been identified for 7 of the 22 groups (Table 2). The Tn*9*-borne *catI* gene has been found in seven genera of Gram-negative bacteria. Besides chromosomal locations, the *catI* gene is often detected on large plasmids that carry additional resistance genes. The plasmid-borne gene *catII* is frequently associated with Cm^r *Haemophilus* sp. (62), but has also been found on plasmids from *Photobacterium damselae* and *Agrobacterium tumefaciens*. The gene *catIII* from *Shigella flexneri* has also been detected in bacteria different from *Enterobacteriaceae*. This gene represents part of plasmid-borne multi-resistance gene clusters in *Mannheimia* spp. (63) and uncultured eubacteria. The staphylococcal *cat*-carrying plasmids pC221, pC223/pSCS7 and pC194 are small plasmids of <5 kb in size that only mediate Cm^r (64). These small plasmids have also been isolated in *Bacillus* sp. (64). Naturally occurring co-integrates between pC221 and pS194, a small staphylococcal streptomycin resistance plasmid, have also been detected. Plasmids similar to pC221 can also recombine with larger plasmids to form new resistance plasmids that have a broader host range, extended transfer abilities and carry additional resistance genes like the conjugative plasmid pIP501 which has a pC221-like *cat* gene, the macrolide resistance gene *erm*(B) and the Tc^r gene *tet*(M) (65). The *cat* genes of the pC221 group have been detected in *Staphylococcus* sp., *Streptococcus agalactiae*, *Enterococcus faecalis* and *Bacillus subtilis*, while the *cat* gene of the pC223 has been found on plasmids from *Staphylococcus* sp., *Listeria monocytogenes*, *Lactococcus lactis* and *Enterococcus faecium*. The pC194-like *cat* genes have been identified in *Staphylococcus aureus*, *Enterococcus faecium*, *Lactobacillus reuteri,* and *Streptococcus suis*.

The *catB1*gene from *Agrobacterium tumefaciens*, the *catB7* gene from *Pseudomonas aeruginosa* and the *catB9* gene from *Vibrio cholerae* have been found exclusively on the chromosome. In contrast, the Tn*2424*-borne *catB2* gene has been detected on plasmids from Gram-negative enteric genera and in the chromosome of *Shewanella oneidensis*. The closely related *catB3* – *catB8* genes are usually located on plasmids and are widespread among various Gram-negative genera (Table 2).

The first two groups of specific exporter genes are most widespread among Gram-negative bacteria (Table 2). The cassette-borne *cmlA* group is frequently found on multiresistance integrons or associated with transposons located on conjugative and/or nonconjugative plasmids in *Pseudomonas aeruginosa* and various enteric genera. The genes *fexA* and *cfr* are associated with staphylococcal plasmids. It should be noted that independent acquisition of mobile elements carrying *cat* genes, *cmlA* genes, or *floR* genes can lead to the simultaneous occurrence of more than one type of Cm^r gene in the same bacterium. Thus, multiresistant isolates of *Salmonella enterica* serovar Typhimurium DT104 var. Copenhagen carry a *catI* gene in addition to a *floR* gene (66), while *catA2* and *catA3* genes were detected on the same plasmids of *Klebsiella pneumoniae* (67) and *catA3* together with *floR* on a plasmid from *Pasteurella [Bibersteinia] trehalosi* (68).

5 Conclusion

Bacterial resistance to tetracycline and/or chloramphenicol due to acquisition of new genes and/or mutation of existing genes has increased over the last 30 years. Resistance levels vary by geography and by species, but many pathogenic and opportunistic bacteria are resistant to one or both of these antibiotics. Acquired genes are often associated with mobile elements which provide flexibility to host bacteria and help in the spread and distribution of these genes across diverse bacterial populations. Multiple antibiotic resistance genes can be clustered on individual mobile elements, which allows for multi-resistance to be transferred increasing the multi-drug resistant population. Unless overall use of antibiotic changes, this trend is likely to continue reducing the usability of current therapies.

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Chapter 16
Fluoroquinolone Resistance in Bacteria

Varsha V. Moudgal and Glenn W. Kaatz

1 Introduction

Quinolones are some of the most widely prescribed antimicrobial agents in the world. For example, levofloxacin sales totaled $1.5 billion in 2005 (Johnson & Johnson Annual Report for 2005; http://www.investor.jnj.com). A detailed discussion of structure-activity relationships is beyond the scope of this chapter, but these agents have undergone several iterations, or "generations," which have consisted of structural modifications to improve potency and spectrum of activity. The first-generation quinolone upon which all subsequent derivatives are based is nalidixic acid (Fig. 1), which was isolated as a by-product during chloroquine synthesis (1). Nalidixic acid actually is a naphthyridone based on the presence of a nitrogen atom at position 8, whereas quinolones generally have a carbon atom at this position. Second-generation drugs, all of which have a fluorine at position 6 of the quinolone nucleus, include norfloxacin, ciprofloxacin, enoxacin, ofloxacin, and pefloxacin and third-generation agents include temafloxacin, levofloxacin, trovafloxacin, gatifloxacin, and moxifloxacin.

Many quinolones have been approved by various regulatory agencies worldwide and some have been withdrawn after widespread use revealed unforeseen toxicities. Examples of this include temafloxacin, which was found to be associated with hypoglycemia and hemolytic-uremic syndrome and trovafloxacin, found to be associated with severe hepatotoxicity (2, 3). Although serious adverse events following quinolone use are relatively rare, some that have been associated with these drugs include prolongation of the QTc interval which can predispose to serious, life-threatening arrhythmias, rash, seizure, glucose intolerance and, as already mentioned, hepatotoxicity (4).

Quinolones are broad-spectrum bactericidal agents active against many Gram-positive and Gram-negative bacteria that target the essential bacterial enzymes DNA gyrase and DNA topoisomerase IV (5). These enzymes are involved in DNA replication and repair and in the presence of a quinolone an intermediate ternary complex consisting of drug, enzyme, and a severed DNA strand is formed. These complexes block further DNA replication leading to cell death. Mutational alterations of the genes encoding DNA gyrase and/or topoisomerase IV in the so-called quinolone resistance determining region, or QRDR, and resulting in critical amino acid substitutions reduce quinolone interaction with each enzyme. These mutations are the basis for high-level, target-based quinolone resistance and will be discussed in detail later in this chapter. Another important mechanism of quinolone resistance is overexpression of membrane-based drug efflux pumps, which also will be discussed subsequently. Such efflux pumps reduce the effective intracellular drug concentration to either a non-inhibitory or borderline inhibitory level, favoring the emergence of target-based mutations and high-level resistance (6–8).

All clinically relevant bacterial species are capable of developing resistance to quinolones, but historically problematic organisms have been *Staphylococcus aureus* and *Pseudomonas aeruginosa*. For many quinolones these organisms tend to have a narrower therapeutic index than other bacteria in that the minimum inhibitory concentration (MIC) and achievable serum levels are relatively close. In this situation subtherapeutic drug levels will exist for long periods of time during therapy, favoring the emergence of point mutations in topoisomerase genes leading to reduced quinolone susceptibility. Newer agents with increased potency against *S. aureus* have helped to reduce this problem but unfortunately the majority of methicillin-resistant *S. aureus* (MRSA) strains recovered from clinical specimens in many areas of the world are already highly quinolone resistant, mainly on the basis of target mutations. Resistance in methicillin-susceptible (MSSA) strains is less problematic, but can be significant in some geographic locales. Over a 7-month period during 2005 we collected more than 200 bloodstream isolates of *S. aureus* from different patients hospitalized in Detroit, Michigan. Of these strains, 65% were MRSA and 35% were MSSA.

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Fig. 1 Structures of selected quinolones. The numbering scheme of the quinolone nucleus is given for nalidixic acid

Norfloxacin resistance (MIC ≥ 16 µg/mL) was observed in 60 and 12% of MRSA and MSSA, respectively (unpublished data). Resistance rates for other areas may differ, but these data illustrate the extent of the problem in *S. aureus*.

In this chapter we will discuss quinolone resistance in both Gram-negative and Gram-positive bacteria. We will not address resistance to these agents in *Mycobacterium tuberculosis* since they are not frequently used to treat infections caused by this organism. However, many of the mechanisms that will be discussed here have also been found to exist in *M. tuberculosis* (9, 10). We will conclude with a short discussion on the means to limit quinolone resistance and perhaps to overcome some pre-existent resistance by use of efflux pump inhibitors.

2 Gram-Negative Bacteria

Gram-negative bacteria are an important cause of morbidity and mortality. The increasing antibacterial resistance observed in many Gram-negative organisms parallels the increasing use and abuse of antimicrobial agents, and this is certainly true for the quinolones (11). Until 1998 it was thought that quinolone resistance in Gram-negatives occurred either by way of target alteration or active drug extrusion by membrane-based efflux pumps. A third mechanism described more recently involves the Qnr protein, the gene for which is plasmid-encoded and thus transferable (12). This mechanism of quinolone resistance is addressed in detail elsewhere in this volume and will only be briefly described in this chapter.

2.1 Target-Mediated Resistance

As already mentioned, the targets of quinolones are the essential bacterial enzymes: DNA gyrase and topoisomerase IV. DNA gyrase, the major type II topoisomerase in bacteria and initially described by Gellert et al. is a heterotetramer composed of two pairs of subunits (A and B) encoded by the *gyrA* and *gyrB* genes, respectively (13). The GyrA subunits bind to DNA and the GyrB subunits are ATPases. The main function of this enzyme is to maintain negative supercoiling via DNA strand breakage and rejoining, a function that facilitates the movement of DNA through replication and transcription complexes. Negative supercoiling is essential for initiation of DNA replication and introduction of supercoils depends on the binding of ATP to gyrase with subsequent ATP hydrolysis (14). Thus, this process is sensitive to changes in membrane energetics. DNA gyrase also helps remove knots and in the bending and folding of DNA. Following the discovery of DNA gyrase, it was ascertained that this enzyme is a target of quinolones (15).

Kato et al. discovered DNA topoisomerase IV, a heterotetrameric enzyme composed of two subunit pairs encoded by the *parC* and *parE* genes (16). ParC and ParE are homologous with GyrA and GyrB, respectively, with a high degree of amino acid conservation in the QRDR regions. The principle function of topoisomerase IV appears to be its ability to decatenate linked daughter chromosomes at the terminal stages of DNA replication (17). Despite DNA gyrase and topoisomerase IV sharing considerable amino acid sequence similarity, they have distinct mechanisms of action. One of the important differences seems to be that DNA gyrase wraps DNA around itself, while topoisomerase IV does not (18). Given the homology between DNA gyrase and topoisomerase IV the latter enzyme was thought to also be a quinolone target, which has now been demonstrated clearly (19, 20).

As mentioned previously, quinolones bind to DNA-DNA gyrase and DNA-topoisomerase IV complexes and cause a conformational change in the enzyme structure (21, 22). They also alter the enzyme-bound DNA itself (23, 24). In the presence of quinolones the topoisomerases become trapped on DNA and the resultant quinolone-enzyme-DNA ternary complex forms a physical barrier at the replication fork, inhibiting further DNA replication which results in cell death (25).

In Gram-negative bacteria the primary target for most quinolones is DNA gyrase, with topoisomerase IV being a secondary target (19, 26). In contrast, in most Gram-positive bacteria and for most quinolones topoisomerase IV is the primary target (27, 28). These differences are thought to be due to the differential affinity of quinolones for the two enzymes in each respective background (29). Quinolone resistance occurs in a stepwise fashion as a result of the accumulation of mutations resulting in amino acid substitutions mainly in *gyrA* and *parC*. Less commonly, mutations occur in *gyrB* and *parE* that can contribute to reduced quinolone susceptibility (26, 30). Additional MIC increases are seen when a "first-step" mutant, having a critical amino acid substitution in the primary target, acquires a "second-step" mutation resulting in an amino acid substitution in the secondary target enzyme. Many topoisomerase mutations in *E. coli*, as well as many other Gram-negative bacteria, have been shown to correlate with raised quinolone MICs (Table 1).

Analyses of *gyrA* mutants have revealed that most of the quinolone-resistance conferring mutations cluster near the 5′ end of the gene in the QRDR region. For *E. coli*, this region includes codons 67–106 and for other species the region homologous to this (30, 31). Very near the QRDR is the codon for the active site tyrosine (codon 122). Tyrosine-122 binds covalently to DNA when the enzyme breaks the phosphodiester bonds of DNA, forming a phosphotyrosine linkage (32). Single *gyrB* mutants appear to be less resistant to quinolones than single *gyrA* mutants. In *E. coli* only two *gyrB* mutations have been recognized (Table 2). Only Asp426→Asn confers resistance to quinolones, whereas Lys447→Glu results in an increase in quinolone susceptibility (31).

Within topoisomerase IV, mutations in *parC* occur more frequently than those in *parE*. As mentioned previously, topoisomerase IV generally is the secondary quinolone target in *E. coli* and other Gram-negative organisms. *gyrA-parC* double mutants exhibit a higher level of quinolone resistance than *gyrA* single mutants, with the highest levels of resistance found in the mutants with two *gyrA* and two *parC* mutations. The reverse generally is true in Gram-positive organisms, where the first mutations are usually seen in the topoisomerase IV genes with the gyrase genes being the secondary targets.

Table 1 Topoisomerase amino acid substitutions associated with reduced quinolone susceptibility in *E. coli*

GyrA	GyrB	ParC	ParE
Ala51→Val	Asp426→Asn	Gly78→Asp	Leu445→His
Ala67→Ser	Lys447→Glu	Ser80→Arg, Ile, Leu	
Gly81→Cys, Asp		Glu84→Gly, Lys, Val	
Asp82→Gly			
Ser83→Leu, Trp, Ala, Val			
Ala84→Pro, Val			
Asp87→Ala, Asn, Gly, His, Tyr, Val			
Gln106→Arg, His			

Data are from (30, 31)

Table 2 Topoisomerase amino acid substitutions associated with reduced quinolone susceptibility in *S. aureus*

GyrA	GyrB	ParC (GrlA)	ParE (GrlB)
Ser84→Ala, Leu, Lys, Val	Asp437→Asn	Lys23→Asn	Pro25→His
Ser85→Pro	Arg458→Glu	Val41→Gly	Ser410→Pro
Glu86→ Lys, Gly	Glu477→Ala	Arg43→Cys	Glu422→Asp
Glu88→Lys, Val		Ile45→Met	Asp432→Asn, Gly, Val
Gly106→Asp		Ala48→Thr	Pro451→Gln, Ser
		Ser52→Arg	Asn470→Asp
		Asp69→Tyr	Glu472→Lys, Val
		Gly78→Cys	His478→Tyr
		Ser80→Phe, Tyr	
		Ser81→Pro	
		Glu84→Ala, Gly, Leu, Lys, Tyr, Val	
		His103→Tyr	
		Ala116→Glu, Pro	
		Pro157→Leu	
		Ala176→Gly, Thr	

Data are from (30, 31)

2.2 Decreased Outer Membrane Permeability

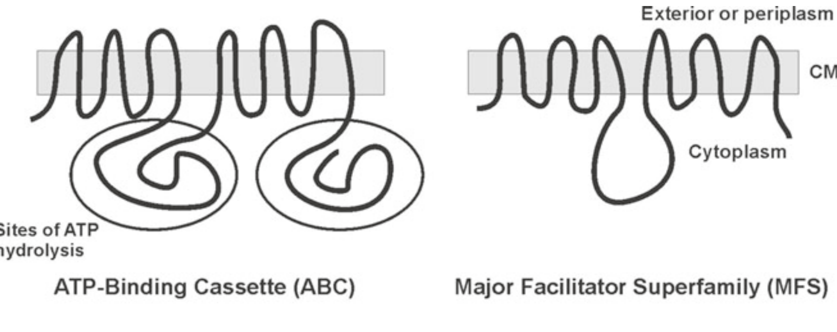
Quinolones must traverse the outer membrane, periplasmic space, cell wall, and cytoplasmic membrane of Gram-negative organisms to reach their topoisomerase targets. The porous bacterial cell wall does not impede the diffusion of small molecules such as quinolones and will not be considered further. The outer membrane may provide a rather formidable barrier, however, and in conjunction with efflux pumps (see below) can result in significant quinolone resistance (30, 31). Quinolones traverse this structure by two mechanisms, which include diffusion across the lipid bilayer and passage through pore-forming proteins called porins. Porins are protein channels that allow influx and egress of hydrophilic molecules. All quinolones may cross the outer membrane through the porins, but diffusion across the lipid bilayer is dependent on the hydrophobicity of the molecule. The more hydrophobic quinolones such as nalidixic acid are capable of traversing the lipid bilayer, whereas the more hydrophilic compounds such as ciprofloxacin are more dependent on porins (33, 34). Three main porins are found in *E. coli* and consist of OmpF, OmpC, and OmpA. Loss of porins by mutational inactivation of structural genes often manifests as a decrease in quinolone susceptibility, but this effect is significantly amplified in the presence of drug efflux. *E. coli* mutants with reduced amounts of OmpF, the most abundant porin, exhibit low-level quinolone resistance (35). Other unrelated drugs such as tetracyclines, chloramphenicol, and some β-lactams also utilize this porin and hence OmpF-deficient mutants also demonstrate resistance to these other agents due to decreased drug accumulation (36). Chromosomal loci such as *marRAB* and *soxRS* encode transcriptional factors that regulate OmpF expression in *E. coli* (37). Overexpression of *marA* and *soxS* results in post-transcriptional repression of OmpF and thus quinolone resistance by increasing the expression of *micF*, an antisense regulator (37, 38). More on the roles of MarA and SoxS in quinolone resistance will be presented in the next section.

The permeability of the outer membranes of *Pseudomonas aeruginosa* and *Acinetobacter baumanii* may account for some of their intrinsic resistance to various antibiotics, including quinolones. The *P. aeruginosa* outer membrane has very poor permeability to hydrophilic molecules, approximately 100-fold less than that of the *E. coli* outer membrane (39).

2.3 Efflux-Related Resistance

Gram-negative bacteria tend to be resistant to a wider range of antimicrobial agents compared to Gram-positive species. The outer membrane is one reason for this in that it acts as a barrier to the penetration of hydrophilic molecules. This mechanism generally confers only low-level reduced susceptibility. Membrane-based efflux pumps contribute more significantly to innate drug insensitivity. Bacterial efflux pumps can be divided into five families based on structural characteristics, mechanisms of action and source of energy for the transport process. These include primary transporters that depend on ATP hydrolysis for drug export (ATP-binding cassette, or ABC pumps) and secondary transporters that require an intact proton motive force (pmf) across the cell membrane for their function (major facilitator superfamily [MFS], resistance-nodulation-division [RND], small multidrug resistance [SMR], and the multidrug and toxic compound extrusion [MATE] families) (Fig. 2). Efflux pumps may be quite specific with respect to substrates transported, with a clinically relevant example being the various MFS tetracycline efflux pumps found in Gram-negative and Gram-positive bacteria (40). Circumvention of the resistance generated by specific drug pumps is as simple as providing alternative therapy with agents not affected by the pump in question. Multidrug efflux pumps, which have an apparent lack in substrate specificity, are capable of extruding numerous structurally dissimilar compounds, creating a multidrug resistant (MDR) phenotype, and can pose a very formidable therapeutic challenge (Table 3). Drug efflux can lead to subtherapeutic intracellular concentrations of an antibiotic substrate, thereby setting up the ideal milieu for the development of chromosomal mutations that confer high-level antibiotic resistance.

Efflux-related quinolone resistance has been identified in virtually all medically important Gram-negative organisms,



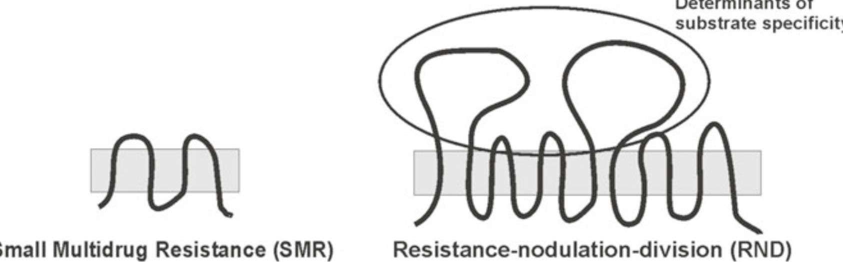


Fig. 2 Schematic illustrating the general structural characteristics of each family of bacterial efflux pump. The sites at which ATP hydrolysis occurs in ABC pumps are indicated. MATE pumps do not necessarily have the large central loop that is characteristic of members of the MFS and some MFS proteins have 14 membrane-spanning segments. Substrate specificity for RND pumps such as AcrB and perhaps other pumps of this family lie in the two large periplasmic loops. The cytoplasmic membrane is shown in *gray* and the cytoplasm and exterior/periplasm are as indicated

Table 3 Selected bacterial multidrug efflux pumps

Pump	Family	Organism	Selected substrates[a]
Gram-negative			
AcrB	RND	*E. coli*	FQ, BL, CM, TCN, TI
MdfA	MFS	*E. coli*	FQ, CM, EM, TCN
MexB	RND	*P. aeruginosa*	FQ, BL, CM, TCN, TI, TM
MexD	RND	*P. aeruginosa*	FQ, CM, EM, TCN, TI, TM
MexF	RND	*P. aeruginosa*	FQ, CM, TM
MexY	RND	*P. aeruginosa*	FQ, AF, AG, EB, EM
SmeE	RND	*S. maltophilia*	FQ, CM, TCN
NorM	MATE	*V. parahaemolyticus*	FQ, EB
Gram-positive			
NorA	MFS	*S. aureus*	FQ, AF, BAC, CT, EB, TPP
PmrA	MFS	*S. pneumoniae*	FQ, EB
Bmr	MFS	*B. subtilis*	FQ, AF, EB, TPP
Blt	MFS	*B. subtilis*	FQ, AF, EB, TPP
MepA	MATE	*S. aureus*	FQ, BAC, DQ, EB, TPP, PT
LmrA	ABC	*L. lactis*	FQ, AG, BL, CM, TCN

[a]*AF* acriflavine; *AG* aminoglycosides; *BAC* benzalkonium chloride; *BL* beta-lactams; *CM* chloramphenicol; *CT* cetrimide; *DQ* dequalinium; *EB* ethidium bromide; *EM* erythromycin; *FQ* fluoroquinolones; *PT* pentamidine; *TCN* tetracycline; *TI* tigecycline; *TM* trimethoprim; *TPP* tetraphenylphosphonium

including *E. coli* and *P. aeruginosa* (41). Pump-related resistance to quinolones is due to the activity of multidrug pumps; no pumps having quinolones as sole substrates have been described. These pumps are capable of transporting several antimicrobial agents and thus cross-resistance to multiple antibiotics can be observed in their presence. It has already been mentioned that the relatively impermeable Gram-negative outer membrane, which limits drug entry, works synergistically with efflux pumps capable of extruding drugs that do gain access to the cytoplasmic membrane (30, 31). The RND-type pumps of Gram-negative organisms are composed of three different subunits, which include the pump protein itself, which is a transmembrane protein having 12 membrane-spanning alpha helices or transmembrane segments (TMS), an outer membrane pore-forming channel or porin, and a periplasmic membrane fusion protein (MFP) that links the other two.

The AcrB pump is the predominant quinolone efflux system of *E. coli* (42). This pump is a member of the RND family and utilizes TolC as its outer membrane channel, to which it is associated by the AcrA MFP (43). AcrB has a broad substrate profile including quinolones, tetracyclines, chloramphenicol, ampicillin, nalidixic acid, rifampin and dyes and disinfectants. The expression of *acrAB*, which is transcribed as an operon, is governed by at least two global regulatory systems, the *marRAB* and *soxRS* loci; both systems positively regulate the production of AcrAB. Multiple antibiotic resistant (Mar-type) mutants of *E. coli* have mutations in the *marRAB* operon (44). The Mar phenotype is induced following exposure to a variety of chemicals with aromatic rings, including salicylate. The most common location for mutations conferring the Mar phenotype is in *marR*, which encodes for the repressor of the *marRAB* operon. *E.coli soxRS* mutants exhibit a similar resistance phenotype to *marR* mutants. Increased quantities of MarA and SoxS upregulate *acrAB* and down-regulate the production of the OmpF porin

channel. These changes lead to multiple antibiotic resistance by these synergistic mechanisms.

The crystal structure of AcrB in the presence and absence of substrates was recently solved (45). These data indicate that the pump acquires substrates from the outer leaflet of the cytoplasmic membrane; however, acquisition of substrate from the cytoplasm may also occur. Substrate specificity of AcrB seems to lie in its large periplasmic loops (Fig. 2) (46).

In *P. aeruginosa* the main multidrug efflux system (including quinolones) is the *mexAB-oprM* operon, which encodes proteins homologous to AcrAB-TolC in *E. coli. mexCD-oprJ*, *mexEF-oprN,* and *mexXY-oprM* are three additional multidrug resistance operons found in *P. aeruginosa*. Each of these operons encodes for a set of three proteins similar in structure and function to MexAB-OprM and all are RND type efflux pumps. Like AcrAB-TolC, the most striking characteristic of these pump systems is their broad substrate specificity. The substrate profile for MexAB-OprM includes quinolones, chloramphenicol, nalidixic acid, trimethoprim, tetracyclines (including tigecycline), dyes, disinfectants and organic solvents (Table 3). Most wild-type strains of *P. aeruginosa* express MexAB-OprM constitutively, which contributes to the intrinsic multidrug resistant nature of this organism (47). Overexpression of efflux pumps due to chromosomal mutations in the promoter region of the pump genes or in the gene encoding the regulator for pump gene expression can cause clinically relevant resistance to antimicrobial agent substrates. There are a number of laboratories actively searching for compounds capable of efflux pump inhibition, which could restore clinically relevant activity of substrate antibiotics.

Multidrug efflux pumps having quinolones as substrates have been identified in many other Gram-negative bacteria. Examples include the SmeDEF RND pump system of *Stenotrophomonas maltophilia*, the NorM and BexA MATE pumps of *Vibrio parahaemolyticus* and *Bacteroides thetaiotaomicron*, respectively, and the VceAB MFS pump of *V. cholerae* (48–51) (Table 3). Overexpression of these pumps in either their natural or a heterologous background results in increased MICs for a variety of quinolones.

2.4 Plasmid-Mediated Quinolone Resistance

In 1998 Martínez-Martínez et al. reported quinolone resistance to be expressed in the presence of pMG252, a plasmid belonging to incompatibility group IncC (12). This plasmid mediates low-level quinolone resistance (to both nalidixic acid and more modern quinolones) and has a broad host range. Subsequently, the gene responsible for quinolone resistance was identified and named *qnr* (52). Qnr “protects” both DNA gyrase and topoisomerase IV from quinolone inhibition (52–54). Prevalence studies have revealed that among quinolone-resistant strains of *E. coli* recovered in Shanghai, China, 7.7% contained the *qnrA* gene (55). In the United States, *qnrA* was present in 11.1% of quinolone-resistant *Klebsiella pneumoniae* strains but not in any of the tested *E. coli* strains (56). Further investigation led to the discovery that *qnrA* was present in clinical strains of *Enterobacter* spp. (57). Thus, the *qnrA* gene is widely distributed and contributes to quinolone resistance in Enterobacteriaceae. More recently, a new *qnrA*-related gene called *qnrB* was discovered in a strain of *K. pneumoniae* that had less than 40% amino acid sequence identity with *qnrA* (58). Although *qnr* confers relatively low-level quinolone resistance, its presence may facilitate selection of other quinolone mutations leading to high-level resistance. Further discussion of this novel quinolone resistance mechanism can be found elsewhere in this volume.

2.5 Enzymatic Modification of Quinolones

Being synthetic substances, the occurrence of natural degradation systems in bacteria seemed unlikely. However, fungi capable of degrading ciprofloxacin and the veterinary fluoroquinolone enrofloxacin have been identified (59, 60). Recently, a plasmid-associated gene recovered from a clinical *E. coli* strain was found to encode an aminoglycoside acetyltransferase that could also acetylate selected fluoroquinolones and compromise their antimicrobial activity (61). The effect of acetylation was relatively small, as exemplified by expressing the gene in question (aac[6′]-Ib-cr) from a plasmid in an *E. coli* background. Norfloxacin and ciprofloxacin MICs were increased fourfold, whereas those of levofloxacin and gemifloxacin were unaffected. The MIC increases were not clinically significant, but the existence of a plasmid-based and naturally occurring enzyme capable of modifying quinolones is worrisome as widespread dissemination is possible. The combination of this resistance mechanism with others causing borderline MIC increases, such as efflux pumps or single QRDR mutations, may result in a clinically relevant fully resistant organism.

3 Gram-Positive Bacteria

Fewer quinolone resistance mechanisms are found in Gram-positive bacteria than those identified in Gram-negatives. The lack of an outer membrane results in no permeability issues beyond those posed by the cytoplasmic membrane and no Qnr-like proteins or quinolone-modifying enzymes have been identified in this group of organisms. The mechanisms of quinolone resistance that have been recognized include target-based mutations and drug efflux. Studies done in vitro provide

evidence that inhibition of efflux pumps reduces the emergence of topoisomerase mutations in both *S. aureus* and *Streptococcus pneumoniae*, suggesting that efflux pumps play a critical role in the evolution of high-level quinolone resistance (6, 7).

3.1 Target-Mediated Resistance

Similar to the situation in Gram-negative bacteria, mutations in the QRDR regions of mainly *gyrA* and *parC (grlA* in *S. aureus)* resulting in amino acid substitutions is the main mechanism of quinolone resistance in Gram-positive bacteria. In general, GrlA is the primary quinolone target in Gram-positives and single amino acid substitutions in this enzyme can result in clinically relevant resistance (62). Accumulation of QRDR mutations first in *parC* and then in *gyrA* typically results in very high MICs. Topoisomerase amino acid substitutions correlating with quinolone resistance in *S. aureus* are presented in Table 2.

3.2 Efflux-Related Resistance

Examination of genome data available for *Enterococcus faecalis, S. aureus*, *S. epidermidis,* and *Streptococcus pneumoniae* reveals coding regions for several putative drug transport proteins (http://www.membranetransport.org). Many of these proteins are homologous with known multidrug transporters for which quinolones are substrates. Several of the most extensively studied Gram-positive drug pumps will be discussed in this section.

NorA is a chromosomally encoded 12 TMS *S. aureus* multidrug pump having broad substrate specificity that includes antiseptic compounds as well as quinolones (63). As are all MFS pumps, its activity is dependent on the pmf (64). Knockout mutations have revealed that NorA contributes to quinolone susceptibility in wild-type strains in that elimination of the gene results in MIC reductions for norfloxacin and ciprofloxacin (65, 66). Overexpression of *norA*, either by way of a regulatory mutation or expression from a multicopy plasmid in the laboratory, results in modest MIC increases for selected quinolones as well as many other structurally unrelated drugs, mainly hydrophobic cations (67, 68).

The understanding of *norA* regulation is incomplete. Recent work has identified the MgrA protein, which apparently binds upstream of *norA* repressing its expression (69, 70). MgrA is not a specific regulator of *norA* expression but rather is a global regulator that, in addition to affecting *norA* transcription, also affects the transcription of other pump-encoding genes (including *norB* and *norC*; see below), autolytic regulators, murein hydrolases and virulence factors such as alpha toxin, coagulase and nuclease (70–73).

NorB and NorC are two 14 TMS MFS multidrug transporters that are quite similar to each other on the basis of 70% amino acid sequence homology (74, 75). The substrate profile of NorB includes a variety of quinolones (norfloxacin, ciprofloxacin, sparfloxacin, moxifloxacin, gemifloxacin, garenoxacin, and premafloxacin), tetraphenylphosphonium bromide, cetrimide, and ethidium bromide, many of which also are substrates for NorA. NorC seems capable of effluxing a similar set of quinolone substrates with the exception of gemifloxacin. Further studies will be required to elucidate the reason(s) for this difference, but it may be related to differences in substrate binding sites. Transcriptional profiling experiments have shown that MgrA represses the expression of *norC*, but augments that of *norB* (73).

A novel *S. aureus* 14 TMS MFS multidrug efflux pump, MdeA, was recently described (76). When overexpressed in *S. aureus* MdeA confers resistance to an intriguing array of substrates including norfloxacin, ethidium bromide, benzalkonium chloride, virginiamycin, novobiocin, fusidic acid and augments EtBr efflux (76, 77). Expression of *mdeA* in wild-type strains is low, but spontaneous mutants having increased transcription are selectable in vitro. These mutants, which have reduced susceptibility to MdeA substrates, were found to have mutations in the *mdeA* promoter but further details regarding the regulation of *mdeA* expression are not available.

Although not considered a human pathogen, several multidrug transporters of *Bacillus subtilis* have been extensively studied and have contributed greatly to our knowledge of the regulation and function of MFS proteins. Bmr is a 12 TMS MFS MDR transporter having 44% amino acid identity with NorA and a similar substrate profile (78, 79). The expression of *bmr* is regulated by the binding to its promoter of BmrR, a transcriptional activator protein encoded by a gene immediately downstream from *bmr* (80). The crystal structure BmrR in the presence and absence of substrates has been solved and has revealed that Bmr substrates bind to BmrR via hydrophobic and electrostatic interactions, which in turn facilitate BmrR binding to the *bmr* promoter and induction of *bmr* transcription (81).

Blt is a second 12 TMS MFS MDR transporter of *B. subtilis* that has a similar substrate profile to those of NorA and Bmr (82). The expression of *blt* is enhanced in a similar manner to that of *bmr* by the binding of the transcriptional activator BltR (encoded by *bltR*, found immediately upstream of *blt*) to the *blt* promoter. This binding is thought to be improved by the interaction of substrates with BltR, although the specific activator substrates have not been identified. Interestingly, *blt* is not expressed in wild-type cells.

In addition to the specific regulators of *bmr* and *blt* transcription just described, the expression of these genes also is

affected by MtaN, a global transcriptional regulator that interacts with the *bmr* and *blt* promoters stimulating their transcription (83). MtaN consists of the N-terminal 109 residues of a larger protein, Mta (257 residues); the intact parent protein does not activate *bmr* or *blt* transcription. It is hypothesized that upon interacting with an inducer (as yet unidentified), the N- and C-terminal domains of Mta are functionally separated allowing it to function as a transcriptional activator.

Bmr3 is a 14 TMS MDR pump that confers reduced susceptibility to only select quinolones and puromycin when overexpressed (84). The bmr3 gene is likely poorly expressed and does not contribute to intrinsic drug resistance because when it is disrupted the norfloxacin MIC is unchanged from that of a parent strain.

PmrA is an MFS transporter found in *S. pneumoniae* (85). Disruption of *pmrA* results in increased quinolone susceptibility and reduced efflux of ethidium bromide, indicating that at least some quinolones are substrates for this pump and that it is a multidrug transporter. The contribution of PmrA to quinolone susceptibility in clinical strains is uncertain as overexpression does not necessarily result in any change in quinolone susceptibility (86).

EmeA is a NorA homologue identified by probing the *Enterococcus faecalis* V583 genome data (87). It is a multidrug pump that can transport norfloxacin and ethidium bromide and when deleted susceptibility to acriflavine and ciprofloxacin increases, suggesting that these compounds also are substrates. The contribution of EmeA to intrinsic quinolone susceptibility in clinical isolates of *E. faecalis* is unknown.

The MATE family of efflux proteins is the most recently described and the least well characterized. MATE pumps function for the most part by an unusual sodium ion:drug antiport mechanism and have been found mainly in Gram-negative bacteria, with two examples also reported in Gram-positives (88–91). MATE family proteins are similar in size to MFS transporters and are typically arranged into 12 TMSs, but they have no sequence similarity to any MFS proteins (Fig. 2). Substrates can be variable between different MATE pumps but can include cationic dyes, aminoglycosides, anticancer agents, and quinolones. Gene inactivation studies have demonstrated that MATE pump genes can be expressed at sufficient levels to affect MICs for pump substrates in wild-type cells and along with other pumps and alternative resistance mechanisms can contribute to reduced susceptibility to clinically relevant drugs such as FQs (50).

The regulation of MATE pump expression is not well understood. The MepA pump of *S. aureus* is repressed by MepR, a MarR-like protein encoded immediately upstream of *mepA* (90, 92). MepA substrates appear to bind to MepR, reducing its binding to the *mepA* promoter resulting in augmented *mepA* expression. MepR also is autoregulatory in that it represses the expression of its own gene. However, relief of *mepR* repression in the presence of MepA substrates is much less than that observed for *mepA*. The mechanism(s) of this apparent paradox are yet to be worked out, but the end result is significant relief of *mepA* and relative maintenance of *mepR* repression, leading to increased MepA protein unimpeded by MepR when the need for detoxification exists.

Lactococcus lactis is generally not considered a human pathogen but is extensively used in the dairy industry. However, like the study of multidrug pumps in *B. subtilis*, the study of such pumps in *L. lactis* has added significantly to our knowledge of how these pumps work. At least one true infection with *L. lactis* has been described making drug pumps of this organism that are capable of effluxing quinolones, in combination with other quinolone resistance mechanisms, potentially relevant clinically (93). At this time the only pump capable of transporting quinolones in *L. lactis* is LmrA, which is unique among bacterial efflux pumps capable of transporting quinolones in that it is an ABC transporter homologous with the human multidrug transporter P-glycoprotein (94). In addition to transporting quinolones it also is capable of effluxing chemotherapeutic agents such as daunorubicin.

4 Means to Limit or Overcome Quinolone Resistance

As mentioned previously, quinolones are among the most commonly prescribed antimicrobial agents. It is not infrequent that they are used inappropriately, with an example being the prescription of levofloxacin for viral upper respiratory tract infections. Education of primary care physicians regarding the seriousness of the antimicrobial agent resistance problem in general, and that of quinolones in particular, and encouraging them to not succumb to pressure to prescribe antimicrobial treatment for infections that are most likely viral in nature will help to reduce selective pressure. The dissemination of well-conceived guidelines for the proper use of these drugs and the institution of formulary restrictions are other methods by which inappropriate quinolone use might be reduced.

Once resistance to a particular antimicrobial agent reaches a critical prevalence, the utility of that drug becomes severely compromised. Most often, alternative therapy will be prescribed. Much work has been done on the development of compounds that block multidrug efflux pumps of both Gram-negative and -positive organisms, many of which have quinolones as substrates (efflux pump inhibitors, or EPIs) (95). Increased efflux often is the first step along the pathway towards high-level quinolone resistance and inhibition of this process may prevent such mutants from appearing. In addition, if efflux is the only mechanism of quinolone resis-

tance the combination of such a drug with EPI may result in the recovery of clinically useful activity of that drug. It has been shown in vitro that target-based resistance mutations occur much less frequently when an EPI is present in addition to the quinolone (6, 7, 96). Recently, an IND was filed to study the combination of an EPI (MP-601,205) with a quinolone for therapy of pulmonary infections in patients with cystic fibrosis. This will be the first clinical trial involving an EPI and its results are anxiously awaited.

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Chapter 17
Plasmid-Mediated Quinolone Resistance

George A. Jacoby

In the 1990s quinolone resistance increased in parallel with increased quinolone utilization (1) and also with the emergence of plasmid-mediated quinolone resistance. The first type of plasmid-mediated resistance was discovered in a clinical strain of *Klebsiella pneumoniae* isolated at the University of Alabama in 1994 that transferred low-level quinolone resistance along with resistance to several other antibiotics to *Escherichia coli* and other Gram-negative organisms (2). In *E. coli* the plasmid caused an eight- to 32-fold decrease in susceptibility for nalidixic acid and for all fluoroquinolones tested. Although the increased minimum inhibitory concentration (MIC) may remain in the susceptible range as defined by the CLSI, the presence of the plasmid raised the mutant protective concentration (3, 4) and facilitated the selection of truly quinolone-resistant mutants (2).

The responsible gene was termed *qnr* and coded for a 218 amino acid protein belonging to the pentapeptide repeat family that was shown with purified components to prevent inhibition of DNA gyrase and topoisomerase IV by ciprofloxacin (5, 6). Qnr protein bound to both topoisomerases and to their subunits and decreased the binding of gyrase to DNA (6, 7). Whether quinolone binding to gyrase is also affected is not yet known. Another pentapeptide repeat protein that protects against ciprofloxacin and that is encoded by a chromosomal gene in *Mycobacterium tuberculosis* folds into a helical structure similar in size, shape and, charge to B-form DNA (8). Qnr might adopt a similar structure but differs from the *M. tuberculosis* protein by having a glycine residue that divides the protein into two domains and by readily demonstrating topoisomerase protection in vitro (5).

Qnr plasmids have been found around the world in a variety of *Enterobacteriaceae* (9, 10). The original *qnr* gene has been renamed *qnrA* to accommodate related plasmid-mediated pentapeptide repeat proteins QnrS found in *Shigella flexneri* from Japan (11) and QnrB discovered in *K. pneumoniae* from India (12). QnrA, QnrB, and QnrS have less than 60% of their amino acids in common. Variants of each type differing in only a few amino acids are also known and define QnrA1-6, QnrB1-6, and QnrS1-2. Table 1 shows that despite the amino acid variability plasmids encoding QnrA, QnrB, or QnrS mediate similar quinolone MIC values, which do not reach the CLSI breakpoint for resistance for QnrB with nalidixic acid and for all three proteins with ciprofloxacin.

Genes for *qnrA* and *qnrB* have been found in sul1-type integrons, although they lack the 59-base elements usually associated with mobile gene cassettes (14). The *qnrA1* gene has invariably been found downstream from IS*CR1* (formerly known as ORF513), a novel gene-capturing element (15), in multiresistance plasmids from widely different parts of the world (Fig. 1). *qnrB* is associated with IS*CR1* in some plasmids (19) and has also been found near what may be a similar element termed ORF1005 (12). *qnrS1* is not linked to such genes, but rather to Tn*3* or IS-type elements (13, 20, 21). A likely origin of the *qnrA* genes is the chromosome of an aquatic bacterium, *Shewanella algae,* where Poirel and co-workers discovered closely related genes, one of which, *qnrA3,* was subsequently found on a plasmid in *Salmonella enterica* serotype Enteritidis from Hong Kong (22, 23). Genes with more than 80% similarity to *qnrS* have also been found in the genome of *Vibrio splendidus* (24), and other marine organisms have more distantly related genes (11, 25, 26). Their natural function is unknown.

qnr genes have not as yet been found on plasmids from strains isolated before 1994 (27, 28). The genes are often linked to extended-spectrum or AmpC-type β-lactamases. In a sample of 313 ceftazidime-resistant *Enterobacteriaceae* collected in the United States between 1999 and 2004, *qnr* genes were present in 20% of *K. pneumoniae* isolates, 31% of *Enterobacter* sp. isolates and 4% of *E. coli* isolates (29). *qnrA* and *qnrB* were equally common. *qnrS* was not found in this sample, but has been found in similar strains from France and Vietnam and in non-Typhi serotypes of *Salmonella enterica* from the United States (13). Hospital outbreaks of *qnr* containing *Enterobacteriaceae* have been reported from the United Kingdom (30) and the Netherlands (31).

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The second type of plasmid-mediated quinolone resistance is specific for agents with an unsubstituted amino nitrogen on the piperazinyl substituent, such as ciprofloxacin and norfloxacin, which are modified by acetylation (32). The responsible enzyme is a plasmid-determined variant of aminoglycoside acetyltransferase AAC(6′)-Ib with two amino acid changes which are essential for this new activity. Acetylation decreases the antibacterial potency raising the ciprofloxacin MIC and, as with Qnr, increasing the mutant protective concentration as well. In a set of 78 quinolone-resistant clinical *E. coli* isolates from Shanghai, six strains carried *qnrA,* 36 strains carried AAC(6′)-Ib-cr (the ciprofloxacin resistance variant), and four strains had both resistance mechanisms (32). In *E. coli* isolates from the United States the AAC(6′)-Ib-cr variant is also more common than Qnr (33), but it provides a lower MIC and is specific for certain quinolones.

Table 1 Resistance produced by Qnr proteins

Plasmid in *E. coli J53*	Qnr protein	MIC (μg/mL)[a]	
		Nalidixic acid[b]	Ciprofloxacin[c]
R⁻		4	0.008
pMG252	QnrA1	32	0.5
pMG298	QnrB1	16	1
pMG306	QnrS1	32	0.5

[a]Data from (12, 13)

[b]The CLSI MIC breakpoints for nalidixic acid are ≤8 μg/mL susceptible and ≥32 μg/mL resistant

[c]The CLSI MIC breakpoints for ciprofloxacin are ≤1 μg/mL susceptible and ≥4 μg/mL resistant

The third type of plasmid-mediated quinolone resistance involves efflux pumps of the resistance-nodulation-cell division (RND) (34, 35) or the major facilitator superfamily (MFS) (36, 37). The prevalence of this mechanism and its association with *qnr, aac(6′)-Ib-cr*, or higher-level quinolone resistance remains to be determined.

At least the first two plasmid-mediated resistance mechanisms have been found in clinical isolates testing susceptible by CLSI criteria. Because Qnr and AAC(6′)-Ib-cr often occur in strains resistant to other antibiotics, quinolone therapy

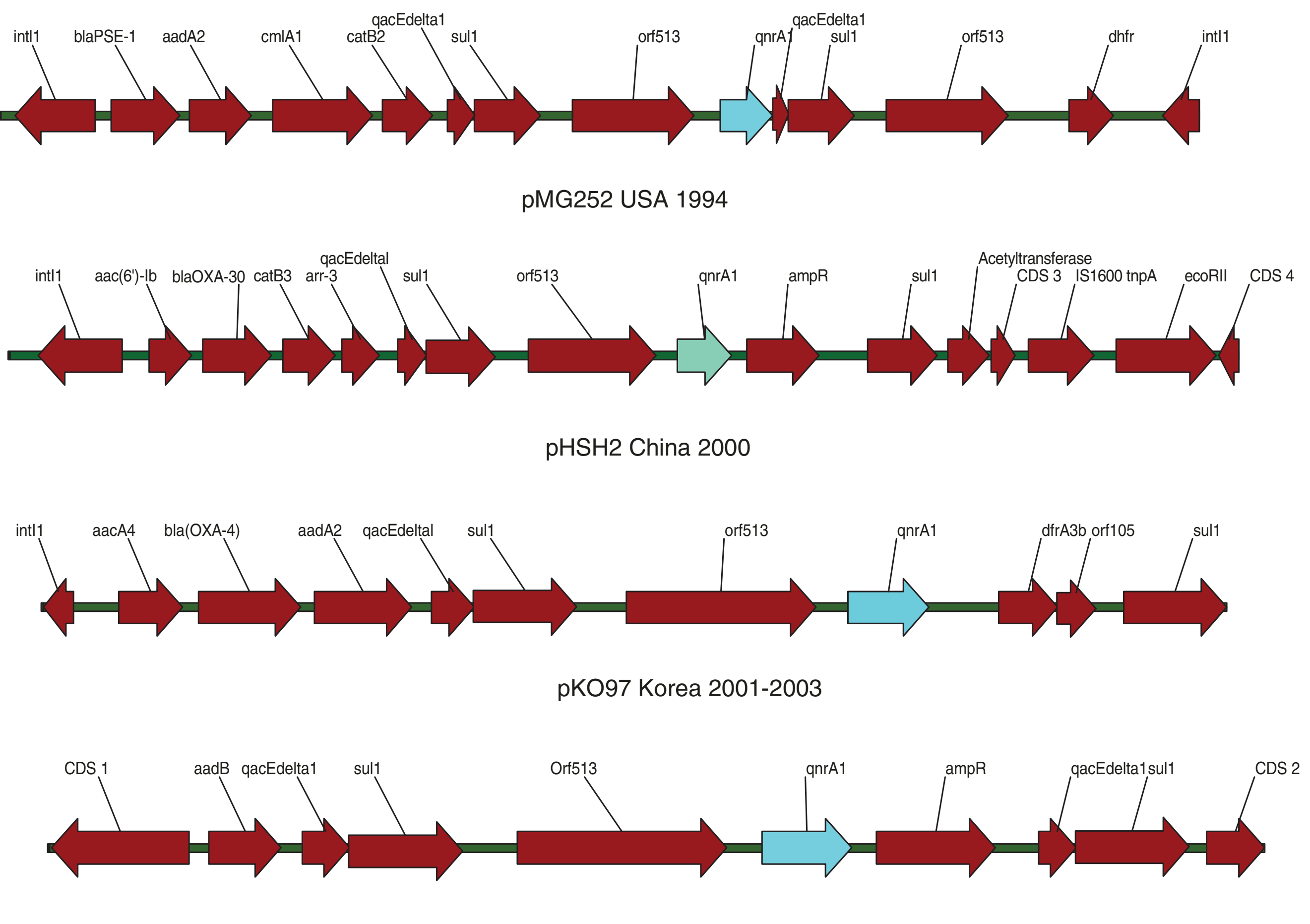

Fig. 1 *qnrA1* sul1-type integrons. The structure of integrons containing *qnrA1* from the United States (Jacoby, unpublished), China (16), Korea (17) and the Netherlands (18) are shown. Plasmid pHSH2 also carries the gene for *aac(6′)-Ib-cr*. Orf513 has been renamed IS*CR1* (15)

may appear to be an attractive option. Such strains have enhanced potential for higher-level resistance development with consequent failure to respond. In particular Qnr has been shown to act additively with mutations in *gyrA, gyrB, parC,* or *omp* (38). Even though Qnr, AAC(6′)-Ib-cr and the efflux pumps by themselves provide only low-level resistance, they thus contribute to the rising prevalence of quinolone resistance.

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Chapter 18
Macrolides and Lincosamides

Annie Canu and Roland Leclercq

1 Introduction

The structurally unrelated antimicrobials, macrolides, lincosamides, and streptogramins are grouped into a single family, the MLS family. This classification is justified by a similar, although not identical, mechanism of action.

Macrolides are composed of a minimum of two amino and/or neutral sugars attached to a lactone ring of variable size (1) (Fig. 1). Erythromycin is a mixture of antibiotics that includes erythromycin A which is the active compound and has a 14-membered lactone ring with two sugars, L-cladinose and an amino sugar. Other commercially available macrolides derived from erythromycin A include clarithromycin, dirithromycin, roxithromycin, and azithromycin which have an enlarged 15-membered ring resulting from a nitrogen insertion. The structural modifications of erythromycin A resulted in improved pharmacokinetic profiles and better tolerance, but cross-resistance between members of this class of antimicrobials was still observed. Certain 16-membered ring macrolides are also available in a few countries (spiramycin, josamycin, midecamycin, and miocamycin) or for veterinary use (tylosin). The recently developed ketolides, telithromycin and cethromycin (ABT773), are derived from clarithromycin and have two major modifications, replacement of L-cladinose by a keto-function and an 11-12-carbamate extension with an arylalkyl modification in telithromycin.

Lincosamides form a small group of antibiotics of naturally occurring compounds or semi-synthetic derivatives that contain an amino acid, a proline residue, attached by a peptide bond to a galactoside ring (2) (Fig. 1). Lincomycin is a naturally occurring lincosamide. Clindamycin (7-chloro-7-deoxy-lincomycin), a semi-synthetic derivative of lincomycin in which a hydroxyl group has been replaced by chlorine, is the most important in clinical use. This minor difference in the structure of the molecules results in a noteworthy increase of the molecule affinity for its target (3).

2 Mode of Action of Macrolides and Lincosamides

Macrolides and lincosamides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and ultimately inhibit microbial growth (1, 2).

The ribosome is composed of two subunits 30S and 50S built with RNA and proteins, which assemble to produce a functional structure for protein synthesis. Each part undertakes a specific function. The small subunit 30S decodes mRNA. In the large 50S part, the protein is formed by the polymerization of amino acids according to the genetic code. tRNA molecules carry the amino acids. Ribosomes possess three tRNA binding sites A, P, and E, hosting the aminoacyl-tRNA, the peptidyl-tRNA, and the exiting tRNA, respectively. Each elongation cycle involves the advancement of the mRNA together with A → P → E site passage of the tRNA molecule driven by GTPase activity (4).

The 50S subunit is formed in part by 23S rRNA which is organized into six domains. The domain V loop called peptidyl transferase center (PTC) contains the active site of peptide bond formation (5, 6). This PTC loop is positioned at the bottom of a cavity located at the interface of the two subunits, adjacent to the entrance of the peptide tunnel. This tunnel crosses the 50S subunit and emerges on the back of the ribosome. Recently, three-dimensional molecular structure of the ribosome was revealed by electron-cryomicroscopic studies and at atomic level by RX crystallography at high resolution (7). From three bacterial species (*Thermus thermophilus*, *Haloarcula marismortui*, and *Deinococcus radiodurans*) chosen as a model for the high stability of their ribosomes, much has been learned about the antibiotics that inhibit ribosome function. Although some differences may occur in the ribosomal binding of macrolides and lincosamides according to bacterial species, common features have been found (8, 9).

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Fig. 1 Structure of selected macrolides (erythromycin, clarithromycin, azithromycin, and telithromycin) and lincosamides (lincomycin/clindamycin)

The binding sites for the MLS antibiotics are located in the PTC or in the near vicinity of the PTC at the beginning of the peptide tunnel, before it is constricted by the ribosomal proteins L4 and L22 (10). The common nucleotide moieties involved in hydrogen bond interactions of the 23S rRNA with macrolides and clindamycin are the nitrogen bases of the nucleotide residues A2058, a crucial MLS-binding site, and A2059 (10). However, each class of drugs forms its own unique set of interactions with specific additional nucleotides. According to its position, the antibiotic inhibits peptide bond formation or peptide nascent chain progression. All the macrolides attach their lactone ring inside the peptide tunnel at the upper portion, and can protrude their appendage into the PTC cavity (11). The mechanism of action depends on their size and sugar components (12). Important contacts are formed between the C5 monosaccharide (desosamine) or disaccharide side chain of 14-15-16-membered macrolides and rRNA (13). The shape of desosamine sugar of the macrolactone ring in erythromycin fits exactly with that of the cavity formed by several nucleotides including A2058 and this interaction is considered to be required for ribosome binding (14). The telithromycin macrolactone ring had additional hydrogen bond and hydrophobic interactions involving the three keto group and two nucleotide residues of PTC. Several telithromycin and erythromycin binding sites within the 23S RNA overlap exactly. Telithromycin binds ten times more strongly to ribosomes than the parent macrolide erythromycin, largely because of the alkyl–aryl substituent extending from the macrolactone ring position 11 and 12 that generates a hydrogen bond with the nucleotide U2609 (8).

Both macrolides and ketolides act by producing a steric blockage of the ribosome exit tunnel hence hampering the progression of nascent peptide (10).

Clindamycin binds in an elongated conformation oriented with its long axis more or less parallel to the axis of the exit tunnel. Its prolin residue occupies the same cleft as the site A substrate puromycin and blocks PTC activity by hampering the binding of transfer RNA to the A site. Clindamycin interacts directly with the A and P sites and blocks the formation of peptide bond by disturbing the positioning of tRNA in A and P sites (8).

The overlapping of some binding sites may explain why macrolides and clindamycin bind competitively to the ribosome and why modification of binding sites confers cross-resistance.

3 Spectrum of Activity

MICs of macrolides and clindamycin for pathogenic bacteria are shown in Table 1. Macrolides have a spectrum of activity limited to Gram-positive cocci and bacilli, notably staphylococci, hemolytic streptococci, and pneumococci, and Gram-negative cocci. Gram-negative bacilli are generally resistant with the exception of some clinically important

Table 1 MICs of macrolides and lincosamides for susceptible pathogenic bacteria

Bacterial species	MIC 50 (μg/ml)					
	Ery[a]	Cla	Azi	Tel	Lin	Cli
Aerobes						
Gram-positive organisms						
Staphylococcus aureus	0.25	0.25	1	0.04	0.5	0.1
Staphylococcus epidermidis	0.25	0.12	0.5	0.04	0.5	0.2
Streptococcus pyogenes	0.06	0.015	0.06	0.06	0.06	0.03
Streptococcus pneumoniae	0.06	0.015	0.06	0.03	0.25	0.06
Streptococcus viridans	0.06	0.015	0.06	0.03	0.25	0.1
Corynebacterium diphteriae	0.008	0.004	0.015	0.004	0.5	<0.5
Gram-negative bacteria						
Campylobacter jejuni	1	1	0.12	1	>8	>8
Haemophilus influenzae	4	4	1		32	8
Helicobacter pylori	0.25	0.015	0.25		4	0.5
Intra-cellular pathogens						
Legionella pneumoniae	0.25	0.03	0.12	0.03	16	12
Chlamydia pneumoniae	0.12	0.12	0.12	0.12	–	–
Mycoplasma pneumoniae	0.015	0.004	0.003		4	1
Anaerobes						
Bacteroides fragilis	16	2	8	16	1	0.1
Prevotella sp.	0.5	0.06	0.12	0.12	0.25	0.01
Fusobacterium spp.	64	16	8	16	0.5	<0.1
Actinomyces sp.	0.03	0.03	<0.01	<0.01	0.25	0.06
Propionibacterium	0.01	<0.01	0.03	<0.01	0.5	0.03
Clostridium perfringens	1	0.5	0.5	0.12	0.5	0.1
Peptostreptococcus spp.	4	2	4	0.06	0.5	0.05

[a]*Azi* azithromycin; *Cla* clarithromycin; *Cli* clindamycin; *Ery* erythromycin; *Lin* lincomycin; *Tel* telithromycin

genera, i.e., *Bordetella pertussis*, *Campylobacter*, *Chlamydia*, *Helicobacter*, and *Legionella*.

Lincosamides have a spectrum of activity closely related to that of macrolides, despite their different structure. *Enterococcus faecalis* has an intrinsic resistance to clindamycin and lincomycin which is shared with other species of enterococci, such as *Enterococcus avium*, *Enterococcus gallinarum*, and *Enterococcus casseliflavus*. By contrast, *Enterococcus faecium*, *Enterococcus hirae*, and *Enterococcus durans* are intrinsically susceptible to lincosamides.

A particular feature of clindamycin is its activity against anaerobic bacteria, in particular, *Clostridium* spp., *Peptostreptococcus* spp., and Gram-negative rods. However, incidence of acquired resistance is now relatively high in *Bacteroides fragilis*. Also, *Clostridium sporogenes*, *Clostridium tertium*, and *Clostridium difficile* are frequently resistant to clindamycin. Clindamycin has some activity against *Toxoplasma gondii* and *Pneumocystis carinii*.

4 Mechanisms of Resistance to Lincosamides and Clinical Implications

Resistance to macrolides and lincosamides can be mediated by multiple mechanisms including target modification, enzymatic drug inactivation, and active efflux. Target modification encompasses methylation of A2058, which is, as previously mentioned, a key residue with which macrolides and lincosamides interact, and mutations in 23S rRNA or in conserved regions of ribosomal proteins L4 and L22. In pathogenic microorganisms, the impact of these mechanisms is unequal in terms of incidence and of clinical implications. Modification of the ribosomal target confers broad-spectrum resistance to macrolides and lincosamides, whereas enzymatic modification affects only structurally related antibiotics. These mechanisms have been found in the antibiotic producers, which often combine several approaches to protect themselves against the antimicrobial that they produce.

4.1 Ribosomal Methylation

4.1.1 *erm* Genes

Ribosomal modification by methylation was the first mechanism of resistance to macrolides elucidated. This mechanism results from the acquisition of an *erm* gene (erythromycin ribosome methylase) usually carried by plasmids or transposons in pathogenic bacteria. Biochemical studies indicated that the *erm* genes encode methylases which methylate bacterial 23S rRNA at a single site, adenine at position 2058 (15). As a consequence of methylation, the activity of

antibiotics that have the A2058 nucleotide as a key nucleotide for their binding to the ribosome is impaired. The overlapping binding sites of macrolides, lincosamides, and streptogramins B in 23S rRNA account for cross-resistance to the three classes of drugs, which gave its name to the MLS_B resistance phenotype. A wide range of microorganisms that are targets for macrolides and lincosamides express Erm methylases.

Nearly 40 *erm* genes have been reported so far. Four major classes are detected in pathogenic microorganisms (http://faculty.washington.edu/marilynr/): *erm*(A), *erm*(B), *erm*(C), and *erm*(F). *erm*(A), and *erm*(C) typically are staphylococcal gene classes. Genes belonging to the *erm*(B) class and to a subclass of the *erm*(A) gene class previously called *ermTR* are widespread in streptococci and in enterococci, whereas the *erm*(F) class genes are detected in *Bacteroides* species and other anaerobic bacteria. However, although each class is relatively confined to a bacterial genus, it is not strictly genus specific. For instance, *erm*(B) genes may be found in staphylococci and anaerobes.

Although all members of the *erm* family methylate the adenine of 23S rRNA located at position A2058, they differ by their capacity to monomethylate or dimethylate this nucleotide position. The major Erm methylases detected in pathogens, Erm(A), Erm(B), and Erm(C), generally function as dimethylases that confer a high-level cross-resistance to MLS_B drugs (including telithromycin). However, Erm(B) in a pneumococcus background may function as a monomethylase rather than as a dimethylase (16). In fact, this makes a difference for the ketolides telithromycin and cethromycin, which are weakly affected by monomethylation, but not for erythromycin and clindamycin which are poorly active whether the ribosome is mono- or dimethylated.

4.1.2 Regulation of Erm Genes Expression

Inducible Resistance

MLS_B resistance may be constitutively or inducibly expressed (17, 18). In inducible resistance, the bacteria produce inactive mRNA that is unable to encode methylase. In the model of the staphylococcal gene *erm*(C), the inactivity of the mRNA is due to the structure of its 5′ end which has a set of inverted repeats which sequester the initiation sequences (ribosome-binding site and initiation codon) for the methylase by base-pairing in the absence of erythromycin (18). Thus, the methylase cannot be produced since the initiation motifs for translation of the enzyme are not accessible to the ribosomes. Induction is related to the presence of an open-reading frame encoding a short 14-amino-acid peptide upstream of the *erm*(C) structural gene. In the presence of low concentrations of erythromycin, binding of the antibiotic to a ribosome translating the leader peptide causes the ribosome to stall. Ribosome stalling probably induces destabilization of the pairing and conformational rearrangements in the mRNA which would then unmask the initiation sequences for the methylase, allowing synthesis to proceed by available ribosomes. This *erm*(C) regulation model designated as post-transcriptional (or translational) attenuation would also account for the regulation of the *erm*(A) and *erm*(B) determinants (18).

For a given attenuator, the inducing capacity of the macrolides depends on the antibiotic structure. Whereas 14-membered macrolides (erythromycin, roxithromycin, and clarithromycin) and 15-membered macrolides (azithromycin) are inducers for the production of most Erm methylases, ketolides and lincosamides are generally not. Mutations in the attenuator may modify the induction pattern. In particular, lincosamides may become inducers in the case of mutation of the attenuator. This feature has been reported in laboratory mutants (19) and rarely for clinical isolates of *S. aureus* (20).

In staphylococci that typically contain *erm*(A) or *erm*(C) genes, inducible resistance leads to dissociated phenotypes of resistance between inducers (erythromycin) that are not active and noninducers (clindamycin) that remain active. The phenotype of MLS_B inducible resistance expressed by staphylococci is characteristic, provided that the strains are tested by the disk-diffusion technique. A blunting of the clindamycin inhibition zone, similar to the shape of the letter D and referred as to a D-shaped zone, can be observed, provided that a disk of erythromycin is placed nearby (Fig. 2b).

Which holds true for staphylococci is not for streptococci that usually harbor *erm*(B) genes. Indeed, the inducible *erm*(B) gene generally confers a cross-resistance to erythromycin and clindamycin, which differs from the dissociated resistance conferred by the staphylococcal *erm*(A) and *erm*(C) genes. The particular expression of *erm*(B) might be related to methylation of various proportions of ribosomes even in the absence of erythromycin (16). This paradox could be explained by a nonstringent control of the expression of the methylase by the *erm*(B) attenuator. Fusion of the mutated *erm*(B) attenuator with a *lacZ* reporter gene has confirmed that the expression of the methylase can be partly derepressed in certain strains (21). By contrast, the control of methylase expression by the staphylococcal *erm*(A) and *erm*(C) methylases appears more strict. Other additional features, such as differences in the promoter strength or in the copy number of the *erm*(B) gene, may also account for the various levels of ribosomal methylation. The presence of basal levels of methylase appears sufficient to confer resistance to lincosamides, explaining the cross-resistance between macrolides and lincosamides in streptococci containing inducible *erm*(B) genes (20). Another gene, *erm*(TR), which is a subset of the

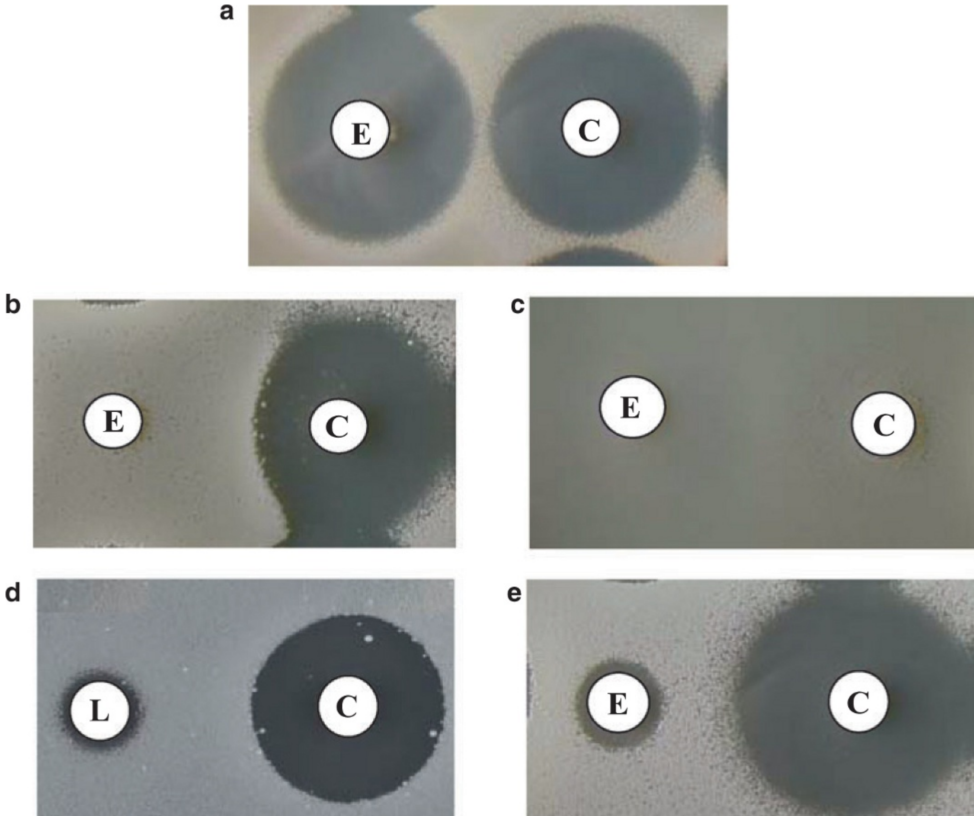

Fig. 2 Phenotypes of resistance to macrolides and clindamycin in *S. aureus*. (**a**) *S. aureus* susceptible to erythromycin and clindamycin; (**b**) *S. aureus* containing an *erm*(C) gene inducibly expressed (a D-shaped zone can be observed for the clindamycin zone of inhibition on the edge closest to the erythromycin zone of inhibition); (**c**) *S. aureus* containing an *erm*(C) gene constitutively expressed; (**d**) *S. aureus* containing an *lnu*(A) gene responsible for inactivation of lincosamides; (**e**) *S. aureus* resistant to erythromycin by efflux (*msr*(A) gene) (note the absence of D-shaped zone). *C* clindamycin; *E* erythromycin; *L* lincomycin

erm(A) gene class, is spread in beta-hemolytic streptococci. The expression of the gene resembles that of the staphylococcal *erm*(A) gene (17).

Constitutive Resistance

In constitutive expression, active methylase mRNA is produced in the absence of an inducer and the strains express cross-resistance to MLS_B antibiotics, regardless of the nature of the *erm* gene (Fig. 2c).

In the laboratory, mutants derived from inducible strains of staphylococci and expressing constitutive MLS_B resistance can be selected on agar plates containing inhibitory concentrations of clindamycin at frequencies varying between 10^{-6} and 10^{-8}, depending on the strain (17, 22). In addition, clinical isolates constitutively resistant to erythromycin are widespread, especially in methicillin-resistant staphylococci. It has been shown both in laboratory mutants and in clinical isolates that constitutive expression is due to deletions, duplications, or point mutations in the attenuator sequence leading to derepressed production of the methylase (18).

Similarly, in vitro selection by clindamycin of constitutive resistance at a frequency of 10^{-7} has been reported in a clinical isolate of *Streptococcus pyogenes* UCN1 inducibly resistant to erythromycin and harboring an *erm*(TR) gene (subset of the *erm*(A) gene class) (23).

4.1.3 Clindamycin for Infections Due to *S. aureus* with the MLS_B Inducible Phenotype?

The use of clindamycin for the treatment of an infection due to an inducibly resistant strain of *S. aureus* is not devoid of risk. As mentioned previously, constitutive mutants can be selected in vitro in the presence of clindamycin at a relatively high frequency. Bacterial inocula exceeding 10^7 cfu can be found in mediastinitis and in certain lower respiratory tract infections. The risk to patients is illustrated by reports of selection of constitutive mutants during the course of

Table 2 Failures of clindamycin therapy in infections due to *S. aureus* with inducible resistance to macrolides–lincosamides–streptogramins B-type (after ref. 28)

No. of patients treated with clindamycin	No. of failures	No. of MLS_B constitutive isolates selected	Reference
3	2	1	(20)
2	2	2	(21)
3	1	1	(22)
2	2	1	(23)
1	1	1	(24)
1	1	1	(25)

clindamycin therapy administered to patients with severe infections due to inducibly erythromycin-resistant *S. aureus* (22, 24–29). However, clinical evidence regarding the risk of emergence of clindamycin resistance is based only on a few case reports which are summarized in Table 2 and there are also reports of successful use of clindamycin in treating patients with D-test-positive isolates. Most data come from pediatric patients. This is due to the fact that infections due to community-acquired methicillin-resistant *S. aureus* (CA-MRSA) are increasing in this population, that many CA-MRSA have an inducible MLS_B phenotype, and that clindamycin is an interesting alternative to vancomycin for the treatment of CA-MRSA infections.

So far, although it seems reasonable to discourage the use of clindamycin in deep-seated infections or in infections with heavy bacterial inoculum which increases the risk for selection of constitutive mutants, we have no criteria to confidently predict the success or the failure of clindamycin therapy in infections due to MLS_B inducible staphylococci.

We need more prospective studies of cases of staphylococcal or hemolytic streptococcal infections treated with clindamycin to better define the role of this antimicrobial in infections due to microorganisms with various macrolide resistance phenotypes.

4.2 Ribosomal Mutations

Studies with mutants obtained in the laboratory and reports of clinical isolates have revealed that several structures participating in the binding of macrolides, domains V and II of 23S rRNA and proteins L4 and L22, can display mutations responsible for macrolide/lincosamide resistance. The resistance phenotype conferred by alterations in the ribosomal target varies according to the nature of the mutated structure, but there is generally cross-resistance between macrolides and lincosamides. In addition, since bacteria generally have several copies of the *rrl* gene for 23S rRNA, susceptibility to macrolides and lincosamides varies according to the number of mutated copies and decreases as the number of the mutated gene copies increases (30). Ribosomal mutations are rare in clinical isolates. They have been mostly reported in staphylococci and streptococci (17), but appear more related to the use of macrolides than to the use of lincosamides.

4.3 Enzymatic Modification of Macrolides

Unlike target modification, inactivation of MLS_B antibiotics confers resistance to structurally related antibiotics only. Esterases, phosphotransferases, acetyltransferases, hydrolases, and nucleotidyltransferases have been identified in strains resistant to macrolides or lincosamides.

Members of the family Enterobacteriaceae which are highly resistant to erythromycin have been reported. Most of the strains were isolated from stool or blood cultures during selective digestive tract decontamination in neutropenic patients (31). The isolates inactivate the lactone ring of 14-membered ring macrolides by production of eythromycin esterases or a macrolide 2′-phosphotransferase that add phosphate to the 2′-hydroxyl group of desosamine or mycaminose (32).

mph(A) and *mph*(B) encode two different macrolide phosphotransferases in enteric bacteria. Also, two types (I and II) of esterases encoded by *ere*(A) and *ere*(B) (erythromycin esterase) genes, respectively, have been found (31, 33, 34). The G + C content of *ere*(B) (36%), unlike that of *ere*(A) (50%), is significantly different from the base composition of the *Escherichia coli* chromosome (50%). This observation suggests that *ere*(B) is of exogenous origin, possibly a Gram-positive coccus. However, macrolide-inactivating enzymes have been rarely detected in Gram-positive cocci.

An *mph*(C) gene, distinct from *mph*(A) and *mph*(B), has been described in a few strains of *S. aureus* (35). The esterase gene *ere*(B) was detected in 5 of 851 isolates (0.6%) of erythromycin-resistant and methicillin-resistant *S. aureus* strains collected from 24 European hospitals (36). No *ere*(A) gene could be detected in this collection.

4.4 Enzymatic Modification of Lincosamides

Specific resistance to lincosamides is due to enzymatic inactivation of those antibiotics. Phosphorylation and nucleotidylation of the hydroxyl group at position 3 of lincosamides have been detected in several species of *Streptomyces*. In both animal and human isolates, lincosamide nucleotidyltransferases encoded by *lnu* genes (formerly *lin*) were reported. In clinical isolates, eight *lnu* genes have been described: *lnu*(A), *lnu*(A′), *lnu*(B), *lnu*(B-like), *lnu*(A_{N2}), *lnu*(C), *lnu*(D), and *linF* (37–42). The *O*-nucleotidyltransferases encoded by these genes inactivate lincosamides by adenylylation (38).

lnu(A) and *lnu*(A′) have been reported in *Staphylococcus haemolyticus* and *S. aureus*, respectively (37). They encode two isoenzymes of 161 amino acids differing by 14 amino acids. An *lnu*(A_{N2}) gene homologous to *lnu*(A) and *lnu*(A′) (55% of identity) was evidenced in *Bacteroides* spp. (40). This gene would be carried by a mobilizable transposon.

The *lnu*(B) gene from *E. faecium* does not display homology with the other *lnu* genes and is carried by a large conjugative plasmid (38). More recently, an *lnu*(B-like) gene (79% identity with *lnu*(B)) and an *linF* gene (34.9% identity with *lnu*(B)) were identified in *Eubacterium* and *E. coli*, respectively (39).

The *lnu*(C) gene was characterized in the clinical isolate *Streptococcus agalactiae* UCN36 (41). The gene was located on a genetic element named TnLnu that bore a homologue of the IS*1* transposase gene and which was delineated by imperfect inverted repeats.

The *lnu*(D) gene was characterized in a clinical isolate of *Streptococcus uberis* responsible for a case of bovine mastitis (42).

The precise site of nucleotidylation of lincomycin and clindamycin was characterized for proteins LnuA and LnuB. The LnuA nucleotidyltransferase modifies a hydroxyl group of clindamycin and lincomycin at positions 3 and 4, respectively. By contrast, LnuB modifies a hydroxyl at position 3 in both clindamycin and lincomycin (38).

4.4.1 Expression of the lnu Genes

Although LnuA, LnuB, LnuC, and LnuD nucleotidyltransferases inactivate in vitro more efficiently clindamycin than lincomycin, the corresponding genes confer resistance to lincomycin (MICs from 16 to 32 μg/ml) but not to clindamycin (MICs from 0.06 to 0.12 μg/ml), the so-called L phenotype (37, 38, 41, 42) (Fig. 2d). By contrast, when the *lnu*(A), *lnu*(A′), *lnu*(B), *lnu*(C), and *lnu*(D) genes were cloned into *E. coli*, they conferred cross-resistance to lincomycin and clindamycin (37, 38, 41, 42). A similar phenotype was observed for the *linF* gene in *E. coli* (39). The reason for the difference in phenotypic expression of the resistance determinant in the two backgrounds remains unexplained. Hypothetically, the difference between the two lincosamides might be related to differences in relative affinities of clindamycin and lincomycin for the ribosomes of Gram-positive and Gram-negative organisms and for the Lnu enzymes: clindamycin might have better affinity for the Gram-positive ribosomes than for LnuC, explaining why its activity is maintained.

Although the activity of clindamycin against the Gram-positive hosts of the *lnu* gene was only weakly affected by the mechanism of resistance, a 100-fold increase in the bacterial inoculum led to a three-dilution increase in the MIC of clindamycin for *S. agalactiae* UCN36 containing *lnu*(C) (41) and the bactericidal activity of clindamycin (already weak against susceptible strains) was totally abolished against a staphylococcal strain with *lnu*(A) (37).

4.5 Efflux

Efflux has been reported as responsible for the intrinsic resistance to macrolides and lincosamides of *E. coli* and, putatively, as responsible for the intrinsic resistance of *E. faecalis* to lincosamides and streptogramins A-type. In *E. coli*, inactivation of the tripartite pump AcrAB-TolC renders this organism susceptible to erythromycin and clindamycin (43). In *E. faecalis* OG1RF, cross-resistance to lincosamides and streptogramins A-type, defining the Lsa phenotype, has been related to the expression of a chromosomal *lsa* gene, which appears to be species specific (44). Inactivation of the *lsa* gene resulted in susceptibility to clindamycin, dalfopristin, and quinupristin–dalfopristin, whereas complementation with a recombinant plasmid bearing an intact *lsa* gene restored resistance to clindamycin and dalfopristin. The Lsa protein shows similarities to members of a superfamily of transport-related proteins known as ABC transporters. ABC proteins are capable of transporting both small and large molecules in response to ATP hydrolysis. The ABC transporter system requires two ATP-binding domains located in the cytoplasm that interact with two hydrophobic domains consisting generally in six transmembrane segments. The four core components of an ABC transporter can be synthesized as individual proteins or be fused into multifunctional polypeptides in a variety of combinations. Conserved motifs that are used to define ABC domains have been identified in Lsa. However, no transmembrane partner has been found associated with the Lsa protein and the efflux mechanism has not been proven. In addition, the environment of the *lsa* gene is important for its expression since changes in different regions of the *E. faecalis lsa* locus influence the ability of cloned *lsa* to confer resistance to lincosamides and streptogramins A-type (45).

Active efflux has been reported as an acquired mechanism of resistance to macrolides in clinical isolates of Gram-positive organisms. In particular, efflux pumps *msr*(A) responsible for the MS phenotype (resistance to erythromycin and streptogramins B) in staphylococci and *mef*(A) responsible for the M phenotype (resistance to erythromycin) in streptococci which belong to the ABC transporter family and to the Major Facilitator Superfamily, respectively, are widely spread (46). These mechanisms do not affect lincosamides. The activity of ketolides is affected by *mef*(A) only at a very low level, probably not clinically significant. *mef*(A) is borne by a transposon (47, 48) and has been described in a variety of species, mostly *S. pneumoniae* and *S. pyogenes*, but also

S. agalactiae, *viridans* streptococci, *Streptococcus milleri*, *Streptococcus mitis*, Groups C, F, and G streptococci, *Micrococcus luteus*, *Corynebacterium* spp., *Enterococcus* spp., and Gram-negative bacilli (http://faculty.washington.edu/marilynr/ermweb4.pdf). *S. pneumoniae* or *S. pyogenes* strains harboring *mef*(A) generally have MICs against clarithromycin, azithromycin, and erythromycin of 1–32 μg/ml versus an MIC range of 0.03–0.5 μg/ml for telithromycin in *S. pneumoniae* (46).

The *msr(A)* gene is usually found in staphylococci but has also been detected in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* (49).

Acquired efflux of lincosamides seems to be limited to a LSA phenotype detected in *S. aureus* and similar to that reported as intrinsic in *E. faecalis*. This phenotype is due to the acquisition of plasmid genes *vga*(A) and *vga*(Av) which confer a low-level resistance to lincomycin, clindamycin, and streptogramins A-type (50). Homology of the deduced sequences of the Vga proteins with those of ABC transporters suggests that resistance is due to efflux. A similar LSA phenotype has been recently reported from *S. agalactiae* isolates from New Zealand (51) and in *E. faecium* (52).

5 Report of Susceptibility Tests by the Laboratory

5.1 *Staphylococci*

Both clindamycin and erythromycin have to be tested. As noted above, resistance to both erythromycin and clindamycin relates to constitutive MLS_B resistance and is easily recognized. Dissociated susceptibility results for erythromycin and clindamycin require the attention of the clinical microbiology laboratory. The following cases can be discussed.

5.1.1 Strains Resistant to Erythromycin but Susceptible to Clindamycin

When clindamycin is active, the identification of the phenotype is required. The MLS_B inducible resistance can be detected only by methods showing induction of clindamycin resistance. As previously mentioned, the disk-diffusion method is an easy method to detect this phenotype by placing an erythromycin disk near to a clindamycin disk on an agar growth medium, using a standard disk dispenser (53). The presence of a D-shape zone is the signature of the MLS_B inducible phenotype (Fig. 2b). This approach is recommended by the 2004 CLSI susceptibility testing standards. When staphylococci are tested using a broth-based method (including an automated instrument), the CLSI recommends placing erythromycin and clindamycin disks nearly 15 mm apart (center to center) on the blood agar plate that is used to control the purity of the bacterial inoculum (54, 55).

Isolates displaying a D-shaped zone, therefore inducibly resistant to MLS_B antibiotics, should be reported as clindamycin resistant by the laboratory (54). However, the clinical laboratory may add the comment that "this isolate is presumed to be resistant based on detection of inducible clindamycin resistance. Clindamycin may still be effective in some patients." The final decision to treat or not treat the patient with clindamycin should be based on the analysis of each specific case and if a clindamycin therapy is started, it requires close follow-up of the patient for failure.

In the absence of D-shaped zone, the staphylococcal isolate is presumably resistant to erythromycin by active efflux (acquisition of the *msr*(A) gene) (Fig. 2e). Since clindamycin is neither an inducer nor a substrate for the pump, the isolate can safely be reported as susceptible to clindamycin.

Strains of *S. aureus* ATCC strain BAA-977 containing *erm*(A) and ATCC BAA-976, which harbors the efflux pump encoded by *msr*(A), have recently been recommended as positive and negative control organisms, respectively (56).

5.1.2 Strains Susceptible to Erythromycin but Resistant to Lincosamides

This dissociated phenotype of resistance is rare in *S. aureus*, found in less than 1% of the strains, but is more frequent in coagulase-negative staphylococci, with frequencies ranging from 1 to 7% of strains depending on the staphylococcal species (37). On account of the rarity of this phenotype in streptococci and staphylococci and on its intrinsic nature in *Neisseria* spp. and in most enterococi, identification of the isolate should be checked.

Two phenotypes of resistance should be distinguished.

The LSA type of resistance is detected as a resistance or an intermediate susceptibility to both clindamycin and lincomycin.

The L phenotype resistance can be identified only if lincomycin is tested since MIC of clindamycin or zone size diameter for the disk of clindamycin remain within the range of those for a susceptible isolate. This phenotype can be easily identified by testing lincomycin and clindamycin which display an unusual dissociated susceptibility to clindamycin and resistance to lincomycin. By the disk-diffusion technique, lincosamide inactivation can be easily predicted by observing the appearance of the clindamycin inhibition zone edge. A sharply demarcated edge correlates with the production of lincosamide nucleotidyltransferases (Fig. 2d). There is no recommendation for the interpretation of the result for clindamycin and the clinical relevance is unknown.

5.2 Other Organisms

For streptococci, concerns about the activity of clindamycin against isolates susceptible to this antibiotic but with an inducible MLS_B phenotype could also be raised. However, routine testing for inducible resistance for pneumococci is not recommended since the isolates containing an inducible *erm*(B) gene usually display cross-resistance between erythromycin and clindamycin, as mentioned above. Only rare isolates with an inducible MLS_B phenotype are susceptible to clindamycin and clinical significance has not been established. The same observation can be made for beta-hemolytic streptococci containing an inducible *erm*(B) gene. However, beta-hemolytic streptococci might contain an inducible *erm*(TR) gene that is expressed similarly to the inducible *erm*(A) gene, with a positive D-shaped zone test. In this case, although no clinical failure has been reported, the use of clindamycin does not seem safe. Isolates of *S. pneumoniae* or *S. pyogenes* expressing the efflux pump MefA remain fully susceptible to clindamycin.

Resistance to clindamycin in *Bacteroides fragilis* is frequent (generally more than 30% of isolates) and is mostly due to ribosomal methylation (MLS_B phenotype) mostly by *erm*(F), *erm*(G), and *erm*(B) genes. The resistance is often expressed at a high level. However, expression of resistance may be inducible and full expression of resistance may be delayed. In case of susceptibility to clindamycin, the final result should not be read before a full 48-h period of incubation.

C. perfringens is rarely resistant to clindamycin. Again, resistant isolates express an MLS_B phenotype which, in some cases of inducible expression, can be detected only after 48 h of incubation.

6 Conclusion

Favorable properties of macrolides and clindamycin, in terms of tissue distribution, convenient oral or intravenous dosing, and low cost explain why these antibiotics, available for more than 40 years, remain widely used. However, a multiplicity of mechanisms have emerged in streptococci, staphylococci, and *Bacteroides* spp. that confer resistance to this group of antimicrobials and lead to complex resistance phenotypes. Identification of the corresponding resistance mechanisms has a clinical importance as regards to the use of macrolides and clindamycin. The clinical relevance of the inducible MLS_B type of resistance for activity of clindamycin still remains to be fully evaluated.

The incidence of resistance to macrolides and lincosamides has not been discussed in this chapter. It is highly variable according to the country and even within a country. The frequencies of resistance to clindamycin cannot be deduced from those to erythromycin since cross-resistance is unpredictable. In particular, efflux mechanisms affect the activity of erythromycin but not that of clindamycin, both in streptococci and staphylococci. The reverse is also true for other mechanisms of resistance. Therefore, specific survey of macrolide and lincosamide resistance in pathogens is required. Both surveillance of the incidence of resistance of the respective prevalence of the various resistance mechanisms is justified by the rapid variations in resistance observed in several countries.

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Chapter 19
Mechanism of Resistance in Metronidazole

Abhay Dhand and David R. Snydman

Metronidazole [1-(2 hydroxyethyl)-2-methyl-5-nitroimidazole] was introduced in the 1960s. Since then it has been drug of choice for human infections caused by various anaerobic and microaerophilic bacteria (Bacteroides, Clostridia, Helicobacter) and parasites (Trichomonas, Giardia, Entamoeba). Other Gram-positive anaerobes (e.g., lactobacilli, propionobacterium acnes, majority of the periodontal pathogens, peptostreptococci) are known to be inherently resistant to metronidazole. Virtually all the anaerobic Gram-negative rods are known to be susceptible to metronidazole.

Sensitivity testing for anaerobes is not performed routinely. Therefore, resistance to metronidazole is under-reported. With improvements in molecular detection, increasing resistance rates are being noted. This emerging resistance to metronidazole poses various diagnostic and therapeutic dilemmas. Mechanisms of resistance are being defined, and a better understanding is the key for prevention of resistance and improved management of these infections.

1 Antimicrobial Mechanism of Action

5-Nitroimidazole is administered as a prodrug. It enters the cell by passive diffusion and is activated in either the cytoplasm in bacteria, Entamoeba, and Giardia, or in a specialized organelle called hydrogenosome in Trichomonas. Activation to its cytotoxic form occurs via transfer of an electron from various donors to the nitro group, which converts it to a nitroso free-radical form. This toxic metabolite interacts primarily with DNA, RNA, or intracellular proteins, leading to DNA strand breakage, inhibited repair, and disrupted transcription. If the disruption of DNA is faster than its repair, it ultimately leads to cell death.

The selective toxicity and effectiveness of metronidazole depend on the cytoplasmic environment in the anaerobic and microaerophilic organisms, which provides a sufficiently low redox potential environment required for the activation of the drug. Metronidazole has very low reduction potential (E_{17} – 486 mV) and will be activated only in conditions where low redox status in maintained. Oxygen has higher affinity for an electron than metronidazole (E_{17} –150 mV). Therefore, oxygen can either successfully compete with 5-nitroimidazole for the electron from the electron carrier or be able to remove the electron from the activated nitroso group, thereby re-forming the parent drug – a phenomenon known as *futile cycling*. Similarly, downregulation of various intracellular electron donors may prevent activation of the prodrug, and therefore the lack of efficacy.

2 Mechanism of Resistance

The proposed mechanisms of resistance are

1. Decreased drug uptake or increased efflux
2. Decreased drug activation/change in the biological target
3. Increased oxygen scavenging capabilities (SOD/catalase/peroxidase)
4. Enhanced activity of DNA repair enzymes

2.1 *Bacteroides*

Metronidazole resistant (MTZ-R) *Bacteroides fragilis* was first reported in a patient with Crohn's disease after long-term therapy with metronidazole (1). Metronidazole resistance in *Bacteroides* spp. is quite rare but has been reported in several countries (2, 3). Metronidazole resistance among *Bacteroides* spp. is of concern, as these species can also be resistant to a wide variety of antimicrobial agents including β-lactams, tetracycline, clindamycin, cefoxitin, and imipenem (4).

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Breuil et al. and Reysset et al. showed that all Bacteroides strains that were resistant to 5-nitroimidazole harbored a genetic determinant, which was either plasmid borne or on the chromosome (5, 6). This resistance was shown to be transferable by a conjugation-like process to susceptible strains with a frequency ranging from 10^{-3} to 10^{-7} per donor. These genetic determinants have been shown to be specific nitroimidazole-resistant genes (*nim*), presumably encoding a nitroimidazole reductase that converted nitroimidazole to aminoimidazole, thereby avoiding the formation of toxic nitroso radicals that are essential for antimicrobial activity. So far seven *nim* genes (*nim* A,B,C,D,E,F,G) have been described. These genes are commonly transcribed from promoters located within different insertion elements. Gal and Brazier studied 50 resistant isolates and found the *nim*A gene was the most common, followed by *nim*B and *nim*E (7). Although the presence of a *nim* gene does not always equate to therapeutic resistance, prolonged exposure of *nim*-gene carrying *Bacteroides* spp. to metronidazole can select therapeutic resistance. Diniz et al. used a combination of proteomics for identification of differentially expressed proteins and other genes involved in the adaptive response to metronidazole (8). Protein profile of resistant strains showed upregulation of lactate dehydrogenase and downregulation of flavodoxin and impaired enzymatic activity of pyruvate–ferrodoxin oxidase reducatse. They also suggested that multiple enzymes involved in oxidation/reduction and electron transfer reactions may be important in activation of MTZ and possible mechanisms of inducing resistance. This supports the idea that there is no one specific gene for MTZ resistance and multiple possible pathways for resistance exist.

2.2 *Helicobacter pylori*

High rates of metronidazole resistance in *H. pylori* have also been reported worldwide. In Western Europe 20–45% isolates of *H. pylori* have been reported as MTZ-R. This rate is higher in developing countries, within immigrant populations, and in young women who may have received this agent in the past for parasitic infections or gynecologic infections (9–11). Thompson and Blaser showed that inactivation of *recA* (a gene needed for generalized DNA repair and recombination) severely impaired the ability of *H. pylori* mutants to survive treatment with UV light, ciprofloxacin, and metronidazole (12). Expression of a cloned *recA* gene obtained from a resistant strain of *H. pylori* in *E. coli* raised its level of resistance (12). Smith and Edwards showed that a relationship existed between the intracellular oxygen-scavenging ability of *H. pylori* and sensitivity of the bacterium to metronidazole. MTZ-R strains of *H. pylori* possessed considerably lower soluble cytosolic NADH oxidase activity than MTZ-S strains (13). Goodwin et al. first demonstrated that a major mechanism of MTZ resistance in *H. pylori* is due to null mutations in the *rdxA* gene, which encodes an oxygen-insensitive NAD(P)H nitroreductase (14). Using a cosmid cloning approach in MTZ-R strains, they identified an open reading frame (ORF) that had protein level homology to classical oxygen-insensitive NAD(P)H nitroreductases. An *H. pylori* gene corresponding to this ORF was designated *rdxA*. In a series of elegant experiments Goodwin et al. also showed that *E. coli* (normally MTZ-R) was rendered MTZ-S by a functional *rdxA* gene, introduction of *rdxA* on a shuttle vector plasmid into formerly MTZ-R *H. pylori* rendered it MTZ-S, and replacement of *rdxA* in MTZ-S *H. pylori* with a rdxA::camR null insertion allele resulted in MTZ-R phenotype (14). Kwon et al. reported role of an additional gene *frxA*, which encodes NAD(P)H flavin oxidoreductase, in MTZ resistance in *H pylori* (15). Using a lambda phage genomic library, they identified an MTZ nitroreductase encoding gene, NAD(P)H flavinoxidoreductase (*frxA*). Frame shift mutations leading to premature termination of frxA protein were associated with metronidazole resistance in *H. pylori*. This was further confirmed by insertion activation of *frxA* and/or *rdxA* genes. In addition, cloned *frxA* gene expressed in *E. coli* showed nitroreductase activity and rendered normally metronidazole-resistant *E. coli* sensitive. Strains carrying *frxA* null alleles enhanced MTZ resistance in *rdxA* deficient cells. Also, inactivation of genes that encode ferridoxin-like protein (fdxB) along with previously described *frxA* and *rdxA* genes increased the MIC of MTZ-S strains (16). This suggested that multiple possible factors might be involved in high-level resistance to MTZ. Jeong et al. suggested two types of MTZ-S strains by genetic (mutational) and molecular tests on the basis of the need for inactivation of *rdxA* alone or along with *frxA* gene to render *H. pylori* resistant (17). Subsequent work suggested that *rdxA* gene might play a major role in the high-level resistance to metronidazole (18).

2.3 *Trichomonas*

The first report of resistance appeared in *Trichomonas vaginalis* about two years after introduction of metronidazole (19). Recently, there has been an increase in the recognition of metronidazole-resistant trichomoniasis associated with increase in therapeutic failures (20). In trichomonads, activation of MTZ occurs within specialized organelles, the hydrogenosome, which contains pyruvate:ferrodoxin oxidoreductase (PFO) and ferrodoxin. PFO catalyzes the decarboxylation of pyruvate to acetyl CoA, transferring the electron to ferrodoxin. MTZ replaces protons as the acceptor of electrons donated by ferrodoxin. In the absence of the drug, these protons would normally be reduced to molecular hydrogen

by hydrogenase. Yarlett, Yarlett, and Lloyd provided evidence that the reductive activation of metronidazole is diminished in resistant strains relative to drug-sensitive strains (21, 22). Quon et al. examined the intracellular levels of ferrodoxin and its mRNA in four clinically resistant strains and demonstrated decreased levels of ferrodoxin and its mRNA. This was attributed to reduced transcription of the ferrodoxin gene as determined by nuclear run-on assays (23). Cerkasovova et al. noted that Tritrichomonas foetus strains that are highly resistant to MTZ lack detectable enzymatic activity for pyruvate:ferrodoxin oxidoredutase and hydrogenase (24). The molecular basis for these altered enzyme activities has not been established.

2.4 *Clostridium* spp.

Clostridium species are usually sensitive but *C. ramnosum* may require higher concentrations for inhibition (25, 26). There are reports of high-level resistance to metronidazole in *C. difficile* isolates from horses (27). There is one report of documented resistance (high minimum inhibitory concentration (MIC) with therapeutic failure) in a *C. difficile* isolate in a patient with *C. difficle*-associated diarrhea. (28)

Santengelo et al. developed *E. coli* F19recA, nitrate reductase-deficient mutant that was rendered MTZ-S by isolating and expressing *C. acetobutylicum* genes on recombinant plasmids. Further tests on these isolates revealed that flavodoxin and hydrogenase genes were responsible for electron transfer system, suggesting its possible role in metronidazole resistance (29). Church et al. provided biochemical evidence that hydrogenase 1 of *C. pasteuranicum* plays a critical enzymatic role in the reduction of metronidazole via a ferrodoxin-linked mechanism (30, 31).

2.5 *Entamoeba and Giardia*

Drug-resistant Giardia isolates have been grown from patients with therapeutic failure with metronidazole. There is no reported clinical resistance in Entamoeba, but resistant strains have been generated in vitro in various laboratories.

Purified PFOR and ferrodoxin have been shown to activate MTZ in vitro. Upcroft and Upcroft characterized biochemical markers in a clinically resistant isolate and showed that PFOR is downregulated up to fivefold. Ferrodoxin 1, which is the next electron acceptor in the transport chain, is also downregulated about seven times (32). Increased efflux of the drug also might be responsible in protecting the parasite.

Entamoeba produces superoxide dismutase (SOD), catalase, and peroxidase for detoxification of oxygen and its breakdown products. Only one 2-oxoacid oxidoreductase, PFOR, has been detected in Entamoeba and it is predominantly membrane bound. Upcroft and Upcroft showed marked increase in superoxide dismutase activity in MTZ-resistant *E. histolytica*, while PFOR activity remained constant (32). Wassmann et al. confirmed the lack of change in PFOR activity in resistant strains. They also showed increased expression of iron-containing FE-SOD and peroxiredoxin, while the expression of flavin reductase and ferrodoxin1 was decreased (33).

3 Cross-Resistance

There is documented cross-resistance between all the currently used 5-nitroimidazole drugs and their worldwide availability (32, 34).

4 Mechanism of Spread of Resistance

Although both plasmid-mediated and chromosomally mediated resistance has been described, the transfer to metronidazole-sensitive Bacteroides species does not yet appear to be a problem. Also, a combination of several mechanisms may be required for emergence of high-level resistance in various organisms that might lead to therapeutic failures.

5 Alternative Agents

5.1 *Helicobacter Pylori*

Virtually all *H. pylori* isolates are susceptible in vitro to a variety of antimicrobial agents, including bismuth salts, amoxicillin, macrolides, nitrofurans, tetracyclines, and aminoglycosides. Combination therapy with a bismuth salt and two antibiotics has been widely used. After treatment failure, a second course of triple therapy may still be effective; alternatively, a regimen not including imidazoles may be used.

5.2 *Trichomonas Vaginalis*

If infection persists in a patient treated with a 7-day regimen and re-infection can be ruled out, other options include treating with 2 g of metronidazole orally daily for 3–5 days; 1–2 g of metronidazole daily for 14 days along with 500 mg intravaginally

daily; high-dose intravenous metronidazole (35), intravaginal paromomycin (36, 37), and tinidazole, which has recently been approved by the FDA. Tinidazole has been shown to be effective in some cases of metronidazole-resistant *T. vaginalis* infection (38). Crowell et al. found that although in vitro activities of metronidazole and tinidazole against the parasite are highly correlated, the tinidazole does have lower MICs than metronidazole (34).

5.3 Giardia

Some success has been noticed with quinacrine and albendazole in combination with metronidazole in cases of giardiasis treatment failures (39).

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Chapter 20
Glycopeptide Resistance in Enterococci

Bruno Périchon and Patrice Courvalin

1 Enterococci

Enterococci are part of the normal intestinal flora of humans and various animals. They are found in the feces of a high proportion of healthy adults. Enterococci are able to grow in variable environmental conditions at temperatures from 10 to 45°C, in hypotonic, hypertonic, acidic, or alkaline media, under anaerobic or aerobic conditions. *Enterococcus faecalis* and *E. faecium* are the two major species of enterococci and represent more than 95% of clinical isolates (1–3). *E. faecalis* is more prevalent than *E. faecium* and accounts for 57–77% of clinical isolates (4). Enterococci are opportunistic pathogens and can be responsible for endocarditis and urinary tract infections, as well as intra-abdominal and pelvic sepsis and surgical wound infections (5). They present intrinsic resistance to several classes of antibiotics such as β-lactams (due to the low affinity of the vital penicillin-binding proteins (PBP) for penicillins) (6, 7), and to low concentrations of aminoglycosides, clindamycin, and trimethoprim–sulfamethoxazole (3). Furthermore, they easily become resistant to other antibiotics by mutation or acquisition of foreign genetic material carried by conjugative transposons, pheromone-response plasmids, and broad-host-range plasmids. Resistance to higher levels of penicillins, by overproduction or alteration of PBP5 (8, 9) or by synthesis of a β-lactamase (10), and to aminoglycosides, chloramphenicol, macrolides-lincosamides-steptogramins, tetracycline, fluoroquinolones, rifampin, as well as to the glycopeptides, has been described (11). Therefore, treatment of enterococcal infections is often difficult.

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2 Glycopeptides

Glycopeptide antibiotics, such as vancomycin and teicoplanin, are active against many important Gram-positive pathogens. The structure of glycopeptides is based on a heptapeptide domain in which five amino acid residues are common to all glycopeptides (12, 13). The biologically active part of the molecule is located in the structure containing seven amino acid residues (13). Vancomycin, produced by *Amycolatopsis orientalis*, was the first glycopeptide used in the treatment of serious infections due to Gram-positive bacteria. Glycopeptides act by inhibiting cell-wall formation (Fig. 1). They bind to the D-alanyl-D-alanine (D-Ala-D-Ala) *C*-terminal dipeptide, synthesized by the D-Ala:D-Ala ligase (Ddl), of pentapeptide peptidoglycan precursors and inhibit transglycosylation and transpeptidation reactions that occur outside the cytoplasmic membrane (15). Thus, incorporation of the precursors into the bacterial cell wall is prevented (13). Binding of vancomycin to its target is stabilized by five hydrogen bonds between the pentapeptide and the antibiotic (12). Gram-negative bacteria are insensitive to this group of antibiotics because of the outer membrane which is impermeable to glycopeptides.

3 Glycopeptide Resistance in Enterococci

The first enterococcal isolates resistant to high levels of vancomycin and teicoplanin were reported in 1988 (16, 17). Currently, the proportion of clinical glycopeptide-resistant enterococci (GRE) from intensive care units in the US has reached more than 26% of the isolates tested (18). In Europe, where most GRE are *E. faecium*, the prevalence in hospitals is much lower (2.2%) (1).

Glycopeptide resistance is due to the replacement of the normal peptidoglycan precursors by modified precursors ending in D-Ala-D-lactate (D-Ala-D-Lac) or D-Ala-D-serine (D-Ala-D-Ser) in place of D-Ala-D-Ala. This alteration is

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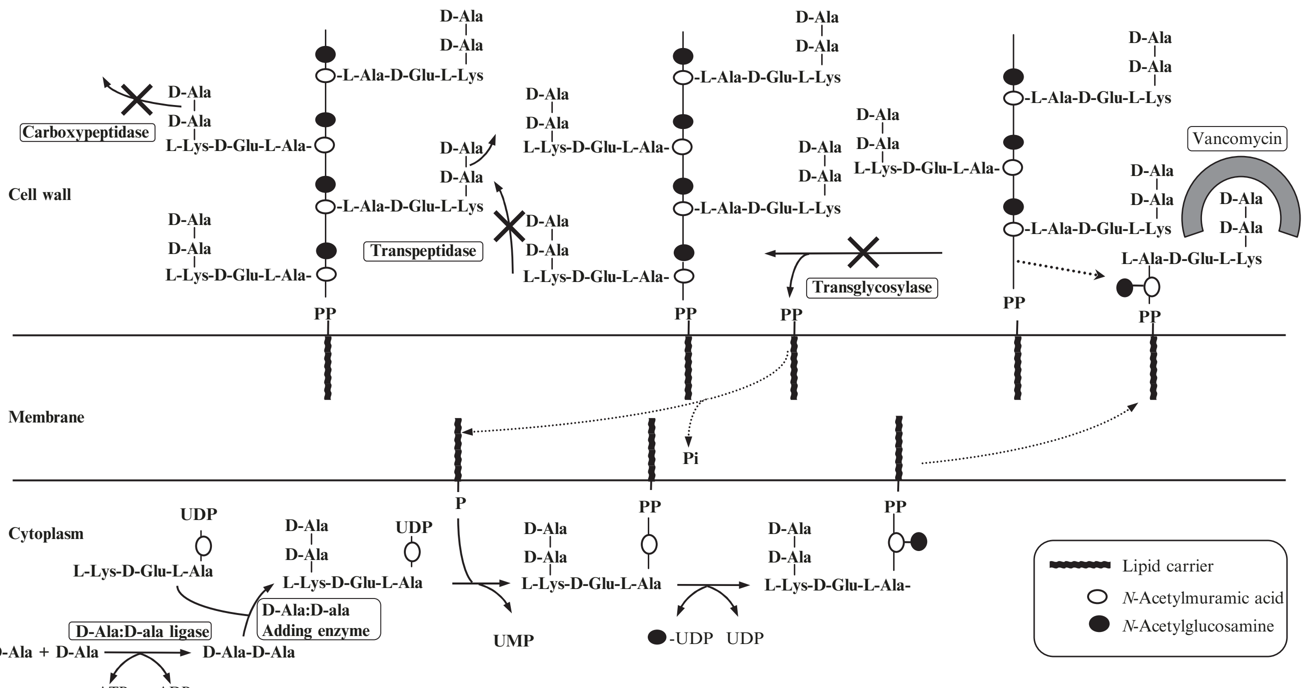


Fig. 1 Schematic representation of the mode of action of vancomycin on peptidoglycan biosynthesis (from 14). Binding of vancomycin on the *C*-terminal D-Ala-D-Ala prevents the transglycosylation, transpeptidation, and carboxypeptidation steps

responsible for diminished binding affinity of glycopeptides for their target. In the case of precursors ending in D-Ala-D-Lac, the affinity is 1,000-fold lower because the substitution eliminates a critical hydrogen bond (Fig. 2) (19). The replacement of D-Ala by D-Ser should not affect the number of hydrogen bonds that can be formed between vancomycin and the altered precursor, but the binding affinity is altered (sevenfold lower) (20), probably because of conformational changes (Fig. 2). In addition to production of modified peptidoglycan precursors, resistant strains are also able to eliminate the precursors normally synthesized by the host. Combination of these two pathways, synthesis of modified precursors and elimination of classical precursors, leads to resistance. Therefore, resistance to glycopeptides is a complex system involving several genes.

4 The *van* Alphabet

Six types of glycopeptide resistance have been described to date in enterococci: five are acquired (VanA, B, D, E, and G) (14, 21–23) and one, VanC, is an intrinsic property of *Enterococcus gallinarum, E. casseliflavus*, and *E. flavescens* (24, 25). The minimum inhibitory concentration (MIC) ranges of vancomycin and teicoplanin against the various types overlap (Table 1): classification of glycopeptide resistance is now based on the primary sequence of the structural gene for the resistance ligase rather than on the levels of resistance to glycopeptides.

4.1 Glycopeptide Resistance Due to Synthesis of Modified Peptidoglycan Precursors Ending in D-Ala-D-Lac

4.1.1 VanA

VanA was the first type of glycopeptide resistance described (16, 17) and has, since then, been the most extensively studied. VanA-type strains are characterized by a high level of resistance to both vancomycin and teicoplanin due to synthesis of modified peptidoglycan precursors ending in D-Ala-D-Lac (Table 1). The *vanA* gene cluster, generally located on transposon Tn*1546* (26) or related elements (27), can be found on both transferable and nontransferable plasmids as well as on the bacterial chromosome (Table 1). It has been reported mainly in *E. faecium* and *E. faecalis* but also in *E. avium* (28), *E. durans* (29–31), *E. gallinarum*, and *E. casseliflavus* (32), as well as in *Bacillus circulans* (33). Recently, the *vanA* gene cluster was found in nine isolates of *Staphylococcus aureus* (34–37).

Tn*1546* is composed of nine genes: two encode a transposase and a resolvase, responsible for the movements of the element, and the remaining seven genes are involved in expression and regulation of glycopeptide resistance (Fig. 3a). The *vanH*, *vanA*, and *vanX* genes code for proteins that are necessary for expression of resistance (Fig. 3b). VanH is a dehydrogenase that converts pyruvate to D-Lac (38); VanA is a ligase that uses D-Lac and a D-Ala residue to

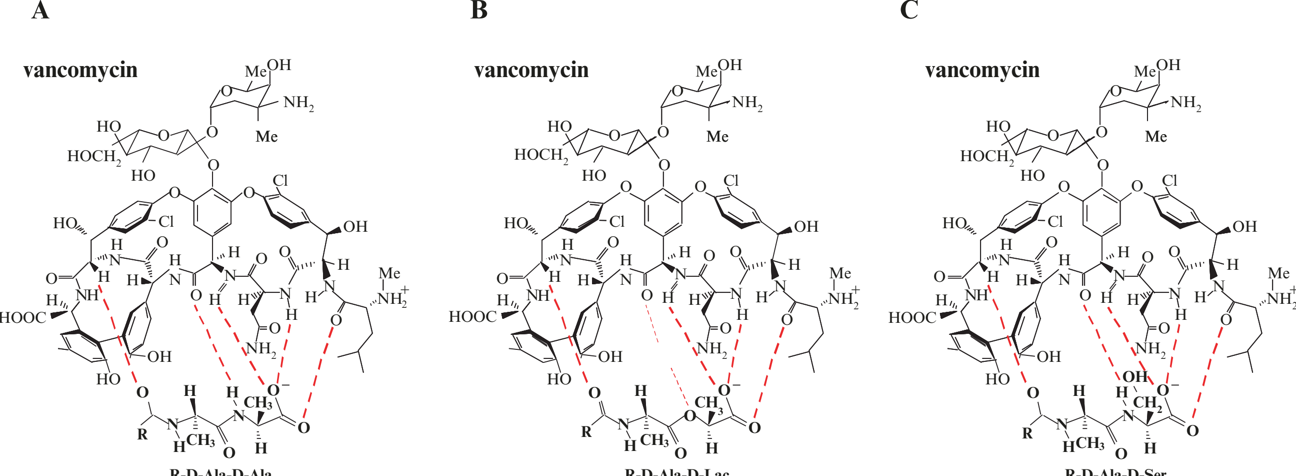


Fig. 2 Interactions between vancomycin and (**a**) *N*-acetyl-D-Ala-D-Ala, (**b**) *N*-acetyl D-Ala-D-Lac, (**c**) *N*-acetyl-D-Ala-D-Ser. Hydrogen bonds are indicated by *dotted lines*. With the D-Ala-D-Lac depsipeptide, a central hydrogen bond is missing because of substitution of a NH group by an oxygen and repulsion between the two oxygens and 3-D alteration of the target; with the D-Ala-D-Ser pentapeptide, replacement of a CH_3 group by a CH_2OH group is responsible for conformational changes

Table 1 Glycopeptide resistance in enterococci

Resistance	Acquired					Intrinsic
Type	VanA	VanB	VanD	VanE	VanG	VanC
MIC (mg/L)						
Vancomycin	64–1,000	4–1,000	64–128	8–32	8–16	2–32
Teicoplanin	16–512	0.5–1	4–64	0.5	0.5	0.5–1
Expression	Inducible		Constitutive	Inducible	Inducible	Constitutive Inducible
Location	3Plasmid / Chromosome [FX]		Chromosome	Chromosome	Chromosome [FX]	Chromosome
Modified target		D-Ala-D-Lac			D-Ala-D-Ser	

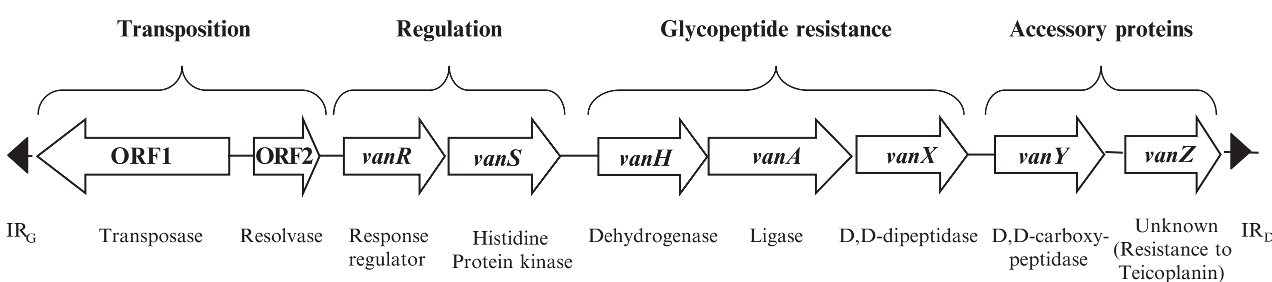
synthesize the depsipeptide D-Ala-D-Lac, which is incorporated into the peptidoglycan precursors in place of D-Ala-D-Ala (19); and VanX is a D,D-dipeptidase that hydrolyses the dipeptide D-Ala-D-Ala formed by the endogenous chromosomal D-Ala:D-Ala ligase (Ddl) (39, 40) thereby reducing the level of normal peptidoglycan precursors ending in D-Ala-D-Ala. The penicillin-insensitive D,D-carboxypeptidase, VanY, not essential for resistance, cleaves the D-Ala *C*-terminal residue of the pentapeptide precursors synthesized from the D-Ala-D-Ala dipeptide that has escaped VanX hydrolysis (14). Vancomycin has no affinity for the resulting tetrapeptide precursors. The *vanZ* gene confers low-level resistance to teicoplanin by an unknown mechanism (41).

Expression of glycopeptide resistance is regulated by two genes, *vanR* and *vanS*, located upstream from *vanH*. The genes code for a two-component regulatory system (42–45). VanS is a membrane-associated sensor that contains, in the *C*-terminal cytoplasmic domain, a histidine residue which is phosphorylated in response to the presence of glycopeptides in the medium. VanR acts as a transcriptional activator that can be phosphorylated on an aspartate residue by acquisition of the phosphoryl group of the activated VanS. In summary, in the presence of glycopeptides in the culture medium, a signal leads to autophosphorylation of VanS on a specific histidine residue and the phosphoryl group is then transferred to a specific aspartate residue of VanR (Fig 4). The resistance and regulatory genes are transcribed from two distinct promoters, P_H and P_R, respectively, that are coordinately regulated (46). Phospho-VanR binds to P_H and P_R and activates transcription of the two sets of genes (47).

The *vanA* gene cluster can harbor insertion sequences, such as IS*1216V* or IS*3*-like, in the transposition genes or contain alterations downstream from *vanX* and point mutations in *orf1*, *vanS*, *vanA*, *vanX*, and *vanY* (48–50).

4.1.2 VanB

The VanB type is characterized by a variable level of resistance to vancomycin only (Table 1). The *vanB* operon also confers resistance by production of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac and its organization is similar to that of *vanA* (Fig. 5a). The *vanB* cluster is composed of resistance genes ($vanH_B$–$vanB$–$vanX_B$), with deduced



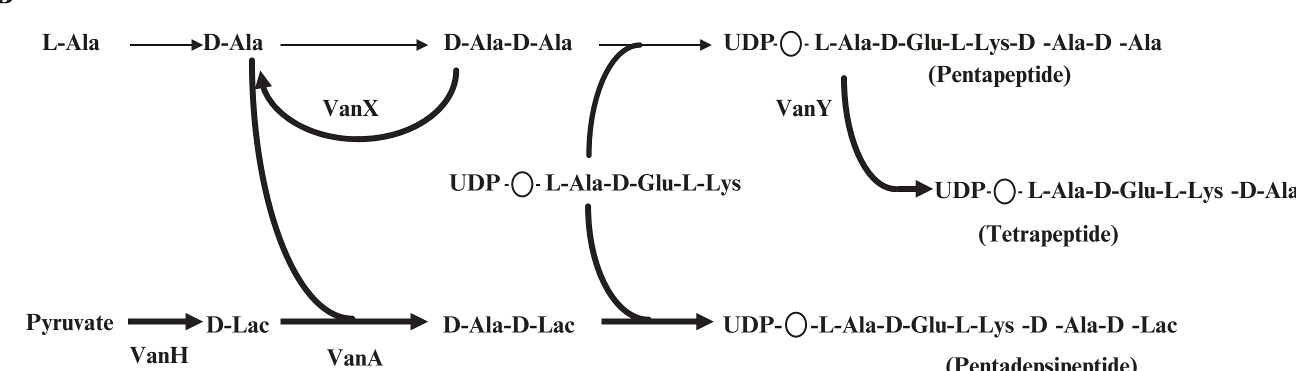


Fig. 3 (**a**) Organization of Tn*1546*. IR_G and IR_D indicate inverted repeat sequences at the ends of the transposon. (**b**) Schematic representation of the synthesis of peptidoglycan precursors in a VanA-type resistant strain after induction with glycopeptides. Ddl, D-Ala:D-Ala ligase, O*N*-acetylmuramic acid

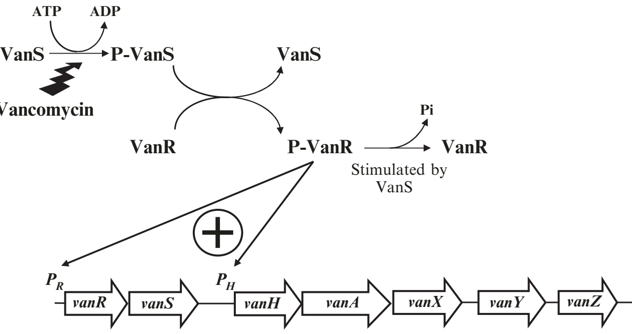


Fig. 4 Schematic representation of activation of the P_R and P_H promoters of the *vanA* operon by phospho-VanR after induction with vancomycin

amino acid sequences highly similar to those of VanH–VanA–VanX, of the accessory $vanY_B$ gene, and of regulatory genes ($vanR_B$–$vanS_B$) (51). The function of *vanW* is unknown.

The $VanR_B$–$VanS_B$ system displays only limited sequence identity with VanR–VanS (34% and 23%, respectively). The phenotypic difference between VanA- and VanB- type resistance is due to the fact that teicoplanin is not an inducer for expression of glycopeptide resistance in VanB-type strains. It has been demonstrated that, in common with VanS, purified $VanS_B$ also acts as both a histidine protein kinase and a phospho-$VanR_B$ phosphatase (52).

The *vanB* gene cluster is carried by large conjugative elements that are transferable from chromosome to chromosome (53). These elements contain transposons such as Tn*1547* found in a 250-kb genetic element (54) or Tn*5382* (55, 56). The *vanB* element can also be located on plasmids (Table 1). Tn*1549*, located on a plasmid related to pAD1 (57), contains 30 open reading frames (ORFs) organized into three functional regions as observed in the *Tn916* family of conjugative transposons (58). These regions are implicated in (a) the excision–integration process, (b) vancomycin resistance, and (c) conjugative transfer. Interestingly, analysis of the base composition indicated that the origin of the left end of the transposon is different from that of the two other functional regions.

Three subtypes, *vanB1*, *vanB2*, and *vanB3* (58–60), of the *vanB* operon can be distinguished on the basis of specific nucleotide sequences in the $vanS_B$–$vanY_B$ intergenic region. There is no correlation between *vanB* subtype and the level of vancomycin resistance.

4.1.3 VanD

VanD-type strains present moderate levels of resistance to vancomycin and teicoplanin. The organization of the *vanD*

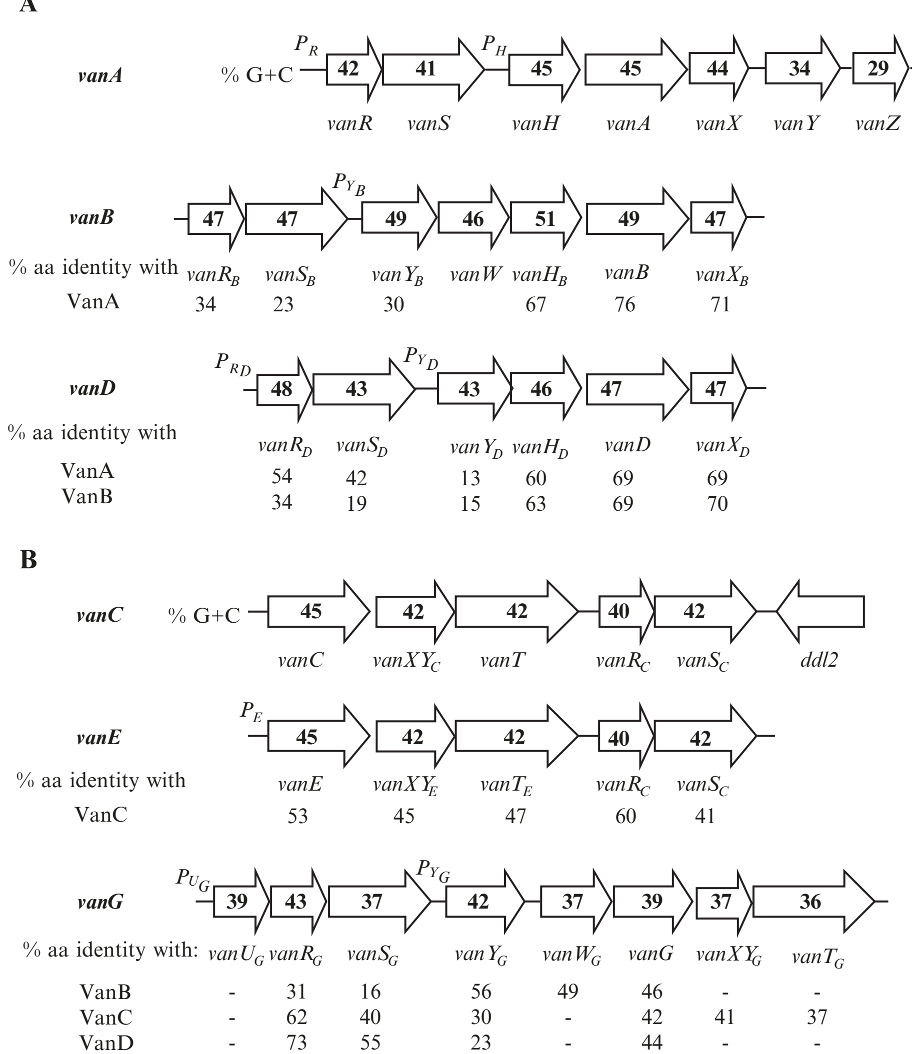


Fig. 5 Comparison of the prototype glycopeptide-resistance gene clusters. Genes implicated in resistance due to synthesis of modified peptidoglycan precursors ending in (**a**) D-Ala-D-Lac (VanA, VanB, and VanD type) or (**b**) D-Ala-D-Ser (VanC, VanE, and VanG type). *Open arrows* represent coding sequences and direction of transcription

operon, which is chromosomally located, is similar to those of *vanA* and *vanB* (Fig. 5a) (21, 61, 62). As in VanA- and VanB-type strains, VanD resistance is due to the synthesis of peptidoglycan precursors that end quasiexclusively in D-Ala-D-Lac (21, 62). Although the biochemical mechanism of resistance is similar to those of VanA and VanB, VanD-type resistance displays some peculiarities. No genes homologous to *vanZ* from the *vanA* operon or *vanW* from the *vanB* operon are present in the *vanD* cluster. As opposed to VanA and VanB, VanD-type resistance is constitutively expressed and is not transferable by conjugation to other enterococci (21, 62, 63). In certain strains, $VanY_D$ D,D-carboxypeptidase activity is inhibited by penicillin G (21, 62–64), whereas VanY and $VanY_B$ activities are insensitive to penicillin G. All the VanD-type strains described to date possess (a) an inactive host Ddl ligase, due to the presence of various mutations in the *ddl* gene, and (b) a mutated $vanS_D$ or $vanR_D$ gene that is responsible for the synthesis of an inactive $VanS_D$ or $VanR_D$ protein and therefore for constitutive expression of glycopeptide resistance (Fig. 6) (61–63, 65). The $VanX_D$ D,D-dipeptidase activity is low in VanD-type strains despite the presence of a putatively functional protein (62). Another intriguing feature is that, in spite of synthesis of peptidoglycan precursors ending essentially in D-Ala-D-Lac, the level of resistance to teicoplanin remains low (Table 1).

4.2 Glycopeptide Resistance Due to Synthesis of Modified Peptidoglycan Precursors Ending in D-Ala-D-Ser

4.2.1 VanC

E. gallinarum and *E. casseliflavus-E. flavescens* are intrinsically resistant to low levels of vancomycin but remain susceptible to teicoplanin (Table 1) (24, 25). Production of peptidoglycan precursors ending in D-Ala-D-Ser is responsible

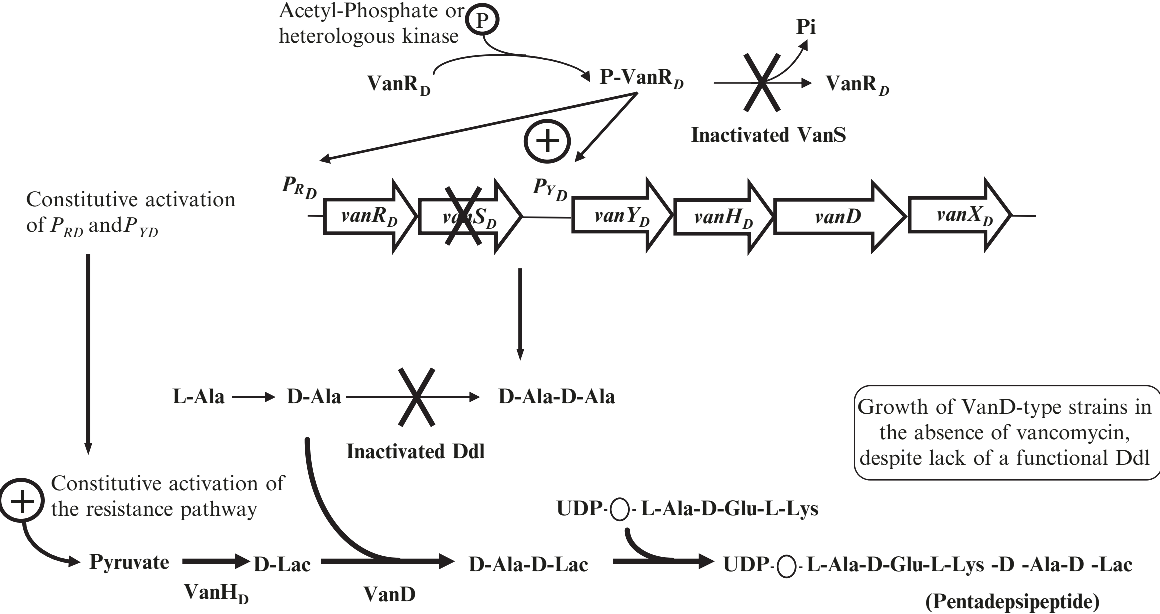


Fig. 6 Schematic representation of constitutive activation of P_{RD} and P_{YD} promoters of the *vanD* operon. Van D-type strains have an impaired D-Ala:D-Ala ligase and a mutation in the *vanS*$_D$ gene which allows growth in the absence of vancomycin

for this type of resistance (20, 66). In *E. gallinarum*, expression of resistance can be constitutive or inducible by vancomycin (67, 68). Three subtypes of the *vanC* genes are known: *vanC-1* for *E. gallinarum*, *vanC-2* for *E. casseliflavus*, and *vanC-3* for *E. flavescens* (24, 69). The organization of the *vanC* operon, which is chromosomally located and not transferable, differs from those of *vanA* and *vanB* (Fig. 5b). Three gene products, VanC, VanXY$_C$, and VanT$_C$, are required for resistance (70–72). VanC is a ligase that synthesizes the dipeptide D-Ala-D-Ser which replaces D-Ala-D-Ala in late peptidoglycan precursors (20). As already mentioned, in VanA- and VanB-type strains, hydrolysis of precursors ending in D-Ala is achieved by two enzymes, a D,D-dipeptidase and a D,D-carboxypeptidase, encoded by two separate genes (*vanX*/*vanX*$_B$ and *vanY*/*vanY*$_B$, respectively). By contrast, in VanC-type enterococci the two activities are encoded by a single gene, *vanXY*$_C$ (70). Amino acid sequence comparison indicated that VanXY$_C$ is more closely related to VanY than to VanX (70). VanT is a membrane-bound serine racemase in which the cytoplasmic domain is able to convert L-Ser to D-Ser (71, 73). This enzyme also possesses alanine racemase activity. It has been demonstrated that the transmembrane domain of VanT plays a crucial role in VanC-type resistance and that the protein is probably also involved in the uptake of L-Ser from the external medium (73). Expression of the *vanC*, *vanXY*$_C$, and *vanT* genes is regulated by two genes located downstream from *vanT* that encode a two-component regulatory system, VanR$_C$/VanS$_C$ (71). An additional gene, *ddl2*, located downstream from these two regulatory genes and encoding a protein that has structural similarity to D-Ala:D-Ala ligases, was found in the VanC prototype strain, BM4174 (74). Thus, vancomycin-resistant *E. gallinarum* possess at least three ligase genes: two for D-Ala:D-Ala ligases and one for a D-Ala:D-Ser ligase. The *vanC-2* gene cluster of *E. casseliflavus* has been characterized (75). The deduced proteins display high degrees of amino acid identity (71–91%) to those encoded by the *vanC* operon. The *vanC-3* gene cluster displays extensive identity with *vanC-2*, 97–100%, including in the intergenic regions (76). It is therefore difficult to class *E. casseliflavus* and *E. flavescens* as distinct species (76).

4.2.2 VanE

The first VanE-type strain was described in 1999 (22). This clinical isolate exhibits a low level of resistance to vancomycin only not transferable by conjugation (Table 1), and synthesizes peptidoglycan precursors terminating in D-Ala-D-Ser after induction by vancomycin (22). The organization of the *vanE* operon is identical to that of *vanC* (Fig. 5b) (77, 78). As in VanC-type resistance, three genes are required: *vanE*, *vanXY*$_E$, and *vanT*$_E$, encoding, respectively, a ligase responsible for synthesis of the dipeptide D-Ala-D-Ser, a D,D-peptidase, and a serine racemase (77). Two genes, *vanR*$_E$/*vanS*$_E$, coding for a two-component regulatory system, are located downstream from *vanT*$_E$ (76). As demonstrated by Northern experiments and reverse transcription polymerase chain reaction (RT-PCR), the five genes are cotranscribed from a P_E promoter located upstream from *vanE*. Although

the VanS sensor is likely to be inactive owing to the presence of a stop codon in the 5′ portion of the gene, expression of vancomycin resistance is inducible in VanE prototype strain BM4405 (77). Inducibility is probably due to cross-talk with another two-component regulatory system of the host. During 2001–2002, four new VanE-type strains were isolated in Australia (79, 80).

4.2.3 VanG

A new type of glycopeptide resistance, VanG, has been detected (23). Acquired VanG-type resistance is characterized by a low level of resistance to vancomycin (MIC = 16 µg/mL) due to inducible production of modified precursors ending in D-Ala-D-Ser (81). Study of the *vanG* cluster, which is composed of eight genes assigned to a chromosomal location, revealed that its organization differed from that of the other *van* operons (Fig. 5b) (23, 81). The mutated $vanY_G$ gene encodes a truncated D,D-carboxypeptidase most likely inactive; $vanW_G$ encodes a protein of unknown function; the three resistance genes, *vanG*, $vanXY_G$, and $vanT_G$, code for a D-Ala:D-Ser ligase, a bifunctional D,D-peptidase, and a serine racemase, respectively. In contrast to the other *van* operons, there are three regulatory genes, $VanR_G$, $vanS_G$, and also $vanU_G$ which encodes a predicted transcriptional activator. Interestingly, the three genes are constitutively co-transcribed from a P_{UG} promoter, whereas $vanY_G$, $vanW_G$, *vanG*, $vanXY_G$, and $vanT_G$ are co-transcribed in an inducible manner from the P_{YG} promoter (81). This is the first *van* operon to be regulated in that way. VanG resistance is transferable to susceptible *E. faecalis* at a low frequency and the transfer is associated with the movement, from chromosome to chromosome, of large genetic elements of ca. 240 kb (81).

5 Vancomycin-dependent Enterococci

Mutations in the host D-Ala:D-Ala ligase of enterococci are lethal unless an alternative pathway for cell-wall synthesis is present (Fig. 7) (28, 82). Strains of enterococci that require the presence of vancomycin in the culture medium for growth have been isolated in vitro (28, 82–84), in animal models (85), and from patients treated for prolonged periods with vancomycin (86–90). Strains containing a *vanA* or a *vanB* operon are able to survive by producing peptidoglycan precursors ending in D-Ala-D-Lac if a glycopeptide is present in the culture medium to induce expression of the *van* operon. Because of the fact that growth of these strains requires particular conditions, prevalence of vancomycin-dependent enterococci is probably underestimated in routine laboratory analyses. Therefore, they could constitute a reservoir of vancomycin resistance genes that can be transferred to other bacteria. Furthermore, it has been demonstrated, at least in vitro, that these strains can revert to a nondependent, more resistant phenotype (82), suggesting that disruption of vancomycin therapy may not be sufficient to cure patients infected with vancomycin-dependent enterococci.

6 Origin of the Vancomycin Resistance Genes

6.1 *Acquired D-Ala:D-Lac Ligases*

Leuconostoc mesenteroides, *Pediococcus pentosaceus*, and *Lactobacillus casei*, which are intrinsically highly resistant to glycopeptides by production of peptidoglycan precursors ending in D-Lac (67, 91), have been suspected to be the source of resistance ligases producing D-Ala-D-Lac. However,

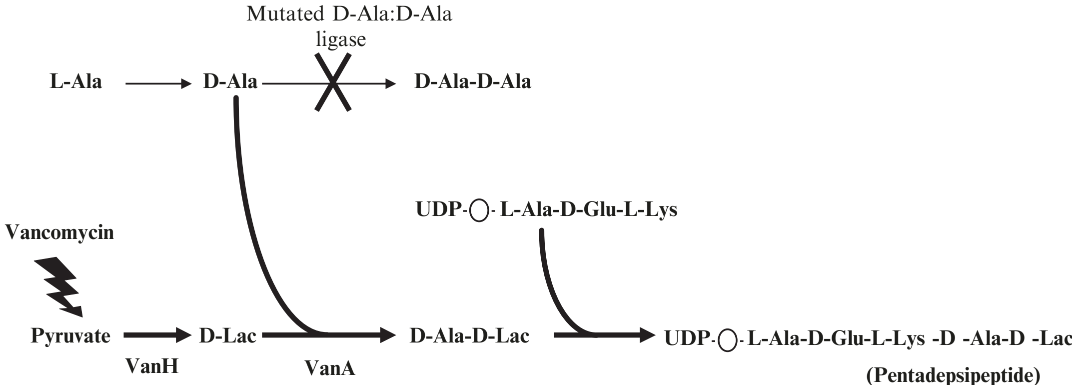


Fig. 7 Schematic representation of the synthesis of peptidoglycan precursors in a vancomycin-dependent strain. Presence of vancomycin in the culture medium is necessary to induce the synthesis of modified peptidoglycan precursors and to allow growth of the bacteria, O*N*-acetylmuramic acid

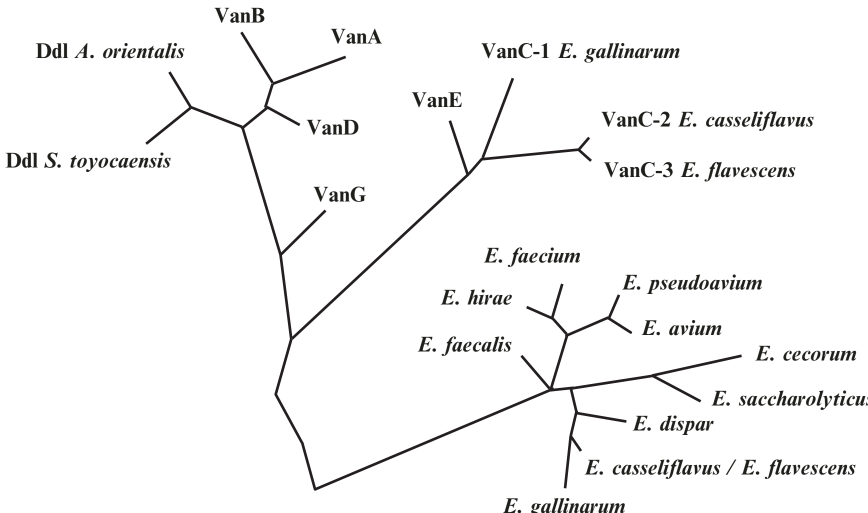


Fig. 8 Phylogenetic tree derived from the alignment of D-Ala:D-Ala, D-Ala:D-Lac, and D-Ala:D-Ser ligases

a phylogenetic tree based on the alignment of the deduced sequences of D-Ala:D-Ala ligases and related enzymes revealed that VanA, VanB, and VanD exhibit only limited identity with D-Ala:D-Lac ligases of these naturally resistant species (Fig. 8).

The glycopeptide-producing organisms, which harbor resistance genes to protect themselves against suicide, could represent a potential source of resistance for human pathogens. Genes coding for homologs of VanH, VanA, and VanX have been found and with the same genetic organization in two glycopeptide-synthesizing organisms, *Amycolatopsis orientalis* C329.2, and *Streptomyces toyocaensis* NRRL15009, which produce vancomycin and the A47934 glycopeptide, respectively (92–95). Furthermore, *vanHAX* homologs have also been detected in producers of chloro-eremomycin, ristocetin, vancomycin, and teicoplanin-avoparcin (95). However, the base composition (G + C content) of the genes composing the *vanA*, *vanB*, and *vanD* clusters is significantly lower than that of the *vanHAX* homologs in the producers, suggesting that acquisition of the genes is probably not a recent event. A vancomycin resistance gene cluster, *vanF*, has been detected in the biopesticide *Paenibacillus popilliae*. This operon is composed of five genes encoding homologs of VanY, VanZ, VanH, VanA, and VanX (96, 97). Orientation and alignment of the genes essential for resistance (*vanH*/*vanH*$_F$, *vanA*/*vanF*, and *vanX*/*vanX*$_F$) are identical in VanF and VanA. The base composition of the three resistance genes of *P. popilliae* is similar to that of the corresponding genes of *vanA* and *vanB*. *P. popilliae* could therefore represent an intermediate in the transfer from the producers to the clinical isolates. Such a transfer could have occurred through a long chain of related organisms so that the first and the last member of this chain are only distantly related. Glycopeptide-resistant *vanA* operons were found in *Paenibacillus* isolated from soil (98). Their level of identity with the enterococcal operons is markedly higher than that of *vanF*. The close similarity of these operons with that of *Enterococcus* suggests that the gene clusters have evolved from a common ancestor or that the *vanA* operons from soil organisms were acquired by enterococci.

The base composition differs also between the essential and the nonessential genes within the *van* operons, suggesting that the genes could originate from different species. The *van* gene clusters may thus have been composed by collecting genes from various sources.

Presence of the *vanB* operon on a Tn*1549*-like element in various anaerobes from the digestive tract was recently demonstrated (99). Furthermore, transfer of the element from *Clostridium symbiosum* to *Enterococcus* spp. was obtained in vitro and in the digestive tract of gnotobiotic mice (100). Anaerobic bacteria, which are also common in soil, could thus be an intermediate in the transfer of VanB-type vancomycin resistance from glycopeptide producers to enterococci.

6.2 Acquired D-Ala:D-Ser Ligases

No glycopeptide producers were found to synthesize peptidoglycan precursors ending in D-Ala-D-Ser, suggesting that the origin of the VanC-, E-, and G-type of resistance is different from that of VanA, B, and D.

The *vanC* and *vanE* gene clusters present a high degree of identity (41–60%) (77). Thus, acquired resistance of the VanE type could be due to acquisition of a chromosomal operon from another species of *Enterococcus* (*E. gallinarum*, *E. casseliflavus*/*flavescens*).

The *vanG* operon appears to be more heterogeneous. VanR$_G$ exhibits the highest identity (73%) with VanR$_D$;

$VanY_G$ exhibits the highest identity with $VanY_B$ (56%); and $vanW_G$ has 49% identity with *vanW* which is present only in the *vanB* operon. The 3´ part of the *vanG* cluster (*vanG*, $vanXY_G$, $vanT_G$) is more closely related to *vanC* and *vanE* than to the corresponding proteins of the other operons (Fig. 5) (81), apart from the VanG D-Ala:D-Ser resistance ligase which is phylogenetically closer to the D-Ala:D-Lac ligases. Thus, the *vanG* operon is composed of genes recruited from various *van* operons.

7 Transfer of VanA-Type Resistance to *S. aureus*

Nine methicillin-resistant *S. aureus* strains exhibiting high or moderate levels of resistance to vancomycin and teicoplanin were isolated in Michigan, Pennsylvania, and New York following acquisition of the *vanA* gene cluster (34–36, 101, 102). In each strain, the VanA-encoding genetic element Tn*1546* was found to be part of a plasmid (102, 103). In one case, the molecular basis for vancomycin resistance acquisition was shown to be due to in vivo intergeneric transfer of a multiresistance conjugative plasmid from an *E. faecalis* strain isolated from the same patient (101). Thus, transfer of glycopeptide resistance from enterococci to *S. aureus*, as already demonstrated in vitro (104), can also occur in vivo. Furthermore, efficient heterologous expression of the glycopeptide resistance genes in the *S. aureus* transconjugants, similar to that observed in the enterococcal donor strain, was demonstrated (37, 105).

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Chapter 21
Streptogramin

Kimberly D. Leuthner and Michael J. Rybak

1 Class

Streptogramins are a naturally occurring class of antibiotics originally isolated from *Streptomyces pristinaspiralis* (6). This family includes many related antibiotics including pristanamycins, ostreomycins, mikamycins, and virginiamycins (25). In Europe, pristinamycin was commercially available as an oral antistaphylococcal agent; however, owing to poor water solubility, an injectable product was not available until 1999. Therefore, clinical experience with pristinamycin has been limited to non-life-threatening infections.

Pristinamycin is actually composed of two primary components, pristinamycin IA and pristinamycin IIA. Pristinamycin IA is a peptidic macrolactone which belongs to the group B streptogramin family. Its molecular weight is approximately 800 Da, and is bacteriostatic when utilized alone. Pristinamycin IIA is a polyunsaturated macrolactone which is a member of the group A streptogramin family. It has a molecular weight of approximately 500 Da, and is also bacteriostatic alone. When utilized in the optimal synergistic ratio, 1:9 and 9:1 for IA and IIA respectively, the combination is bactericidal (31).

Chemical modifications including additions of amino-containing functional groups to the pristinamycin components resulted in compounds which are acid-salt soluble in water. Several of these semisynthetic compounds were evaluated, and on the basis of biologic, toxicologic, and chemical criteria, the first parenteral streptogramin was developed for clinical use. Synercid (RP 59500, King Pharmaceuticals, Inc.) consists of quinupristin (pristinamycin IA) and dalfopristin (pristinamycin IIA) in a 30:70 ratio (w/w) (8). Presently oral formulations of streptogramin compounds are being investigated, including a drug named XRP-2826. Like Synercid, XRP-2826 is a combination compound of two pristinamycin derivatives combined in a 30:70 ratio (w/w) (15, 32).

2 Mechanism of Action

Streptogramin compounds work together to sequentially interrupt bacterial protein synthesis (7). Both group A and group B compounds bind to the 50S ribosomal subunit, but at two separate and distinct sites. Binding of group A streptogramins, including dalfopristin, to the bacterial ribosome interferes with the substrate attachment to the donor and acceptor regions of the peptidyltransferase (46). As a direct result, the ribosome undergoes a conformational change increasing the binding affinity of group B compounds such as quinupristin. Once both streptogramins have attached, a complex is formed that constricts the exit channel of the protein. This narrowing prevents the extrusion of the elongating newly formed proteins, resulting in inhibition of ribosomal function and ultimately cell death (7).

3 Mechanisms of Streptogramin Resistance

Resistance mechanisms to streptogramin compounds are both intrinsic and acquired in nature. Gram-negative organisms, such as *Pseudomonas aeruginosa* and Enterobacteriaceae, are intrinsically resistant to these antibiotics primarily because of interference with entry of the streptogramin molecules into the cell by the outer membrane. Since streptogramins are relatively large hydrophobic molecules, their ability to cross this outer barrier can be impeded in these organisms. Additionally, many of these Gram-negative bacilli have multidrug efflux pumps that can actively expel these compounds and other similarly sized drugs like macrolides (1). Confirming evidence of these efflux pumps have been described in *E. coli* cellular systems devoid of cell walls, which allow the binding of streptogramin and macrolide components (29, 30).

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Other resistance mechanisms have been identified and characterized as well (29–31). Enzymatic modification was first described in *Staphylococcus aureus* organisms in 1975 (28). These isolates were further analyzed and found to contain plasmid-mediated *saa* genes which encode for streptogramin A acetyltransferase, and *sbh* genes for streptogramin B hydrolase, effectively rendering the combination drug ineffective. Genetic analysis of other plasmids demonstrated more genes, such as *sat*, *vatD,* or *vatE* (in enterococci) or *vat* or *vatS* (in staphylococci), that also encode for acetyltransferase enzymes which target the group A streptogramins (3–5, 21). Chromosomally encoded resistance determinants which result in resistance to group A streptogramins and lincosamides have been described in *S. aureus* (termed *lsa*), but the specific resistance mechanism has not been fully described (17).

Many additional genes have been identified in staphylococci which are responsible for resistance to the streptogramin B component, such as quinupristin, including *vgb*, *msr*, and *erm* (21). Plasmid-mediated *vgbA* and *vgbB* encode for a lyase enzyme which is responsible for streptogramin B inactivation. *msr* (macrolide streptogramin resistance) genes, as previously described in *S. epidermidis* isolates (30), encode for an active membrane-bound transport mechanism responsible for removing the streptogramin B compound from the cell. Presently identified, there are two subtypes, *msrA* and *msrB*, which are not usually expressed unless induced by erythromycin (39). Streptogramin A compounds have a plasmid-mediated gene (*vga*) which encodes for an efflux protein specific for group A and related compounds (5). Presently, since organisms that display this type of resistance are rare, the clinical significance of this mechanism is unknown.

The *erm* (erythromycin resistance methylase) genes identified include *ermA*, *ermB*, and *ermC*. These encode for an enzyme responsible for N6-dimethylation of the adenine residue on the 23S rRNA. This addition results in an alteration in the binding site for streptogramin B compounds, along with macrolides and lincosamides (31). Named the MLS_B phenotype, these genes can be either constitutive or inducible in Gram-positive bacteria. In organisms expressing inducible resistance, streptogramin B compounds can retain their activity, whereas organisms constitutively expressing MLS_B are usually resistant to these compounds (31). Since the activity of streptogramin A compounds is not affected by the MLS_B resistance mechanism, synergistic activity may still be present in these organisms when the group A and group B compounds are utilized together. For example, Leclercq et al. (31) were able to demonstrate combined effectiveness of quinupristin/dalfopristin with modal minimum inhibitory concentrations (MICs) of 0.5 μg/mL against constitutively expressed MLS_B-resistant strains of *S. aureus*. Individually, the MICs for quinupristin exceeded 128 μg/mL, with dalfopristin MICs of 4 μg/mL. Since commercially available streptogramin antibiotics are a combination of both group A and group B components, the clinical relevance of MLS_B resistance remains controversial.

Since the commercially available product is a combination of both group A and group B streptogramins, antibiotic activity is usually conserved when inactivating enzymes are present against either of the individual components (33). The clinical importance of inactivating enzymes is presently low. Only 5% of isolates reported from a French hospital demonstrated enzymes capable of modifying streptogramin antibiotics, with 1% or less of these isolates being reported as resistant to pristinamycin (16, 33, 48).

Enterococcus spp. constitute a unique family of organisms. *E. faecalis* and *E. faecium* are both enterococci; however, quinupristin/dalfopristin never demonstrated clinical activity against the *E. faecalis* organisms, despite efficacy against *E. faecium*, thus limiting the clinical use of these streptogramins. Investigations undertaken by Singh et al. (42) attempted to evaluate the difference between the organisms that could explain the difference in susceptibilities. One mutation in the putative transporter of *E. faecalis*, identified as *abc-23*, resulted in reduced susceptibility of these organisms to both quinupristin/dalfopristin and clindamycin. It was determined that mutated activity of the *abc-23* or another gene downstream is required for resistance to quinupristin/dalfopristin and clindamycin to develop. Termed the *LS_A resistance phenotype* (for lincosamide and streptogramin A resistance), this mechanism is believed to be responsible for intrinsic *E. faecalis* resistance, and ultimately the *abc-23* gene was renamed *lsa*. Although not definitive, the genetic sequence of the *lsa* gene is similar to other ATP-mediated efflux pumps, and therefore active transport of the compound from the cell is the likely mechanism for this resistance (13, 42). Confirmation of this mechanism was evaluated by Dina et al. (13) who demonstrated that *E. faecalis* isolates that posess mutated (inactive) *lsa* genes were susceptible to both clindamycin and dalfopristin.

Development of resistance during treatment is always a concern to clinicians. In *S. aureus*, investigations into the selective pressure of quinupristin/dalfopristin were carried out in vitro and in a model of rabbit aortic endocarditis (35). Using an isolate that was known to be susceptible to quinupristin/dalfopristin, Malbruny et al. (35) were able to demonstrate mutations in the L22 ribosomal protein. These mutations resulted in a 4- to 32-fold increase in the organism's MIC of the combination when compared to the original wild-type isolate. The L22 protein plays a role in the assembly of the ribosomal subunit, which is believed to be a site for binding of antibiotics including quinupristin. Genetic sequencing demonstrated a mutation in the *C* terminus of the L22 protein, which resulted in a larger opening to the polypeptide tunnel resulting in ineffective binding of quinupristin.

The resistance to quinupristin in these studies demonstrated a loss of effective synergy between quinupristin and dalfopristin and ultimately resulting in resistance to the combination.

4 Streptogramin Resistance and the Epidemiology of MLS_B

Clinical reports have described the development of streptogramin resistance when patients are being treated with quinupristin/dalfopristin for vancomycin-resistant *E. faecium* (VREF) (12, 20, 34, 38, 43). Additionally, surveillance studies have found clinical isolates of enterococci and/or staphylococci that are streptogramin resistant: e.g., rates in Taiwan as high as 71% for enterococci and 39% for staphylococci (14, 18, 23, 41, 43). While still in compassionate use trials, resistance was documented in 1.8% of patients treated with quinupristin/dalfopristin (14). Similar to other unrelated antimicrobial agents, the development of resistance during therapy appears to be quite low, although this phenomenon has been documented and resulted in treatment failures in patients whose infectious organism expresses MLS_B resistance constitutively (12, 14, 38).

Erythromycin resistance was described in staphylococci isolated in France only a few years after the introduction of the drug (10, 26). Since then, MLS_B resistance has spread worldwide, and has been described in a variety of aerobic and anaerobic organisms. Estimates of the epidemiology of MLS_B resistance in a variety of organisms responsible for human infections have been made. Between 15 and 45% of staphylococci that display MLS_B either constitutively or inducibly has been reported (16, 40), whereas it may be as high as 90% in some methicillin-resistant strains of *S. aureus* (36).

Investigations into the optimization of pharmacodynamics via modification of dosage regimen, such as administration of the drug as continuous infusion, may provide a potential solution to the development of resistance. Additionally, combination therapy, as studied in in vitro simulations utilizing *S. aureus* (MSSA and MRSA), was able to prevent the emergence of quinupristin/dalfopristin resistance (27). Against *E. faecium*, Thal et al. (45) utilized in vitro simulations with a variety of different antimicrobials to examine the prevention of emergence of quinupristin/dalfopristin-resistant mutants. They determined that combinations with clinafloxacin, trovafloxacin, or tetracycline were successful at preventing these mutations. Data from the in vitro model by Aeschlimann et al. (2) supported these findings. Further investigation into combination therapy or optimization of pharmacodynamic parameters is needed for improvement of streptogramin efficacy and prevention of resistance while in clinical use.

The use of streptogramins as growth promoters in the agricultural industry, which possibly resulted in resistance in human infections, is still a concern. Investigations by Welton et al. (47) during October 1995 to April 1996 determined the prevalence of streptogramin resistance in the fecal flora of turkeys to be approximately 25% in *E. faecium*. The examined turkeys were supplied with subtherapeutic virginiamycin for growth promotion, which is believed to contribute to resistance development. In Taiwan, Enterococci have exceedingly high rates of streptogramin resistance (9, 11, 19, 22, 24, 34, 37, 44). Taiwan does not utilize streptogramins clinically, but it is used liberally in the animal husbandry industry there. The European Union has implemented a ban against the use of antibiotics as growth promoters over concerns of the development of resistant infections (45, 47). The United States and Australia are undertaking investigation of a similar ban.

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Chapter 22
Resistance to Linezolid

Dean Shinabarger and George M. Eliopoulos

1 Oxazolidinones: A Brief Description of Chemistry

The first description of oxazolidinones as antibacterials was reported by researchers from the DuPont company in 1987. Compounds Dup-105 and DuP-721 (Fig. 1) were introduced as clinical candidates with good activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus*, in an in vivo animal efficacy model (1). These compounds demonstrated lethal toxicity in animal models and were not further developed (2). Researchers at Pharmacia (now Pfizer) became interested in these molecules and began a chemistry/screening effort to improve the in vitro, in vivo, and safety profiles of oxazolidinones.

The DuPont group had established a structure–activity profile for oxazolidinones (3, 4), but no information was available describing structure–toxicity relationships. Using DuP-721 as the prototype oxazolidinone, the Pharmacia group began modifying the phenyl ring, tracking the in vitro and in vivo activity of the analogs in order to select compounds that were similar to DuP-721 in potency. This effort yielded several molecules, the most exciting of which was PNU-82965 (Fig. 1) (5, 6). This compound contained an indanone ring as a replacement for the phenyl ring and acetyl moieties of Dup-721. A head-to-head comparison of Dup-721 and PNU-82965 in a 30-day rat toxicology study revealed that the latter compound was far superior in safety to Dup-721, thereby establishing the first structure–toxicity relationship for oxazolidinones and paving the way for the synthesis of additional analogs that focused on suitable replacements for the phenyl and acetyl moieties of Dup-721. This effort led to the troponylphenyloxazolidinones, indolinyloxazolidinones, and the piperazinylphenyloxazolidinones subclasses (5). The poor water solubility and poor pharmacokinetic characteristics of the troponyl analogs prevented further exploration of this subclass of compounds, while indolines had a good safety profile but reduced antibacterial activity. Fortunately, the piperazine analogs were superior in all biological activities, plus they were much easier to synthesize. Two clinical candidates emerged from the piperazine chemistry effort in the form of PNU-100592 (eperezolid) and PNU-100766 (linezolid). These two compounds (Fig. 1) were virtually identical in terms of antibacterial activity and in vivo animal efficacy, and both were well tolerated in rat toxicology studies. Eperezolid and linezolid simultaneously entered Phase I clinical testing in late 1994 and early 1995, respectively. The superior pharmacokinetic profile of linezolid resulted in further development, eventually gaining Food and Drug Administration (FDA) approval in March 2000.

2 Mechanism of Action

Because they are truly the first new class of antibiotics to be approved in 30 years, the oxazolidinones have attracted the interest of researchers interested in the mechanism of action of these unique agents. Early work by researchers at DuPont revealed that protein synthesis in growing bacteria was inhibited, resulting in a focus on translation as the primary target of these compounds (7). Continued efforts by the DuPont group resulted in the development of several in vitro assays that measured the effect of oxazolidinones on initiation, elongation, and termination of protein synthesis. However, these compounds failed to inhibit any of the reactions tested, leading to the conclusion that a very early, perhaps immeasurable event in initiation of protein synthesis was the target (8). It was later demonstrated that the amount of mRNA added to the in vitro system played an important role in the mechanism of action in that adding too much mRNA actually *decreased* the potency of the oxazolidinones. Titration of eperezolid (or linezolid) in the presence of varying concentrations of mRNA resulted in an IC_{50} of 15 μM (6 μg/mL), which was very close to the minimum inhibitory concentration

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Fig. 1 Chemical structures of representative oxazolidinones

DuP-105

DuP-721

PNU-82965

PNU-100592 (eperezolid)

PNU-100766 (linezolid)

(MIC) for the linezolid-sensitive *E. coli* strain used to prepare the cell extracts (9). Using the same in vitro translation system, oxazolidinones failed to inhibit either elongation or termination of protein synthesis, once again suggesting that an early event such as initiation was the target.

Cell-free transcription/translation systems have provided the most reproducible data for the evaluation of oxazolidinone potency. Though these compounds are primarily Gram-positive agents, an *E. coli* transcription/translation system generated an IC_{50} of 1.8 µM for linezolid, indicating that Gram-negative ribosomes are sensitive to these compounds. Evaluation of oxazolidinone inhibition in a *S. aureus* cell-free transcription/translation system provided clear evidence of a dose-dependent inhibition by eperezolid, with an IC_{50} value of 30 µM (10) having been reported.

In order to determine the precise molecular mechanism by which oxazolidinones inhibit translation, several groups have examined the ability of these compounds to prevent the formation of the first peptide bond using purified ribosomes. Swaney et al. (11) reported that the binding of *N*-formylmethionyl-tRNA (fMet-tRNA) to either 30S or 70S ribosomes was weakly inhibited by linezolid, suggesting that the unique mechanism of action of oxazolidinones was due in part to their ability to inhibit initiation of protein synthesis. None of the currently marketed antibiotics inhibits this step. Using the potent oxazolidinone PNU-176798, Aoki et al. (12) further demonstrated that fMet-tRNA could compete with oxazolidinone inhibition of first peptide bond synthesis. However, both of these studies revealed that inhibition of initiation was weak, suggesting that the precise conditions for oxazolidinone binding to the ribosome had not been achieved in the in vitro system. Additional kinetic studies by Bobkova et al. (13) and Patel et al. (14) have confirmed that these compounds prevent fMet-tRNA binding to the P site, and may also affect A-site tRNA binding as well.

As discussed above, determining the precise molecular mechanism by which oxazolidinones inhibit protein synthesis has been hampered by in vitro conditions that result in a loss of drug potency. For example, the MIC for linezolid is 4 µg/mL (ca. 12 µM) for *Staphylococcus aureus* cells, and the IC_{50} for cell-free inhibition of translation using a *S. aureus* cell-free extract is 30 µM (9). However, inhibition of fMet-tRNA binding to either 30S or 70S ribosomes from *S. aureus* is weak, producing an IC_{50} of 116 µM (11). Apparently, the purification of ribosomes either results in the loss of a factor that enhances oxazolidinone potency, or the drug is inhibiting an immeasurable intermediate step in first peptide bond synthesis involving positioning of fMet-tRNA on the 50S subunit. Using an azido derivative, Mattasova et al. (15) cross-linked purified *E. coli* ribosomes with an oxazolidinone. Several cross-links were found in the domain V region of the 23S rRNA (U2113, A2114, U2118, A2119, and C2153) plus one additional cross-link at A864 of the 16S rRNA. The 23S rRNA cross-links observed in this study were in the vicinity of the E-site of the ribosome, directly contradicting

studies demonstrating that resistance mutations map to the peptidyl transferase center of the ribosome (16, 17).

A recent cross-linking study utilized a new approach to solve the problem of determining the molecular interaction of oxazolidinones with the ribosome. Using a radioactively tagged photoactive oxazolidinone, Colca et al. (18) grew *S. aureus* cells to exponential phase before exposing the culture to drug and cross-linking the cells in the presence of UV light. Extraction of the cells revealed that the 50S subunit was specifically labeled with oxazolidinone, and the label was primarily found in the 23S rRNA attached to adenine 2602. This base is known to be intimately involved in binding of fMet-tRNA to the ribosome, and mutations at this position are lethal (19). In addition to 23S rRNA, the 50S subunit protein L27 and an elongation factor analog LepA were tagged by the oxazolidinone, further confirming that these compounds target the peptidyl transferase center of the ribosome. Specificity was confirmed by the lack of cross-linking by nonactive enantiomers of oxazolidinones, and the 30S subunit was not specifically labeled at all. Therefore, the evidence to date clearly demonstrates that the oxazolidinones inhibit first peptide bond synthesis through interfering with fmet-tRNA binding/positioning to the 50S subunit of the bacterial ribosome. Several groups are attempting to cocrystallize oxazolidinones with bacterial ribosomes, and the publication of such a structure will greatly aid in the understanding of the unique mechanism of action of this new class of antibiotics.

3 Mechanisms of Resistance

3.1 Mutations Affecting 23S rRNA

The ability of oxazolidinones to inhibit the growth of bacteria containing all known forms of ribosomal resistance to translation inhibitors suggested that these compounds were binding to a unique site on the ribosome (20). Lin et al. (21) were the first to demonstrate that the radioactive oxazolidinone eperezolid bound to the 50S, but not the 30S, subunit of isolated *E. coli* ribosomes. Interestingly, both chloramphenicol and lincomycin could compete with eperezolid for binding, suggesting that oxazolidinones occupied at least a portion of the binding sites for these other two antibiotics. This work was later confirmed by Zhou et al. (22), using nuclear magnetic resonance (NMR) to measure the binding of either eperezolid or PNU-177553 to isolated *E. coli* ribosomal subunits. This NMR study was particularly useful in that the inactive enantiomers of both oxazolidinones failed to bind to the 50S subunit, demonstrating that the binding was specific.

Early attempts to develop oxazolidinone resistance in the laboratory failed to produce cultures with a stable resistance phenotype. Serial passage (23) and gradient plate techniques (24) initially produced isolates that grew at high concentrations of either eperezolid or linezolid, but MIC determinations revealed that the susceptibilities were unchanged from the parent organisms. These studies demonstrated that resistance development to oxazolidinones was not predicted to be rapid, which bode well for the upcoming clinical development of linezolid. The study of oxazolidinone resistance and mechanism of action received a huge boost in 1996 when Zurenko et al. (25) reported the first stable linezolid-resistant isolates of *S. aureus*. Utilizing a spiral gradient technique, it was demonstrated that 20 serial transfers over a 7-week time period was required in order to produce a stable eperezolid MIC of 32 μg/mL, representing a 16-fold increase in resistance to this compound. This isolate (designated 31593) was resistant to linezolid and several other oxazolidinones (data not shown). Repeating the spiral gradient experiment with linezolid produced a stably resistant *S. aureus* clone, designated 31583, exhibiting an MIC of 128 μg/mL and 64 μg/mL for linezolid and eperezolid, respectively.

Taking into account the lack of rapid resistance development and the fact that oxazolidinones bound specifically to the 50S subunit, the 23S and the 16S rRNA genes from isolates 31583 and 31593 were sequenced in an attempt to locate mutations. The results clearly demonstrated that eperezolid resistance resulted in a point mutation at guanine 2576 of the 23S rRNA (26), resulting in a transversion to uracil (G2576U). The linezolid-resistant isolate 31583 also contained a transversion in the 23S rRNA gene, resulting in a guanine to uracil transversion at position 2447 (G2447U). Figure 2 shows that these two mutations were both located in domain V of 23S rRNA, a region of the ribosome responsible for binding of tRNAs and conducting peptidyl transferase activity. The discovery that oxazolidinone resistance requires a point mutation in the 23S rRNA gene shed much light on the reason for the lack of rapid resistance development to these compounds. *S. aureus* contains six rRNA genes for 23S rRNA, thereby requiring a cell to accumulate mutations in some minimal number of genes in order to produce enough resistant ribosomes to permit growth in the presence of high oxazolidinone concentrations. The selective pressure created by the spiral gradient technique allowed selection of a single mutation in one of the 23S rRNA genes, and then that mutation had to be copied to an additional gene, and so on, in order for *S. aureus* to continue to grow in the presence of increasing concentrations of oxazolidinone. *Eco*RI digest of chromosomal DNA from linezolid-resistant 31583 followed by Southern hybridization to identify and elute each of the six 23rRNA genes from *S. aureus* revealed that five of the six genes contained the G2447U mutation, permitting growth in the presence of 128 μg/mL of linezolid (26).

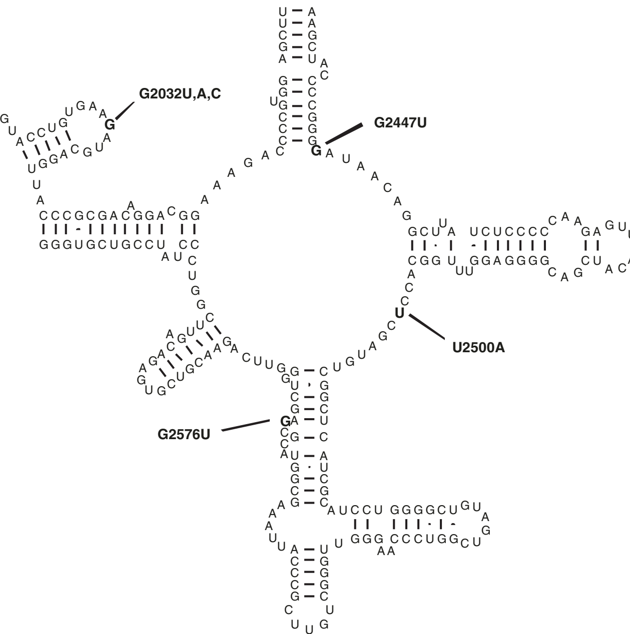

Fig. 2 Resistance mutations in 23S rRNA associated with linezolid resistance as described in the text

In order to select additional linezolid resistance mutations, Kloss et al. (27) utilized the archaebacterium *Halobacterium halobium* as a model for resistance development studies. Because this organism contains a single 23S rRNA gene, linezolid resistance developed very rapidly, resulting in the isolation of seven new point mutations within domain V of the peptidyl transferase center. Interestingly, neither the G2447U nor the G2576U mutations observed with *S. aureus* were isolated in this study, suggesting that not all bacteria could tolerate these mutations and grow in the presence of high concentrations of linezolid. This was further exemplified in a study involving the isolation of a G2032A mutation in *E. coli* (28). Though G2032A is not located in the peptidyl transferase region of 23S rRNA (Fig. 2), recent ribosome crystallography studies have revealed that it does in fact come into contact with the catalytic center of the ribosome. Site-directed mutagenesis of the *E. coli* G2032 to A produced the same level of resistance as the isolate, and changing G2032 to either U or C conferred different levels of resistance.

3.2 Other Mechanisms

Wolter et al. (29) described two clinical isolates of *Streptococcus pneumoniae* that were resistant to macrolides and chloramphenicol and nonsusceptible to linezolid (MIC = 4 μg/mL). Both isolates demonstrated 6-bp deletions resulting in deletions of (two different) amino acids in ribosomal protein L4. Transformants of *S. pneumoniae* strain R6 with DNA from these isolates also revealed this resistance pattern, supporting a causative effect of the deletions.

Methylation at position A2503 of 23S rRNA by a methyltransferase designated Cfr was shown to confer reduced susceptibility to linezolid (MICs, 4–8 μg/mL), as well as to chloramphenicol, lincosamides, pleuromutilins, and streptogramin A compounds (30). The *cfr* gene was found in staphylococci of animal origin, including a porcine isolate of *S. aureus*, in association with other plasmid-mediated genes, conferring resistance to MLS_B compounds by methylation (*erm* (31)), phenicol (*fexA*), and lincosamide (*lsa*(B)) transporters, and a spectinomycin-modifying enzyme (*spc*) (30).

4 Activity of Linezolid Against Clinical Bacterial Isolates

Linezolid inhibits at clinically relevant concentrations the vast majority of Gram-positive bacterial species recovered in the microbiology laboratory (Table 1). MIC breakpoints established by the CLSI for susceptibility are MICs ≤ 2 μg/mL for enterococci and streptococci, including pneumococci, and ≤ 4 μg/mL for staphylococci (Table 2). Using CLSI criteria, large-scale surveys have documented that virtually all staphylococci and streptococci routinely encountered in the clinical microbiology laboratory are susceptible to this agent.

Isolates of common bacterial pathogens including oxacillin-resistant staphylococci and penicillin-resistant pneumococci are as susceptible to linezolid as are their β-lactam-susceptible counterparts. Few surveys recover isolates of these genera that are nonsusceptible to linezolid. Among 405 coagulase-negative staphylococci from Taiwan that were characterized to the species level, 8 of 82 isolates of *Staphylococcus haemolyticus* and 3 of 34 isolates of *S. simulans* were nonsusceptible to linezolid (37).

In these large surveys, >95% of enterococci, including vancomycin-resistant strains, have been susceptible to linezolid. Those few that were not fully susceptible have largely fallen into the intermediate susceptibility range (31, 34). Mutnick et al. (46) reported that resistance to oxazolidinones was not encountered in surveillance studies involving >40,000 Gram-positive cocci collected between 1998 and 2000. Over the subsequent 18-month period ending in June 2002, linezolid resistance was detected in only 8 of >9,800 US isolates collected (0.08%). Styers et al. (47) found only three linezolid-nonsusceptible strains among >14,000 methicillin-resistant *S. aureus* (MRSA) isolates (0.02%) reported to their surveillance network in the US between 1998 and 2005.

The spectrum of other Gram-positive bacteria inhibited by linezolid in vitro is broad (Table 3). *Listeria* spp., *Corynebacterium* spp. and strains of *Bacillus* spp., including *B.*

Table 1 Activity of linezolid in vitro against common Gram-positive bacterial pathogens as determined by large surveys

Organism	No.	MIC_{90} (µg/mL)	% S	% I	%R	References
Staphylococcus aureus						
Oxacillin-susceptible	888	2	100			32
	306	2	100			33
Oxacillin-resistant	334	2	100			32
	193	2	100			33
Any	17,011	2–4[a]			0	34
	755	4	100			35
Coagulase-negative staphylococci						
Oxacillin-susceptible	486	2	100			32
	1,360	2	100			31
Oxacillin-resistant	554	2	100			32
	3,273	2	100			31
Any	6,177	2[a]			0	34
	769	2	100			35
Enterococcus faecalis						
Vancomycin-susceptible	1,798	2	100			32
	2,308	2	96.5		0	31
	460	2	99.6	0.4	0	33
	121	2–2	100			36
Vancomycin-resistant	40	2	100			32
	61	2	95.6		0	31
	81	1–2	100			36
Enterococcus faecium						
Vancomycin-susceptible	333	2	100			32
	310	2	95.5		0	31
	200	2	100	0	0	33
	42	2–2	97.6	2.4	0	36
Vancomycin-resistant	114	2	100			32
	598	2	97.7		0	31
	265	2	95.8	4.2	0	33
	616	1–2	99.5	0.2	0.3	36
Enterococcus spp.	160	2	100			32
	5,103	2		2.8%	0	34
Streptococcus pneumoniae	267	1	100			37
	2,598	1	100			31
	865	1[a]	100			32
	3,362	1–2[a]	100			38
	6,991	1	100			39
	1,057	1	100			34
	998	2	99.2			35
Other streptococci						
β-Hemolytic	367[b]	1	100			32
	318[b]	1	100			40
	419[c]	1	100			41
	397[c]	1	100			40
	72[d]	1[a]	100			40
	2,248	1	100			42
	633	1[a]	100			34
Viridans group	140	2	100			37
	1,152	1	100			42
	355	1–2[a]	100			34

[a] Composite value estimated based on actual MIC_{90}s of subgroups
[b] Group B streptococci
[c] Group A streptococci
[d] Group C, C, and F streptococci
S susceptible; *I* intermediate; *R* resistant

anthracis, are inhibited by the oxazolidinone at concentrations that are potentially achievable in the serum. Relatively high in vitro activity has also been reported in the intrinsically vancomycin-resistant species including lactobacilli, *Leuconostoc* spp. and *Pediococcus* spp. Linezolid inhibits many strains of *Nocardia* spp. and has been used clinically to treat infections caused by these organisms. Activity against mycobacteria is variable. Isolates of *Mycobacterium tuberculosis* have been quite susceptible in vitro, while strains of the *M. avium* complex require substantially higher concentrations for growth inhibition. Linezolid at ≤2 μg/mL inhibited 100% of 47 strains of *M. marinum* and 19 strains of *M. kansasii*, while linezolid at ≤ 8 μg/mL inhibited only 13% of 189 isolates of the *Mycobacterium avium* complex and 54% of 50 *M. chelonae* (55, 56).

Table 2 Susceptibility breakpoints for linezolid

	Interpretative criteria (μg/mL)		
Organization (reference)	Susceptible	Intermediate	Resistant
CLSI (NCCLS) (43)			
Staphylococcus spp.	≤4	–	–
Enterococcus spp.	≤2	4	≥8
Streptococcus pneumoniae	≤2	–	–
Other *Streptococcus* spp.	≤2	–	–
EUCAST (44)			
All bacterial species	≤4	–	>4
BSAC (45)			
Staphylococcus spp.	≤4	–	>4
Enterococcus spp.	≤4	–	>4
Streptococcus pneumoniae	≤2	4	>4
β-Hemolytic streptococci	≤2	4	>4

5 Linezolid Resistance Among Clinical Isolates

5.1 Comparative Clinical Studies

Resistance to linezolid was not encountered in published comparative clinical trials with this agent, which included >800 patients exposed to the agent for treatment of complicated skin and skin structure infections (57), nosocomial

Table 3 In vitro activity of linezolid against other Gram-positive bacteria

		MIC (μg/mL)			
Organism	Number	50%	90%	Range	References
Listeria spp.	39	2	2	1–2	34
	27	2	2	2	48
		2	2	2–4	25
Corynebacterium spp.	102	0.25	0.5	0.12–1	34
	48	0.25	0.5	0.12–1	48
	26	0.5	1	0.125–1	49
Corynebacterium jeikeium	12	0.5	0.5	0.25–0.5	49
Bacillus spp.	99	1	1	0.25–2	34
	23	1	1	0.25–1	48
Bacillus anthracis	18	2	4	1–8	50
Micrococcus spp.	60	1	1	0.5–2	34
	11	1	1	0.5–1	48
Leuconostoc spp.	35	2	2	1–4	37
Pediococcus spp.	8	–	–	0.5–2	37
Lactobacillus spp.	69	1	2	0.06–2	37
	23	4	8	0.5–8	49
	37	4	8	0.5–16	51
Peptostreptococcus spp.	30	0.5	2	0.5–2	49
Clostridium difficile	26	2	8	1–16	49
	18	2	16	2–16	51
Clostridium perfringens	11	2	2	1–4	51
Propionibacterium spp.	15	0.5	1	0.25–1	51
Actinomyces israelii	11	0.5	16	0.125–16	49
Actinomyces spp.	22	0.5	0.5	0.25–1	51
Nocardia asteroides	33	2	4	1–4	52
Nocardia farcina	25	4	4	1–8	52
Mycobacterium tuberculosis	117	0.5	1	≤0.125–1	53
	5	–	–	0.5–2	25
Mycobacterium fortuitum group	69	8	32	0.12–64	54
Mycobacterium chelonae	35	8	16	1–32	54
Mycobacterium abscessus	92	16	32	1–32	54

pneumonia (58), or infections due to methicillin-resistant *Staphylococcus aureus* (59).

Resistance was reported in a study comparing linezolid at two dose levels for treatment of enterococcal infections. In that study, linezolid at the standard adult dose of 600 mg bid was compared with a dose of 200 mg bid of the same drug (60). In the former group, resistance was observed in 2 of 178 treated patients (1.1%). In the low-dose group, resistance was seen in 4 of 153 patients (2.6%).

5.2 Compassionate Use Program

Zurenko et al. first reported resistance to linezolid in two patients infected with *E. faecium*, in whom the site of infection could not be extirpated because of indwelling foreign material and who received protracted courses of linezolid intravenously at a standard dose (61). MICs of the infecting organisms rose from 2 to 16 or 32 μg/mL. The isolates were found to harbor the mutation G2576U in domain V of 23S rRNA (Fig. 2).

Subsequent analysis of 828 linezolid treatment courses administered through compassionate use protocols documented development of resistance in only ten instances (62). All of these (9 *E. faecium* and 1 *E. faecalis*) were found among the 550 infections due to vancomycin-resistant enterococci (1.8% resistance), and in each resistant isolate the same mutation (G2576U) in domain V of 23S rRNA was noted. Emergence of resistance was associated with retained foreign devices or undrained deep collections. In four of these cases, the development of resistance to linezolid in the enterococcus led to failure to respond to therapy. This study also included 18 treatment courses for infections due to mycobacteria and 7 for *Nocardia* spp. infections. Resistance was not observed in these cases.

Resistance to linezolid was not encountered among isolates recovered from 183 patients with *S. aureus* infections treated with linezolid under compassionate use protocols because of intolerance to vancomycin or failure to respond to the glycopeptide (63).

5.3 Independent Observations of Resistance to Linezolid

5.3.1 Enterococci

Gonzales et al. (64) reported five patients treated with linezolid for infections due to vancomycin-resistant *Enterococcus faecium* (VREF) that developed resistance or intermediate resistance to the drug. In this group's early experience, resistance emerged in 2.2% of patients with VREF. Subsequent reports have also documented the emergence of resistance to linezolid in both *E. faecium* and *E. faecalis* from patients treated with the oxazolidinone (65, 66).

Examination of clinical isolates of *E. faecalis* and *E. faecium* with resistance to linezolid has revealed mutations in genes for domain V of 23S rRNA. In particular, the G→U conversion at base pair 2,576 of 23S rRNA reported in the compassionate use experience (61) and from mutants generated in vitro (17, 67) has been encountered in clinical isolates of both species (65, 68–72).

Studies of *Enterococcus* spp. clinical isolates support the role of a gene dosage effect. Two unrelated isolates of linezolid-resistant *E. faecalis* were found to have mutations in 2 of 4 and 4 of 4 copies of the 23S rRNA gene, associated with linezolid MICs of 24 and >256 μg/mL, respectively (71). Another report characterizing a linezolid-resistant clinical isolate of *E. faecalis*, with an MIC of 128 μg/mL, also described mutation in all four copies of 23S rRNA (70).

Examination of a series of *E. faecium* isolates with linezolid MICs ranging from 2 to 64 μg/mL demonstrated a parallel increase in the proportion of mutated copies of the 23S rRNA gene present, from 0/6 in a susceptible isolate, to 4/6 to 5/6 in the most resistant isolates (70). Mutation in only 2/6 copies was sufficient to increase MICs to 32 μg/mL. Another strain of *E. faecium* was found to possess 3/6 mutated copies at base pair 2,576, associated with an MIC of 64 μg/mL, while yet another had 6/6 mutated copies associated with the same level of resistance (66).

5.3.2 Staphylococci

Tsiodras et al. (73) characterized the first linezolid-resistant strain of *S. aureus* recovered in clinical practice (MIC > 32 μg/mL). This isolate was recovered from a patient who had been treated with linezolid for MRSA peritonitis. Curiously, this organism was unrelated to an antecedent MRSA isolate by several criteria and its origin never determined. This isolate contained five copies of the 23S rRNA gene. Each of these copies was individually sequenced and all demonstrated the G2576U mutation in domain V (74). After 15 passages in antibiotic-free medium over a 2-week period, the linezolid MIC of this strain was unchanged at 64 μg/mL.

The second linezolid-resistant strain of MRSA was reported from the UK, recovered from thoracic empyema fluid and a drain site wound of a patient treated with linezolid (75). Analysis of serial isolates, indistinguishable by pulsed field gel electrophoresis (PFGE), revealed that resistant clones (MIC 8–32 μg/mL) emerged from an initial susceptible one (MIC 1–2 μg/mL). The susceptible isolate was wild type at G2576, while 2 of 6 copies of the 23S rRNA gene were mutated (G→T) in an isolate with an MIC of 8 μg/

mL, and 5/6 copies were mutated in the isolate with a linezolid MIC of 32 μg/mL.

The emergence of resistance to linezolid during treatment was documented in another patient with MRSA infection. After successful suppression with linezolid of recurrent MRSA bacteremia associated with an unresectable endovascular focus, isolates with MICs of 8–16 μg/mL were recovered (76). The resistant isolates, which were indistinguishable from the initial susceptible strain (MIC = 2 μg/mL) by PFGE, demonstrated a novel mutation, T2500A (Fig. 2), in two to three copies of the 23S rRNA gene. Mutation at this base pair has previously been associated with oxazolidinone resistance in *Halobacterium halobium* (27). Resistant isolates of this series not only had two to three mutated copies of the 23S rRNA gene, but in two instances, the isolates had lost one of six copies of this gene. Thus, in the more resistant isolates, mutated genes accounted for 40% (2/5 copies) to 50% (3/6 copies) of the 23S rRNA content (76). Several months after discontinuation of linezolid, a linezolid-susceptible (MIC = 4 μg/mL) isolate was recovered that was wild type at this base pair (T2500) in domain V of 23S rRNA.

Paterson et al. (77) studied a linezolid-nonsusceptible and rifampin-resistant bloodstream isolate of MRSA recovered shortly after a course of these antimicrobials in combination for treatment of ventilator-associated MRSA pneumonia. The G2756U mutation was found in 2/5 copies of 23S rRNA in this isolate with a linezolid MIC of 8 μg/mL.

5.3.3 Other Organisms

Through the SENTRY Antimicrobial Surveillance Program, linezolid resistance was detected in one isolate of *Staphylococcus epidermidis* and in one strain of *Streptococcus oralis* (46). Sequencing of domain V in 23S rRNA revealed mutations at G2576U and one or more additional mutations in both isolates. Both patients from whom these strains were recovered had been exposed to linezolid. As described in Sect. 1.3.2, linezolid resistance associated with deletions in ribosomal protein L4 has rarely been encountered among pneumococci.

6 Clinical Significance of Linezolid Resistance

6.1 *Enterococci*

Linezolid-resistant enterococci have caused bloodstream infection, deep intra-abdominal abscesses, and urinary tract infections, and have been isolated from surgical wounds and drains (65, 68, 69, 71, 72, 78).

Restrospective exploration of risk factors for the acquisition of linezolid-resistant VREF was performed utilizing a group of patients who had received this antimicrobial for treatment of VREF infection (79). Four case patients with linezolid-resistant VREF were compared to 26 controls. In univariate analysis, receipt of linezolid before hospitalization, longer courses of linezolid, recent corticosteroid use, and exposure to multiple antibiotics were risk factors for acquisition of a linezolid-resistant VREF. By multivariable analysis, prior exposure to linezolid was independently associated with increased risk of infection with a linezolid-resistant isolate.

Mazur et al. (80) studied serial isolates of *E. faecalis* recovered from one patient who underwent multiple treatment courses with linezolid. Resistant isolates (MICs ≥ 128 μg/mL) were indistinguishable by PFGE from the initial linezolid-susceptible isolates and contained four to six copies of the G2576U mutation. Mutation in all six copies resulted in a growth disadvantage as compared with isolates bearing only four mutated copies when tested in antibiotic-free medium but not in the presence of linezolid, suggesting that fully mutated isolates suffer decreased fitness in the absence of selective pressure.

Notwithstanding any suggestions of reduced fitness of linezolid-resistant enterococci, there has now been documentation of the spread of linezolid-resistant enterococci in the healthcare setting and of acquisition of these organisms by patients who have not been previously exposed to linezolid. Herrero et al. (72) reported recovery of a linezolid-resistant strain of VREF in a liver transplant recipient who had been treated with the oxazolidinone, and the subsequent transmission of this strain to five patients within the same transplantation unit and to a sixth patient on another floor. Isolates from these patients were indistinguishable on PFGE. Except for the index case, these patients had stool colonization only, and did not develop infection that required treatment. In another large outbreak, linezolid-resistant vancomycin-resistant *E. faecium* was recovered from 40 patients, only 15% of whom had received prior therapy with linezolid (81).

Because of the potential acquisition of linezolid-resistant enterococci by patients who have themselves not been exposed to this antimicrobial, several authors have recommended that susceptibility to linezolid be tested in cases where this antimicrobial will be used for treatment of enterococcal infections, especially those due to vancomycin-resistant strains (68, 69, 72, 78).

6.2 *Staphylococci*

The linezolid-resistant methicillin-resistant *S. aureus* isolate studied by Pillai et al. (74), with a linezolid MIC of 64 μg/mL and all five copies of 23S rRNA bearing the G2576U

mutation, grew as well as two linezolid-susceptible MRSA strains in antimicrobial-free broth.

Whether or not linezolid-resistant *S. aureus* are in some way less fit than susceptible strains, they have clearly been isolated from infections of deep tissues. The organisms discussed above were recovered from cultures of blood, peritoneal fluid, and thoracic empyema fluid (73, 75–77). In contrast to the situation with enterococci, we are unaware of any secondary spread of linezolid-resistant MRSA relating to the two strains with which we are familiar (73, 76). One large U.S. medical center reported linezolid resistance among 4.4% of coagulase-negative staphylococci recovered during several months of 2005 (82). Detailed examination of 25 isolates revealed that 84% were genetically related strains of *S. epidermidis*. By multivariate analysis, previous exposure to linezolid and hospitalization in one ward were independently associated with linezolid resistance.

7 Conclusions

Although the mechanisms of oxazolidinone action and resistance in Gram-positive bacteria remain to be fully elucidated, resistance in laboratory mutants and in the rare resistant clinical isolates studied to date has been associated with mutations potentially affecting the peptidyl transferase center in the 50S bacterial ribosome. There has been a general relationship between the proportion of mutated to wild-type copies of 23S rRNA genes and the level of resistance. In large surveys of bacteria recovered from clinical microbiology laboratories, resistance to linezolid has been very uncommon. While there may be a biological cost to linezolid resistance, transmission of linezolid-resistant enterococci within a hospital unit has been reported, with colonization of vulnerable patients, even if they had not received linezolid.

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Chapter 23
Sulfonamides and Trimethoprim

Ola Sköld

1 Introduction

Sulfonamides interfere with the formation of folic acid in bacteria. Since mammalian cells lack the sequence of enzymic reactions leading to folic acid, including that catalyzed by dihydropteroate synthase, the target of sulfonamides, they are dependent on an external source of folic acid. This is the basis for the selective action of sulfonamides on bacteria.

The first demonstration of the antibacterial (antistreptococcal) effect of the chemically synthesized sulfonamides in mice was performed by Gerhard Domagk at the University of Münster in Germany in 1932 (1, 2). This can be regarded as the very first demonstration of the selective antibacterial action of a drug. This work was highly valued and Domagk was nominated for the Nobel Prize in 1939, but since the Nazi regime of that time in Germany did not want any German to receive the Nobel Prize, Nazi officials put pressure on the Nobel Committee at the Karolinska Institute not to award him. The Nobel Committee under its chairman, Folke Henschen, stood up to the pressure and recommended the medical faculty at the Karolinska Institute to award the Nobel Prize to Domagk. Henschen, in his memoirs from 1957, described that when this was announced in October 1939, soldiers came to Domagk's home in Wuppertal in the middle of the night to arrest him and put him in jail. On his rounds the next morning the astonished prison director found Domagk there and asked him: "How come you are here?" Domagk replied: *"Ich habe den Nobelpreis bekommen*." Domagk was not allowed to leave Germany at the time, but finally came to Stockholm in 1947 to receive his Nobel diploma.

Chemically synthesized sulfonamides with Domagk's Prontosil rubrum (4-sulfonamide-2′,4′-diaminoazobenzene, Domagk 1935 (2)) as the first, have been widely used as efficient and inexpensive antibacterial drugs for the treatment of both Gram-positive and Gram-negative pathogens. The many sulfonamide derivatives, that have been in clinical use through the years are identical from a microbiological point of view, but differ in pharmacokinetical properties.

Sulfonamides have not been used much in later years. The distribution of sulfonamides for systemic use as a single drug in Sweden is presently nil. The combination of sulfonamide and trimethoprim is still used, however, but mostly in hospitals and only to a limited extent. The total distribution of this drug combination in the last 4 years in Sweden has been rather constant and has amounted to about 620,000 defined daily doses per year corresponding to less than 0.2 defined daily doses per 1,000 inhabitants and day.

There are three main reasons for the limited use of sulfonamides today. The first is due to side effects, which are quite common in treated patients. Adverse reactions from the skin and the hemopoietic system have led to the restricted use of the trimethoprim-sulfonamide combination also. Systematic clinical studies showed blood dyscrasias, including aplastic anemia, at a frequency of 5.3 per million defined daily doses of sulfonamides, and with a fatality rate of 17% in the affected group (3). Sulfonamides seem to be the most commonly reported drugs for all blood dyscrasias (4). A second reason for the small use of sulfonamides is that after their introduction in the 1930s penicillin and many other efficient antimicrobial agents became available. A third reason finally, would be the rapid resistance development after their introduction in clinical medicine. This ought to mean that sulfonamide resistance in, for example, streptococci and meningococci should have disappeared in the absence of selection pressure. This is not the case, however. Detailed studies on the mechanisms and genetics of this resistance could shed light on the important question of resistance reversibility and compensatory evolution.

In the present clinical situation of increasing resistance to antibacterial agents among pathogens, sulfonamides might have to be reconsidered as remedies against infectious disease with modern vigilance and knowledge of side effects.

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Trimethoprim is related to sulfonamides in the sense that it interferes with folate metabolism. Sulfonamides act by their structural analogy with *p*-aminobenzoic acid, and competitively inhibit the condensation of this folic acid component with 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate to form dihydropteroic acid under the catalysis of dihydropteroate synthase (5–7). Trimethoprim, with its 2,4-diaminopyrimidine structure, on the other hand, is an analogue of the folic acid pterin moiety, and competitively inhibits the reduction of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase, in analogy with the antifolate cytostatic drugs aminopterin and methotrexate. The selective action of trimethoprim on bacterial dihydrofolate reductases, leaving mammalian enzymes untouched, allows the clinical use of trimethoprim as an antibacterial drug (8). As a matter of fact trimethoprim does not interfere with human dihydrofolate reductase even at concentrations 10,000-fold higher than the MIC-values found for most bacteria. There is a structural explanation for this, elucidated by X-ray crystallography studies, showing that trimethoprim fits well into the nucleotide binding site of the dihydrofolate reductase from, for example, *Escherichia coli*, but not in the corresponding site of the mammalian enzyme (9). Trimethoprim has a broad antibacterial spectrum. This can vary slightly in analogues of it, like iclaprim (10), and epiroprim (11). Since sulfonamides and trimethoprim attack successive steps in the same enzymic pathway, there is a synergistic effect, which has been successfully exploited in the broad spectrum combination drug, co-trimoxazole.

Some bacteria like *Campylobacter jejuni* and *Helicobacter pylori* seem to be naturally resistant to trimethoprim. It has turned out that these bacteria lack the gene *fol*A, coding for dihydrofolate reductase on their chromosomes, and thus do not offer any target for the antifolate (12). Tetrahydrofolate-borne one-carbon units are required for RNA- DNA-, and protein synthesis. The main drain on reduced folates in actively dividing bacterial cells is for the methylation of deoxyuridylic acid to form deoxythymidine-5′-monophosphate (thymidylate) under the catalysis of thymidylate synthase (*thy*A). In this process the methylene tetrahydrofolate gets oxidized to dihydrofolate, which is then reduced to tetrahydrofolate by dihydrofolate reductase expressed from *fol*A, which all *thy*A-carrying bacteria also contain. There is, however, a recently discovered alternative pathway for thymidylate synthesis, catalyzed by the product of *thy*X, and which does not involve the oxidation of tetrahydrofolate, but in which reduced flavin nucleotides ($FADH_2$) have an obligatory role (13). The *thy*X-carrying *Campylobacter jejuni* and *Helicobacter pylori* would then seem to be able to do without *fol*A, and thus without the dihydrofolate reductase target of trimethoprim (12).

2 Chromosomal Resistance to Sulfonamides

Spontaneous mutations to sulfonamide resistance changing the *dhps* (*fol*P) gene are easily observed in *Escherichia coli* (14–18). In one of these cases the *fol*P gene had changed by a single base pair, and expressed a dihydropteroate synthase, which was temperature-sensitive, and showed a 150-fold increase in the K_i-value for the binding of sulfathiazole. The K_m-value for the *p*-aminobenzoic acid substrate on the other hand, increased ten times resulting in a less efficient enzyme, which could be regarded as a trade-off for acquiring resistance (19). Sequencing of these spontaneously mutated *fol*P resistance genes showed the change of a Phe to Leu or to iLeu at position 28 in the amino acid sequence of the enzyme (16, 18). The resistance mutations are located at an area of *fol*P, which is highly conserved by different microorganisms (16). Sulfonamides can also function as substrates for dihydropteroate synthase to form an abnormal pterin-sulfonamide product, that cannot participate in folate metabolism. It has been suggested that this could be part of the antibacterial effect by draining dihydropterin pyrophosphate from folate synthesis. The formation of this sulfonamide adduct is much lower in resistance mutants (19).

Sulfonamide resistance is commonly found in clinical isolates of *Campylobacter jejuni*. It is mediated by chromosomal point mutations, but in a more complicated pattern than in the laboratory mutants described above. The *fol*P gene of *Campylobacter jejuni* turned out to be the largest of its kind characterized so far. Its product consists of 390 amino acid residues, and is quite similar (42% identity) to the corresponding enzyme (380 residues) from *Helicobacter pylori*. The *fol*P from a resistant isolate differed by four mutations from that of a corresponding susceptible isolate (20). The ensuing amino acid changes mediated a distinct effect on the sulfonamide sensitivity of the expressed dihydropteroate synthase. The K_i-value for sulfonamide increased from 0.5 μM with the susceptibility enzyme to 500 μM for the resistance one.

In *Streptococcus pneumoniae* sulfonamide resistance is mediated by a different kind of chromosomal change. Several years ago a spontaneous laboratory mutant of this pathogen was found to contain a six-nucleotide repeat in *fol*P mediating the repeat of ile-glu at positions 66 and 67, and extending the helical stretch by two amino acid residues (21). This could significantly alter the tertiary structure of the protein (21). This argument was later put forward by crystallographic studies on dihydropteroate synthase (22). Clinical isolates of sulfonamide resistant *Streptococcus pneumoniae* showed amino acid duplications at several different locations in the protein, indicating that changes to resistance had occurred independently on many occasions (23, 24). None of

these resistant clinical isolates carried the Ile_{66}–Glu_{67} repeat of the laboratory mutant mentioned above, but all had 3- or 6-bp duplications in the same area of the *fol*P gene. In contrast none of the several sulfonamide susceptible isolates had duplications in this region (23). Transformation experiments demonstrated that the duplications were sufficient for conferring the observed sulfonamide resistance (23). The originally characterized Ile_{66}–Glu_{67} repeat (*sul*-d), was eventually found in a clinical isolate of *Streptococcus pneumoniae* from the northwest USA (25). When this repeat was removed by site-directed mutagenesis susceptibility ensued. Kinetic studies on the dihydropteroate synthase showed the K_i for sulfonamide to drop from 18 to 0.4 µM, that is 35-fold, while the K_m for *p*-aminobenzoic acid decreased 2.5-fold. The K_m for pterin pyrophosphate did not change (25). The enzyme characteristics for the mutated strain were identical to those of susceptible strains, demonstrating that the duplication is sufficient for resistance. The fitness cost of resistance seems to be low, as reflected in the small increase in the K_m-value. The small but discernible increase indicates the absence of compensatory mutations. Still it could be enough for counter-selecting resistant strains in the absence of the drug, and might lead to an argument regarding the much-debated problem of drug resistance reversibility.

Also in *Pneumocystis carinii* resistance to sulfonamides seems to be due to a simple chromosomal pattern of mutations hitting the *fol*P of this organism, which causes the life-threatening disease of *Pneumocystis carinii* pneumonia in immunosuppressed patients. Co-trimoxazole, the combination of trimethoprim and sulfonamide (sulfamethoxazole) has been the drug of choice for the prophylaxis and treatment of this disease. Life-long prophylaxis is often recommended for HIV-positive patients. The antipneumocystis effect is mainly due to the sulfonamide component, since studies on the dihydrofolate reductase of this fungus-show trimethoprim to be a very poor inhibitor of this enzyme in *Pneumocystis carinii* (26). Dapsone (4,4′-diamino-diphenyl sulfone), a sulfone drug microbiologically acting as a sulfonamide, is also frequently used for the treatment of this infection. *Pneumocystis carinii* has thus been heavily exposed to sulfonamide with an increasing prevalence of resistance mutations in its *fol*P gene as a consequence. The human *Pneumocystis carinii* cannot be cultured and the dihydropteroate synthase protein is not available for study, but the corresponding *fol*P sequence is known (27, 28) The most common mutations occur at nucleotide positions 165 and 171, leading to thr55ala and pro57ser. They appear either as single or double mutations in the same isolate (29, 30). In later work the important question is whether the recent emergence of resistance mutations is the result of transmission between patients or arise and are selected within the individual patient under the pressure of sulfonamide or dapsone treatment. The latter interpretation was favored, i.e., the mutants are selected within a given patient (31), and the mentioned mutations may be associated with reactivation of the infection (32).

Sulfonamide resistance in clinical isolates of *Staphylococcus aureus* and *Staphylococcus haemolyticus* has been studied and been shown to involve chromosomal mutations in *fol*P in an erratic pattern (33, 34). With *Staphylococcus aureus* the dihydropteroate synthase was purified to homogeneity and subjected to X-ray crystallographic studies (34). In different isolates sequencing could discern four different mutational patterns and identify as many as 14 amino acid changes in the development of resistance. A simple interpretation of their role in resistance has not been possible.

Dapsone (4,4′-diamino-diphenyl sulfone), microbiologically a sulfonamide, has been a standard treatment for leprosy for a long period of time. As could be expected resistance developed, and has actually been known to do so since the 1950s. It has later been defined as chromosomal mutations in the *fol*P of *Mycobacterium leprae*, resulting in thr53ile, thr53ala or pro55leu (35).

The very first experiments demonstrating the selective antibacterial effect of sulfonamides were performed by Gerhart Domagk with *Streptococcus pyogenes* more than 70 years ago (see Sect. 1). The sulfonamides became frequently used, for prophylaxis against streptococcal infections among soldiers in military training camps during World War II. Occasional failures of this prophylaxis was observed to be due to the appearance of resistant streptococcal strains (36). Sulfonamides were replaced by penicillin as antistreptococcal agents in the 1940s, and the mechanism of the mentioned early resistance was described until-later years (37, 38). Sulfonamide-resistant strains of *Streptococcus pyogenes* seem to have been prevalent into present times in spite of the very low or nonexistent systemic use of this drug for decades. This is an interesting illustration of the nonreversibility of resistance in the absence of the selecting effect of the drug. The drug-resistant phenotypes do not seem to have any disadvantage at competition with their drug-susceptible relatives. The sulfonamide-resistant strains studied in (37) varied substantially in resistance with MIC-values of 512–1,024 µg/mL. The mechanism of resistance turned out to be very different from the rather simple mutational *fol*P changes described above. When *fol*P genes in susceptible and highly resistant isolates were compared, a 13.8% difference in nucleotide sequence was observed. This difference is too large to be due to accumulated mutations. The resistance gene must have been introduced by transduction or transformation. The sequence analysis of the complete genome-shows that *Streptococcus pyogenes* contains at least one inducible prophage (39), indicating the possibility of

phage-mediated transduction. Further studies on sulfonamide resistance (38) included sequence determination of the genes neighboring the sulfonamide target, *fol*P, in the folate operon. A comparison between five sulfonamide resistant and one susceptible isolate, the latter showing only a few differences from the sequence available through the genome sequencing project (strain SpM1) (39), demonstrated an overall difference in nucleotide sequence of 15%. More specifically, areas of different nucleotide sequences were scattered over the folate operon in a mosaic fashion, indicating horizontal transfer of genetic material. The *fol*P gene of resistant isolates showed different areas of foreign DNA in different isolates. This imported DNA was identical among three of the five, and between two of the five of the studied resistant isolates. From examining published three-dimensional dihydropteroate synthase structures (40), and conserved amino acids in different known sequences, and also the location of substrate binding, a particular amino acid exchange could be discerned as involved in resistance, at position 213 of the *fol*P product. This is located just after a conserved sequence of Ser-Arg-Lys. In most bacteria this is an Arg as in the sequenced genome of the sulfonamide susceptible *Streptococcus* strain of SpM1 (39). In three of the five sulfonamide-resistant *Streptococcus pyogenes* strains, position 213 is a Gly. A change to Gly by site-directed mutagenesis at this position in a sulfonamide-susceptible strain, resulted in a 50-fold rise in the K_i for sulfonamide, and also in an increased (1.6-fold) K_m for *p*-aminobenzoic acid. In a parallel experiment the Gly was changed to Arg in a resistant strain effecting a 30-fold decrease in K_i. These experiments indicate that a single amino acid change could explain a large part of the resistance property. In two other sulfonamide resistant strains there was an Arg at position 213 like in the susceptible strain. However, they carried another type of sequence change, an insertion of two additional amino acids, Val-Ala after position 67. The removal of the two amino acids by site-directed mutagenesis resulted in an enzyme with a markedly lowered activity, and while the parental strain grew at a sulfonamide concentration of 510 µg/mL, the mutationally changed one showed a MIC value of 64 µg/mL. The two extra amino acids did thus affect resistance but also seem to be involved in forming an efficient enzyme structure in the resistant strain (38).

Chromosomal resistance to sulfonamides in *Neisseria meningitidis* is related to that of *Streptococcus pyogenes* in that its mechanism is based on the horizontal transfer of genetic material. Sulfonamides have been used extensively for prophylaxis and treatment of meningococcal disease since the 1930s. Sulfonamide resistance is commonly observed in clinical isolates of pathogenic *Neisseria meningitidis* today. There even seems to be an association between pathogenicity and sulfonamide resistance and possibly also between mortality rate and resistance (41). Astonishingly large differences in the structure of *fol*P were found between resistant and susceptible strains of *Neisseria meningitidis* (41, 42). Two classes of different resistance determinants were revealed by nucleotide sequence determinations in several clinical isolates. In one of them the *fol*P gene was about 10% different from the corresponding gene in drug-susceptible isolates. From this it could be concluded that resistance had appeared by recombination of horizontally transferred DNA rather than by the accumulation of mutations. In this class of resistant bacteria, strains were found, showing a mosaic *fol*P, in that only the central part corresponded to the resistance gene, while the outer parts were identical to those of susceptible isolates (41). The origin of the resistance gene or gene fragments most likely is in other *Neisseria species*. This interpretation is supported by the finding of an 80-bp sequence identical to the corresponding part of the *fol*P gene in *Neisseria gonorrhoeae*, in the *fol*P of a susceptible isolate of *Neisseria meningitidis* (43).

The mentioned class of resistance genes in *Neisseria meningitidis* is characterized by an insertion of six nucleotides, coding for Ser-Gly, in a highly conserved part of the *fol*P gene (41) Removal of these two amino acids by site-directed mutagenesis resulted in susceptibility (43).

The other mentioned class of sulfonamide resistance determinants lacked the 6 bp insert and showed a lower degree of difference to susceptible isolates (43). Several of these sulfonamide resistance *fol*P genes were identical but distinct from the corresponding susceptibility genes. This again indicates a horizontal transfer of genes followed by recombination (43). A comparison of amino acid sequences of dihydropteroate synthases between those from these resistant strains and those from susceptible ones showed differences at 19 positions. Three of these differences were in amino acids conserved in all known bacterial dihydropteroate synthases (43). One of these is the conserved Phe31, which in the resistance *fol*P is changed to Leu. This same alteration was seen in the described spontaneous mutation to sulfonamide resistance in *E. Coli* (phe28leu) (16). Later, a fourth amino acid change Arg228Ser has been observed to mediate sulfonamide resistance in *Neisseria meningitidis* (44). The three alterations in the meningococcal enzyme, Leu31, Ser84 and Cys194, were subject to site-directed mutagenesis. When the resistance alteration Leu31 was mutated to susceptibility Phe 31 the sulfonamide MIC of the host decreased from about 0.5 mM to less than 0.02 mM. The Phe31 position is localized in a *fol*P area, where six of eleven of the corresponding amino acids are conserved in all known bacterial dihydropteroate synthases. The Cys194 is also located in a very well-conserved area of the enzyme. When this was experimentally changed to the susceptibility Gly, there was a drop in the sulfonamide MIC from more than 0.5 to 0.12 mM. When Ser84 finally, in the resistant strain, was changed to susceptibility Pro, no effect on the sulfonamide MIC of the

host could be observed (43). In further studies on the characteristics of the dihydropteroate synthase resistance the cloned meningococcal genes were expressed in an *E. coli* strain, that had its *fol*P partially deleted (45). This allowed the comparison of resistant dihydropteroate synthases with experimentally mutated variant enzymes in extracts without the interference of chromosomal background activity. The obtained K_i data correlated well with the MIC-data described above (43). A pronounced effect was seen with mutations at position 31. A change of the resistance Leu to the susceptibility Phe caused a more than 300-fold decrease in K_i and a concomitant six to eightfold drop in the K_m for *p*-aminobenzoic acid, measured as pseudo-first order kinetics, since the other substrate, dihydropterinpyrophosphate was added in excess. Alterations of Cys to Gly at position 194 also mediated substantial effects on kinetic characteristics. The experimental change of resistance Ser84 to susceptibility Pro84, did not decrease MIC but in several experiments effected a twofold increase in the K_m for *p*-aminobenzoic acid. The Ser84 could then be interpreted as an amino acid change compensating for the possibly detrimental effect on the enzyme of the other two resistance-mediating amino acid changes.

The same approach was used for studying the other type of sulfonamide resistance mentioned above. At removing the two extra codons Ser195Gly196 a tenfold drop in K_i occurred resulting in susceptibility, but a concomitant tenfold increase in K_m indicated that the two inserted extra codons could not be the sole alteration leading to resistance. Most likely, compensatory mutations have also accumulated in these enzyme genes (45). This idea is further studied in an investigation, where it was seen that sulfonamide resistance was effected also by amino acid changes at position 68 in the dihydropteroate synthase in this class of resistant *Neisseria meningitidis* strains (46). In these resistant strains a Ser or a Leu substituted for the well-conserved Pro at this position in the consensus sequence. When this Ser68 was changed to a Pro in a mutant lacking the Ser-Gly insertion, the already lost sulfonamide resistance was not affected. Instead, the K_m for *p*-aminobenzoic acid was lowered almost tenfold. The amino acid change at position 68 thus seems to be involved in the meningococcal adaptation to sulfonamide resistance. When, the other way around, these results were used in an attempt to create a resistant strain from a susceptible one, the introduction of Ser-Gly had a dramatic effect on the K_m for *p*-aminobenzoic acid in that it increased about 100-fold, while the K_i for sulfonamide increased so little that it did not allow growth in the presence of sulfonamide. This enzyme also performed very poorly when used to complement the *fol*P knock-out mutant mentioned above (45), the generation time doubled to 60 min, when compared to complementation with the unmutated gene (46). Changing also Pro to Ser at position 68 mediated an increase in K_i, but also a K_m increase so large that drug resistance could not be determined (46). The pattern of changes in resistance enzyme thus seems to be more complicated than that described. These observations support the idea that the resistance gene has evolved in another bacterial species and has later been introduced into *Neisseria meningitidis* by transformation and recombination (47). This was further supported by the finding of sulfonamide resistant *Neisseria*-commensals cultivated from throat swabs of outpatients (48). Since the distribution of sulfonamides for systemic use in Sweden, as mentioned above, is nil, and since the combination drug cotrimoxazole is only used in hospitals, it could be concluded that the sampled patients had not been exposed to sulfa drugs. The studied isolates were identified to belong to the *Neisseria subflava*/*Neisseria sicca*/*Neisseria mucosa* group and showed high sulfonamide resistance with MIC-values higher than 256 μg/mL. Their *fol*P genes showed resistance characteristics like those described above for *Neisseria meningitidis*, as, for example, a Leu at position 31 and a Cys at position 194. A new resistance variation was also suggested with a Met at position 66 combined with a Gly-insertion between positions 75 and 76. Experiments were performed to see if resistance could be transferred by natural transformation from these commensals with a pathogenic *Neisseria meningitidis* as recipient. No transfer could, however, be observed, in spite of positive controls showing ready transfer of resistance *fol*P with a *Neisseria meningitidis* strain as a donor (49). A possible explanation could be that the studied isolates lacked the uptake sequence known to be necessary for efficient transformation in *Neisseria* (50).

3 Plasmid-Borne Resistance to Sulfonamides

Sulfonamide is a synthetic antibacterial agent. Resistance by plasmid-mediated drug-degrading or drug-modifying enzymes were not to be expected. Instead nonallelic, drug-resistant variants of the chromosomal dihydropteroate synthase target enzyme have been found to mediate high resistance to sulfonamides (51, 52). Three genes *sul*1, *sul*2 and *sul*3 expressing enzymes of this type are known and characterized (53–56). They are distinct from each other (similarity at the amino acid level is about 40%). Their origins are unknown. Remarkably, only *sul*1 and *sul*2 were found for a long time. In a study from 1991 on 203 human *Enterobacteriaceae* strains from different parts of the world only *sul*1 or *sul*2 or both were found (57). The reason for this could be that there is a constraint on the dihydropteroate synthase structure in discriminating between the normal substrate *p*-aminobenzoic acid and the structurally very similar sulfonamide inhibitor. The enzymes expressed from *sul*1 and *sul*2 bind the normal substrate well, showing low K_m-values

(0.6 µM), and still resist high concentrations of sulfonamide. The *sul*2 enzyme shows a particularly high acuity in distinguishing between *p*-aminobenzoic acid and very high concentrations of sulfonamide. The finding of *sul*3 in swine isolates of *E. coli* and subsequently in human isolates is very interesting in this perspective (56, 58). All three plasmid-borne *sul* genes seem to be mediated by efficient genetic transport mechanisms. The *sul*1 gene is almost always found linked to other resistance genes in the Tn*21* type integron, while *sul*2 is found on small plasmids of the *inc*Q family (e.g., RSF1010), and also on small plasmids of another type, represented by pBP1 (59). The more recently found *sul*3, seems to be part of a composite transposon flanked by the insertion sequences IS*15delta/26* (56). The two genes *sul*1 and *sul*2 used to be found at roughly the same frequency among sulfonamide resistant, Gram-negative, clinical isolates (57). In later years, however, a relative increase in prevalence of *sul*2 has been observed (60). In spite of a very low use of sulfonamides in the United Kingdom, a comparison of large collections of clinically isolated *E. Coli* from 1991 and 1999 showed an increase in sulfonamide resistance during this period, and this was mostly accounted for by an increase in the prevalence of *sul*2, now frequently found on large, conjugative multiresistance plasmids. An explanation for this phenomenon could be the found association between *sul*2 and multiresistance plasmids, allowing selection through the use of other antimicrobial agents (60).

Another recently found location of *sul*2 is in *Haemophilus influenzae* mediating high sulfonamide resistance to this pathogen (61). In the same work high sulfonamide resistance was alternatively mediated by the chromosomal insertion of five amino acids (61).

4 Chromosomal Resistance to Trimethoprim

Resistance to trimethoprim by mutations involving *fol*A, the chromosomal gene expressing the trimethoprim target enzyme, dihydrofolate reductase is known from several pathogenic bacteria. One example of this is a clinical isolate of *Escherichia coli*, which overproduced its chromosomal dihydrofolate reductase several hundred-fold, by a combination of four types of mutations enhancing its expression (62). One was a promoter-up mutation in the −35 region, a second was a 1 bp increase in the distance between −10 region and the start codon, there were also several mutations optimizing the ribosome binding site, and finally there were mutations in the structural gene effecting changes to more frequently used codons. Now, the mere increase in the intracellular enzyme level, could not be expected to decrease the susceptibility of the host more than 1,000-fold to the competitively acting folate analogue of trimethoprim. However, the expressed dihydrofolate reductase also showed a threefold increase in the K_i for the drug, which was thought to be due to the mutational substitution of a Gly for a Try at position 30 of the enzyme protein. The combined action of decreased enzyme susceptibility and enzyme overproduction could then explain the high resistance (MIC > 1,000 µg/mL) observed for the mentioned isolate (62). All the mentioned changes represent a remarkable evolutionary adaptation to the antibacterial action of trimethoprim.

A similar type of chromosomal resistance to trimethoprim has been observed in *Haemophilus influenzae*, where differences in the promoter region and also in the structural gene were seen between trimethoprim susceptible and trimethoprim resistant isolates (63). Different parts of the structural gene in different isolates were changed, also in the C-terminal area, which is not known to participate in substrate or trimethoprim binding. These alterations were suggested to involve changes in the secondary structure mediating a decrease in trimethoprim binding.

Chromosomal resistance to trimethoprim in *Streptococcus pneumoniae* is fairly common (64). Resistant strains were shown to express dihydrofolate reductases, that resisted 50-fold higher concentrations of trimethoprim The 50% inhibitory dose was 3.9–7.3 µM compared to 0.15 µM for the susceptible enzyme. Site-directed mutagenesis revealed that one amino acid change, Ile to Leu at position 100, resulted in the mentioned 50-fold increase in resistance to trimethoprim. Further studies on 11 trimethoprim resistant isolates demonstrated a substantial variability in the nucleotide sequences of their dihydrofolate reductase genes. The resistant isolates could be divided into two groups with six amino acid changes in common. One group showed two extra changes, and the other, six additional changes. This high number of changes indicates horizontal transfer of resistance genes. This interpretation is experimentally supported by the ability of chromosomal DNA from resistant isolates, and cloned PCR products from resistance strains to transform a susceptible strain of *Streptococcus pneumoniae* to trimethoprim resistance (64).

The strange finding of usually plasmid-borne, foreign trimethoprim resistance genes on the chromosome of *Campylobacter jejuni* could, in a way, be classified as chromosomal resistance. Clinically, *Campylobacter jejuni* has always been regarded as endogenously resistant to trimethoprim. In an attempt to study the mechanism of this, it was found that a majority of clinical isolates carried foreign genes expressing drug-resistant variations of dihydrofolate reductase, the target of trimethoprim (65). The found genes *dfr*1 and *dfr*9 are well known (see below) as integron- and transposon-borne genes mediating trimethoprim resistance via plasmids. Remnants of the transposon known to carry *dfr*9 were observed in its context on the *Campylobacter* chromosome and the *dfr*1 was found as an integron cassette (65).

The occurrence of these genes would of course mediate a very high trimethoprim resistance to the bacterium, but as mentioned above it is now known that *Campylobacter jejuni* is really intrinsically resistant to trimethoprim by its different enzymatic mechanism for thymidylate synthesis obviating the need for dihydrofolate reductase, also reflected in that the *fol*A gene is missing from its chromosome (12, 13). The trimethoprim target is thus missing from *Campylobacter jejuni*. The selective value of acquiring the resistance gene *dfr*1 or *dfr*9 (sometimes both, (65)), is then difficult to understand. Speculatively, *Campylobacter jejuni* could take advantage of the *dfr* genes, available through antibacterial selection, for acquiring a better growth potential.

A different type of chromosomal mutations leads to low trimethoprim resistance. Mutations in *thy*A expressing the enzyme thymidylate synthase make *E. coli* cells able to grow in the presence of 8–10 µg/mL of trimethoprim, provided there is an external supply of thymine (66, 67). The inactivated thymidylate synthase makes cells dependent on external thymine, but also relieves dihydrofolate reductase of its main task of regenerating tetrahydrofolate in the formation of N^5-, N^{10}-methylene tetrahydrofolate, which is oxidized in the deoxyuridylate methylation process. The cell can then afford to have a fraction of its dihydrofolate reductase inactivated by trimethoprim. The mentioned concentration of trimethoprim can of course, for the same reason, be used for the selection of spontaneous *thy*A mutants.

5 Plasmid-Borne Resistance to Trimethoprim

As in the case of sulfonamide resistance, plasmid-borne resistance to trimethoprim is mediated by nonallelic, drug-resistant variations of the target enzyme, that is, dihydrofolate reductase. The first of these were found decades ago (68, 69), but newly found ones seem continuously to be added to the list, where now at least 30 different resistance genes (*dfr*:s) expressing these enzymes are to be found. They are numbered consecutively after the first ones found (68, 69). These resistance genes must have moved horizontally into pathogenic bacteria selected by the heavy use of trimethoprim. The precise origin is not known in any case. This mechanism, with an extra resistance-mediating target enzyme is highly prevalent in enterobacteria, where *dfr*1, the one found first, seems to be most common. It occurs in a cassette in both class 1 and class 2 integrons (70). The class 2 integron, with *dfr*1, is borne on transposon Tn*7*, which has spread very successfully, mainly because of its high-frequency insertion into a preferred site on the chromosome of *E. coli* and many other enterobacteria (70). Transposon Tn7 in clinically isolated bacteria is usually located on the chromosome and less frequently on plasmids (71). Among the horizontally moving trimethoprim resistance genes, there is a subclass of four genes, *dfr*2a, *dfr*2b, *dfr*2c and *dfr*2d, which are closely related among themselves, but so different from other trimethoprim resistance genes, that they could not be included in the phylogenetic tree, where the interrelationship of the others could be demonstrated (Fig. 1). Their corresponding polypeptides consist of 78 amino acids which are identical to 67% among themselves, and are active in the form of tetramers showing dihydrofolate reductase activity, that is almost insensitive to trimethoprim IC_{50}s > 1 mM, making hosts so drug resistant that MIC cannot be determined for solubility reasons (73–76). All these group 2 genes have been found as integron cassettes (70, 73). The phylogenetic tree mentioned above and shown

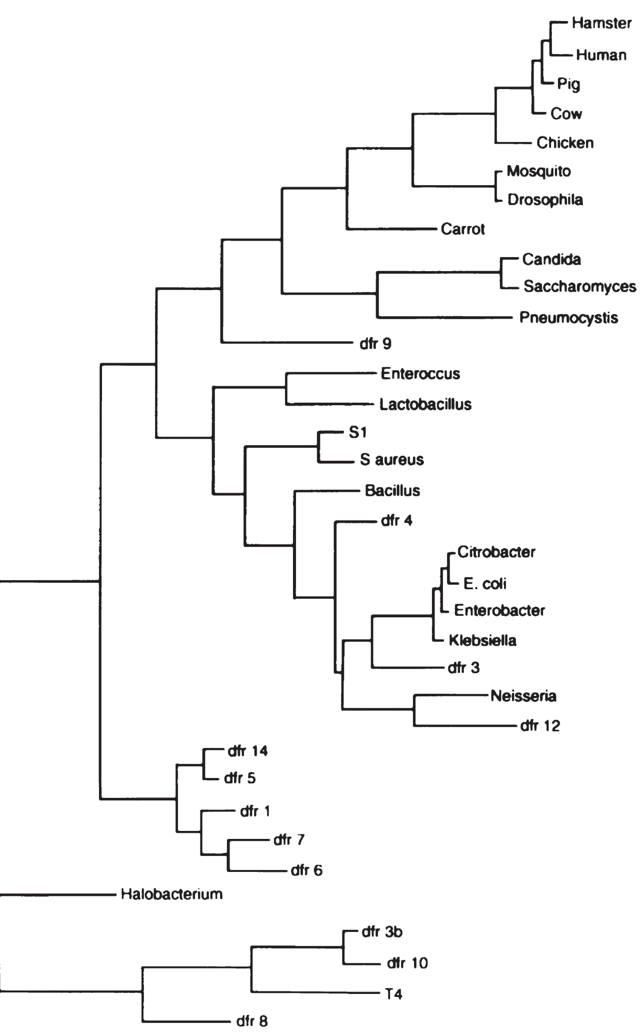

Fig. 1 Phylogenetic tree based on amino acid sequence alignment and parsimony analysis, showing the relationship between different dihydrofolate reductases. Resistance enzymes are marked by dfr and a number. Adapted from (72), where GenEMBL accession numbers to the different sequences can be found

in Fig. 1, relates different dihydrofolate reductases and is based on amino acid alignment and parsimony analysis (72). In this tree, dfr1, dfr5, dfr6, dfr7, and dfr14 form a well-supported group of similar enzymes. Otherwise the trimethoprim resistance enzymes are diverse and scattered all over the tree. This is consistent with the notion that these resistance genes have their origins in a large variety of different organisms. One, however, dfr3, is rather closely related to the chromosomal dihydrofolate reductases of enterobacteria, which could hint at its origin. In staphylococci, extrachromosomally mediated high-level resistance to trimethoprim is effected by the drug-insensitive dihydrofolate reductase S1 borne on the ubiquitous transposon Tn*4003* (77, 78). This trimethoprim-resistant enzyme is almost identical with the chromosomal dihydrofolate reductase of *Staphylococcus epidermidis*. It differs by only three amino acid substitutions, and it has therefore been suggested that a mutated form of the *S. Epidermidis* enzyme has moved horizontally into other staphylococcal species (79). A second trimethoprim-resistant and plasmid-encoded dihydrofolate reductase, S2, was later isolated from *Staphylococcus haemolyticus*. Its similarity with other staphylococcal enzymes indicates, that its origin is similar to that of S1 (80). The S2 enzyme was later found, also in *Listeria monocytogenes* (81). Low intermediate levels of trimethoprim resistance in *Staphylococcus aureus* were observed as a consequence of a Phe98Tyr mutation in the chromosomal dihydrofolate reductase gene (82). This change is identical to one of the differences between S1 and the chromosomal dihydrofolate reductase of *S. Epidermidis* (79).

Further studies of clinically isolated aerobic Gram-negative enterobacteria has extended the list of trimethoprim resistance genes. In a survey of trimethoprim-resistant isolates from commensal fecal flora a gene numbered *dfr*13 was found. Its gene product showed 84% amino acid identity with *dfr*12, and also a similar trimethoprim inhibition profile. It appeared as an integron cassette in a class 1 integron (83). Another, *dfr*15, was also found as a cassette in a class 1 integron, and in a commensal, fecal *Escherichia coli*. Its predicted protein showed 90% amino acid identity with *dfr*1, i.e., the first extrachromosomal trimethoprim resistance enzyme found and it thus belongs to the prevalent group, that can be discerned as a well-supported cluster of similar enzymes in the phylogenetic tree of Fig. 1. (84). Another member of this group, *dfr*17, was observed, again as a cassette in a class 1 integron, and in urinary tract-infecting *Escherichia coli* isolated in Taiwan and Australia, respectively. The *dfr*17 cassette showed 91% identity with the earlier characterized *dfr*7 cassette (85, 86).

An obviously transferable dihydrofolate reductase gene *dfr*F has been observed to be located on the chromosome of highly trimethoprim resistant, clinical isolates of *Enterococcus faecalis* (87). The characterized *dfr*F codes for a predicted polypeptide show 38–64% similarity with other dihydrofolate reductases from Gram-positive and Gram-negative organisms.

One of the resistance enzymes of the phylogenetic tree, dfr9, only distantly related to the earlier mentioned main group (Fig. 1), was originally found expressed from *dfr*9 on large transferable plasmids in isolates of *Escherichia coli* from swine (88). The *dfr*9 was observed at a frequency of 11% among these trimethoprim resistant, veterinary isolates of *E. coli*, but only very rarely among corresponding human isolates (89). The spread of *dfr*9 among swine bacteria is most likely due to the frequent veterinarian prescription of trimethoprim in swine-rearing. A subsequent spread into human commensals might then have taken place (90, 91). The origin of *dfr*9 is unknown, but further study of its surroundings in many plasmids from several strains showed that it is borne on a truncated transposon, Tn*5393*, previously found on a plasmid in the plant pathogen *Erwinia amylovora*, causing fire blight on apple trees (92). This transposon carries two streptomycin resistance genes *str*A and *str*B, and it most probably evolved under the selection pressure of streptomycin ubiquitously used for the control of the mentioned plant disease in many countries (93, 94). The *dfr*9 gene was found inserted in the *str*A gene at the right hand end of Tn*5393*. The occurrence of *dfr*9, expressing trimethoprim resistance in *E. coli* from swine in Sweden, and its location on a genetic structure, closely related to transposon Tn*5393*, originally observed to mediate streptomycin resistance in a plant pathogen in the USA, could be regarded as a powerful demonstration of bacterial adaptation to the heavy use of antibacterial agents in agriculture and stock breeding. Modern pig-rearing in large stables with many animals could be regarded as gigantic genetic laboratories creating very large populations of genetically communicating bacteria, allowing also very rare genetic events to surface, like mobilizing a trimethoprim resistance gene under the selection pressure of the heavy use of this drug in animal husbandry.

As mentioned above, *dfr*9 has been found in a Tn*5393* context also on the chromosome of *Campylobacter jejuni* (65). It could be mentioned that *Campylobacter jejuni* is a commensal in the gut of swine.

6 Conclusions

The study of resistance to sulfonamides and trimethoprim is of interest, in spite of the fact that their clinical importance has diminished dramatically in recent years. The present limited use of sulfonamides is due to the allergic side effects that were already evident several decades ago. For trimethoprim, there has been a steep increase in resistance.

For both drugs the mechanisms of resistance and its spread among pathogenic bacteria reveals a remarkable adaptation to the presence of these antibacterial agents. In the case of sulfonamides, laboratory experiments showed that spontaneous mutations to drug resistance always exacted a trade-off price in the form of a less efficient target enzyme dihydropteroate synthase, that would cause counter selection of its host in the absence of the drug. In sulfonamide-resistant clinical isolates this price seems to be discounted, however, in that compensatory changes in the target enzyme make it as efficient as its wild-type counterpart. Further studies of this phenomenon could be an inroad to the understanding of evolutionary adaptation, which is most important for judging reversibility of resistance and for assessing the future of antibacterial agents in general. Trimethoprim resistance in clinical samples of pathogenic bacteria is most commonly mediated by cassette-borne genes expressing drug-resistant variations of the target enzyme, dihydrofolate reductase. A better understanding of the diverse origins of these genes, and their horizontal transfer as integron-borne cassettes could shed light on the important question of how antibiotic resistance integrons have originated and developed.

Sulfonamides might be forced back into clinical use by the general increase in antibiotic resistance, and then with a better understanding and vigilance regarding the allergic side effects. The selective effect of trimethoprim could possibly be developed further in derivatives like epiroprim and iclaprim mentioned above. That could be a parallel to those new analogs of pyrimethamine and cycloguanil that show inhibitory effect on the dihydrofolate reductase from drug resistant mutants of *Plasmodium falciparum* (95).

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Section D
Fungal Drug Resistance – Mechanisms

Chapter 24
Mechanisms of Action and Resistance of Antimycobacterial Agents

Petros C. Karakousis

1 Introduction

Historically, mycobacterial infections have been associated with significant morbidity and mortality worldwide. In particular, *Mycobacterium tuberculosis* is a highly successful human pathogen, infecting one-third of the world's population and leading to approximately 3 million deaths worldwide annually (1). The organism is unique in its ability to establish persistent infection, requiring prolonged treatment with antimicrobials in order to achieve clinical cure. In general, the goals of antituberculosis therapy include rapid reduction of the massive numbers of actively multiplying bacilli in the diseased host, prevention of acquired drug resistance, and sterilization of infected host tissues to prevent clinical relapse. In order to achieve these goals, currently accepted guidelines recommend administration of multiple active drugs for a minimum duration of 6 months (2). In areas where drug resistance is prevalent and resources permit, *M. tuberculosis* clinical isolates should be routinely tested for susceptibility to first-line antituberculosis agents in order to optimally guide therapy.

Prior to the advent of highly active antiretroviral therapy (HAART), disseminated infection with *M. avium* complex was the most common bacterial opportunistic infection in adults infected with HIV-1 in the developed world, occurring annually in 10–20% of individuals with AIDS (3, 4). The availability of HAART, as well as the use of effective prophylaxis with azithromycin or clarithromycin, has reduced the annual incidence of disseminated *M. avium* complex infection among individuals with advanced HIV disease to less than 1% per year (5). Nevertheless, *M. avium* complex continues to cause disseminated disease in persons with HIV, and patients with advanced immunosuppression who are not receiving or are unable to tolerate HAART. In addition, *M. avium* complex is an important cause of pulmonary infection, particularly in HIV-negative persons with underlying lung disease or other immunosuppression (6). Infections with other mycobacteria, including *M. kansasii*, *M. genavense*, *M. hemophilum*, *M. fortuitum*, *M. xenopi,* and *M. chelonae*, have been reported with increasing frequency, particularly in the setting of HIV infection (7–9).

This chapter will review the mechanisms of action and resistance of the antimycobacterial agents, with emphasis on the four first-line antituberculosis drugs: isoniazid, rifampin, pyrazinamide, and ethambutol. The mechanisms of the action of other drugs used to treat mycobacterial infections, including the fluoroquinolones, the aminoglycosides, and the macrolides, will be reviewed elsewhere in this book. This chapter will focus on specific mutations associated with resistance to antimycobacterial agents in *M. tuberculosis* and *M. avium* complex. It includes mechanistic studies carried out in *M. smegmatis*, which, because of its relatedness to *M. tuberculosis*, its fast-growing nature and lack of pathogenicity, and its relative genetic tractablility, is widely used as a model system to study mycobacterial physiology. The phenomenon of *M. tuberculosis* phenotypic drug resistance will not be addressed in this section, and discussion will be limited to genotypic mechanisms of drug resistance.

2 Isoniazid

Isoniazid (isonicotinic acid hydrazide, INH) has been the most commonly used drug in the armamentarium against *M. tuberculosis* since recognition of its clinical activity by Robitzek and Selikoff in 1952 (10). Consisting of a pyridine ring and a hydrazide group (see Fig. 1), INH is a nicotinamide analog, structurally related to the anti-tuberculosis drugs ethionamide and pyrazinamide (11). Because of its significant potency and bactericidal activity, it has become a critical component of first-line antituberculous chemotherapy. However, in the last two decades, resistance to INH has been reported with increasing frequency, ranging from 3% to as high as 25% of all *M. tuberculosis* isolates from previously

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Fig. 1 Structures of first-line TB drugs. Chemical structures were provided by Dr. Ying Zhang, Johns Hopkins Bloomberg School of Public Health

Isoniazid

Pyrazinamide

Rifampin

Streptomycin

Ethambutol

untreated individuals (12–16) with the highest rates of resistance reported from southeast Asia and the Russian Federation (15, 17).

2.1 Mechanism of Action

Despite the widespread use of INH for more than half a century, its mechanism of action has only recently begun to be elucidated. The drug appears to penetrate host cells readily (18, 19) and diffuses across the *M. tuberculosis* membrane (20, 21). INH is a pro-drug, requiring oxidative activation by the *M. tuberculosis katG*-encoded catalase-peroxidase enzyme (22). The resulting isonicotinoyl radical reacts nonenzymatically with oxidized NAD^+ to generate several different 4-isonicotinoyl-NAD adducts (23). Although the active metabolites of INH have been reported to inhibit multiple essential cellular pathways, including synthesis of nucleic acids (24) and phospholipids (25) and NAD metabolism (26, 27), the primary pathway inhibited by the drug appears to be the synthesis of mycolic acids (28–30) manifesting as a loss of acid-fast staining of the organisms following INH treatment (31).

Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids, which are unique outer cell-wall components of mycobacteria and other Actinomycetales (32). Mycolic acids are covalently attached to arabinogalactan and, together with other lipids of the outer leaflet, constitute a very hydrophobic barrier (33) responsible for resistance to certain drugs (34). Disruption of this hydrophobic barrier is believed to result in a loss of cellular integrity (35). INH interrupts mycolic acid synthesis by binding tightly to the NADH-dependent enoyl acyl carrier protein (ACP) reductase InhA (36) a component of the fatty acid synthase II system of mycobacteria, which is essential for fatty acid elongation (37). Genetic, biochemical, and structural data provide compelling evidence that InhA is the primary target for INH in the mycolic acid synthesis pathway. When transferred on a multicopy plasmid, the wild-type *inhA* gene of *M. tuberculosis* or *M. smegmatis* confers INH resistance to *M. smegmatis* and *M. bovis* BCG (36) as well as to *M. tuberculosis* (38). A missense mutation within the mycobacterial *inhA* gene leading to the amino acid substitution S94A, confers INH resistance to *M. smegmatis* (36) and *M. bovis* (39). In addition, the same single point mutation in *inhA* (S94A) was sufficient to cause fivefold increased resistance to INH and inhibition of mycolic acid biosynthesis in *M. tuberculosis* (40). Interestingly, overexpression of or mutation within *inhA* also confers resistance to the structurally related second-line antituberculosis drug ethionamide in *M. tuberculosis*,

Table 1 Mechanisms of action and resistance of the antimycobacterial agents

Drug/drug class	Cellular process inhibited	Drug target	Resistance mutations in clinical isolates	Frequency	Comments
Isoniazid (INH)	Mycolic acid synthesis	InhA	*katG* (S315T)	50–80%	INH is a pro-drug requiring activation by the *M. tuberculosis* catalase-peroxidase KatG (22)
			inhA	15–34%	
Rifampin	mRNA synthesis	RNA polymerase β subunit	*rpoB* (codons 507–533)	>90%	>90% of rifampin-resistant isolates are also resistant to INH
Pyrazinamide (PZA)	Depletion of membrane energy	Unknown	*pncA*	70–90%	PZA is a pro-drug requiring activation by *M. tuberculosis* pyrazinamidase, which is encoded by *pncA* (133)
Ethambutol	Arabinogalactan synthesis	EmbB	*embB*	50–70%	*embB* mutations may not be sufficient to confer resistance to EMB (174)
Streptomycin	Translation	30S ribosomal subunit	*rpsL* (codons 43 and 88)	~50%	Cross-resistance may not be observed with kanamycin or amikacin
			rrs	~20%	
Amikacin/Kanamycin	Translation	30S ribosomal subunit	*rrs* (codon 1400)		Cross-resistance is observed with capreomycin, but not with streptomycin
Fluoroquinolones	DNA synthesis and transcription	DNA gyrase	*gyrA gyrB*	42–85%	Cross-resistance is generally observed among the fluoroquinolones
Macrolides	Translation	50S ribosomal subunit	23S rRNA gene (Domain V loop)		Mechanisms of action and resistance listed are for *M. avium* complex; *M. tuberculosis* is inherently resistant to the macrolides
Ethionamide	Mycolic acid synthesis	InhA	*ethA inhA*		Ethionamide is a pro-drug requiring activation by the monooxygenase EthA (248, 262)
Capreomycin	Translation	16S rRNA	*rrs*		Cross-resistance is observed with kanamycin/amikacin
Cycloserine	Peptidoglycan synthesis	AlrA Ddl	*alrA*		Mechanisms of resistance have been shown in *M. smegmatis*, but not in *M. tuberculosis*
Paraaminosalicylic acid (PAS)	Folic acid biosynthesis iron uptake?	Unknown	*thyA*		The mechanisms of action and resistance for PAS remain poorly characterized

M. smegmatis, and *M. bovis*, suggesting that *inhA* encodes the target of both INH and ethionamide in these mycobacteria (36). In addition, enoyl reductases, and specifically mycobacterial InhA, have been shown to be targets for the widely used topical disinfectant triclosan, and particular *M. smegmatis* mutants in *inhA* are cross-resistant to INH and triclosan (41). However, although it affects InhA function, INH does not directly interact with InhA. Biochemical and structural studies have shown that InhA catalyzes the NADH-specific reduction of 2-trans-enoyl-ACP, and that the INH-resistant phenotype of the S94A mutant InhA is related to reduced NADH binding (37, 42). X-ray crystallographic and mass spectrometry data revealed that the activated form of INH covalently attaches to the nicotinamide ring of NAD bound within the active site of InhA, causing NADH to dissociate from InhA (42, 43).

Although inhibition of DNA synthesis by INH had been observed long ago (24) only recently was a mechanism of action for this phenomenon proposed. Argyrou and colleagues cloned and overexpressed the *M. tuberculosis* gene encoding dihydrofolate reductase (DHFR) *dfrA* in *M. smegmatis*, and demonstrated a twofold increase in MIC (44). *M. tuberculosis* DHFR was shown to selectively bind and co-crystallize with an active INH metabolite, which is distinct from that which binds InhA (43). However, this work requires further biochemical and genetic confirmation. Mutations in *dfrA* have yet to be reported among INH-resistant clinical isolates of *M. tuberculosis*.

Despite the identification of specific cellular targets in the last 15 years, the precise mechanism by which INH kills *M. tuberculosis* remains elusive. Interestingly, depletion of mycolic acids does not necessarily result in loss of viability in other mycobacteria *in vitro* (45, 46). However, inhibition of mycolic acid synthesis may more severely compromise the intracellular survival of *M. tuberculosis in vivo*. It remains to be shown that inhibition of mycolic acid synthesis is both necessary and sufficient for the highly potent *in vivo* bactericidal activity of INH against *M. tuberculosis*.

2.2 Mechanisms of Drug Resistance

Spontaneous INH resistance may be observed at a rate of 10^{-6} per bacterium per generation in *M. tuberculosis* cultures grown *in vitro* (47). Because INH is the most commonly used antituberculosis drug, resistance to INH occurs more frequently among clinical isolates than resistance to any other agent (48). INH resistance varies geographically (49) and may be as high as 20–30% in some parts of the world (15, 17). Mutations are most commonly detected in the *katG* gene, occurring in 50–80% of INH-resistant clinical isolates, or in the *inhA* gene, accounting for 15–34% of INH resistance (48). Depending on the mutation, the degree of INH resistance may vary from low (0.2 μg/mL) to high (100 μg/mL) (50).

2.3 katG

INH resistance among clinical isolates of *M. tuberculosis* has long been associated with loss of catalase and peroxidase enzyme activities (51). In general, there is a strong inverse correlation between the degree of INH resistance and catalase activity (52). Zhang and colleagues first demonstrated that deficiency in *katG*, which encodes the *M. tuberculosis* catalase-peroxidase enzyme, accounts for the observed resistance to INH in drug-resistant clinical isolates of *M. tuberculosis* (22, 53). Mutations in *katG* reduce the ability of the enzyme to activate the pro-drug INH, thus leading to resistance. The *M. tuberculosis katG* gene is situated in a highly variable and unstable region of the genome, perhaps because of the presence of repetitive DNA sequences (54) thereby potentially predisposing to a high frequency of *katG* mutations. Point mutations in *katG* are more common than deletions in INH-resistant clinical isolates, and a single point mutation resulting in substitution of threonine for serine at residue 315 (S315T) accounts for the majority of INH-resistant clinical isolates (55–57). The S315T mutation is associated with a 50% reduction in catalase and peroxidase activity, and with high-level INH resistance (MIC = 5–10 μg/mL) (52, 58). The recent availability of the crystal structure for *M. tuberculosis* KatG (59) has provided greater insight into the process of INH activation, and may permit a more accurate interpretation of the structural and functional effects of mutations implicated in causing INH resistance in clinical isolates.

2.4 inhA

INH resistance may arise either from mutations in *inhA*, resulting in reduced affinity of the enzyme for NADH without affecting its enoyl reductase activity (60) or in the promoter region of the *mabAinhA* operon (57), resulting in overexpression of the wild-type enzyme. Mutations in the *mabA* promoter region appear to be more frequent, but overexpression of MabA alone does not confer INH resistance in mycobacteria (61). Unlike mutations in *katG*, which can confer low-level or high-level INH resistance, depending on the extent to which catalase-peroxidase enzyme activity is affected, mutations in *inhA* or in the promoter region of its operon usually confer low-level resistance (MIC = 0.2–1 μg/mL) (62).

2.5 Other Genes

With respect to INH resistance, the role of mutations in *kasA*, which encodes a β-ketoacyl ACP synthase of the type II fatty acid synthase system, is controversial. Initial reports identified an association between clinical INH resistance and four independent mutations in *kasA* (63) but subsequent studies reported the presence of three of these mutations in INH-sensitive *M. tuberculosis* strains (64, 65). In addition, although one group reported a fivefold increase in the MIC of INH following *kasA* overexpression in *M. tuberculosis* (66) another group found that overexpression of *kasA* conferred resistance to thiolactomycin, a known KasA inhibitor, but no increased resistance to INH in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* (38). Using radioactive INH, Mdluli and colleagues reported KasA to be covalently associated with INH and ACP in *M. tuberculosis* (63) but Kremer and colleagues used anti-KasA antibodies to show that INH treatment in mycobacteria does not result in significant KasA sequestering, and also demonstrated, in an *in vitro* assay, that activated INH does not inhibit KasA activity (67). Although the preponderance of evidence suggests that InhA, and not KasA, is the primary target of INH in the mycolic acid synthesis pathway, the role of *kasA* mutations in INH resistance of clinical *M. tuberculosis* isolates requires further investigation.

Mutations in *ndh*, encoding a NADH dehydrogenase, were first shown to confer resistance to INH and ethionamide in *M. smegmatis*, as well as to exhibit other phenotypes, including thermosensitive lethality and auxotrophy (68). Subsequently, *ndh* mutations were detected in almost 10% of INH-resistant *M. tuberculosis* clinical isolates, which did not contain mutations in *katG*, *inhA*, or *kasA* (64). Defective NADH dehydrogenase, which normally oxidizes NADH and transfers electrons to quinones of the respiratory chain, could lead to an increased ratio of NADH/NAD, which may interfere with KatG-mediated peroxidation of the drug, or displace the INH/NAD adduct from the InhA active site (68).

Mutations in the promoter region of *ahpC*, leading to overexpression of an alkylhydroperoxide reductase, have

been observed in INH-resistant *M. tuberculosis* (69). Although rarely found in some INH-resistant strains with apparently intact KatG (70), the *ahpC* mutation is usually found in KatG-negative INH-resistant *M. tuberculosis*, presumably as a compensatory mechanism for the loss of catalase-peroxidase activity in such strains (71–73). AhpC does not appear to play a direct role in INH resistance, because *ahpC* overexpression in a wild-type reference strain of *M. tuberculosis* does not appreciably increase the MIC of INH, but mutations in the *ahpC* promoter region may serve as a useful marker for detection of INH resistance (70).

Despite the identification of several genetic mutations associated with resistance to INH, as many as a quarter of all clinical INH-resistant isolates do not have mutations in any of the above genes, suggesting alternative mechanisms of INH resistance.

3 Rifampin and Other Rifamycins

The rifamycins were first isolated in 1957 from *Amycolatopsis* (formerly *Streptomyces*) *mediterranei* as part of an antibiotic screening program in Italy (74). Their discovery and widespread use has revolutionized antituberculosis therapy, allowing for the reduction of the duration of treatment from 18 months to 9 months (75). Although the early bactericidal activity of the rifamycins is inferior to that of INH (76–78), the former are the most potent sterilizing agents available in TB chemotherapy, continuing to kill persistent tubercle bacilli throughout the duration of therapy (79, 80). Rifampin is a broad-spectrum antibiotic and the most widely used rifamycin to treat tuberculosis. Rifabutin, another rifamycin with reduced induction of the hepatic cytochrome P-450 enzyme system, was originally shown to be effective for the prophylaxis (81) and treatment (82) of *M. avium-intracellulare* complex infection in persons with advanced HIV disease. Rifapentine is a rifamycin with favorable pharmacokinetic properties, including substantially greater maximum serum concentration and extended half-life, which permits highly intermittent therapy for HIV-negative patients who do not have cavitation on chest radiograph, and who are sputum culture-negative after 2 months of therapy (83).

3.1 Mechanism of Action

The rifamycins are characterized by a unique chemical structure consisting of an aromatic nucleus linked on both sides by an aliphatic bridge (see Fig. 1) (84). Although structural changes at positions C-21, C-23, C-8, or C-1 may significantly reduce microbiological activity, modifications at C-3 do not alter antituberculous activity. Rifampin is a 3-formyl derivative of rifamycin S; rifabutin is a spiropiperidyl derivative of rifamaycin S; and rifapentine is a cyclopentyl-substituted rifampin (84).

The rifamycins are highly protein-bound in plasma, but easily diffuse across the *M. tuberculosis* cell membrane due to their lipophilic nature (62). The bactericidal activity of the rifamycins has been attributed to their ability to inhibit mRNA synthesis by binding with high affinity to bacterial DNA-dependent RNA polymerase (85). The core structure of RNA polymerase comprising the subunits $\alpha_2\beta\beta'\omega$ is evolutionarily conserved among prokaryotes (86), explaining the antimicrobial activity of the rifamycins against a broad range of bacteria. X-ray crystallographic data examining the interaction of rifampin and RNA polymerase from *Thermus aquaticus* revealed that rifampin exerts its effect by binding in a pocket between two structural domains of the RNA polymerase β subunit and directly blocking the path of the elongating RNA transcript at the 5′ end beyond the second or third nucleotide (87).

Although the molecular target of rifampin has been well characterized, the precise mechanism by which this interaction leads to mycobacterial killing remains unclear. Interestingly, transcriptional inhibition of the toxin-antitoxin *mazEF* module by rifampin was shown to trigger programmed cell death in *Escherichia coli* by reducing cellular levels of the labile antitoxic protein MazE, allowing the unrestrained lethal action of the long-lived toxic protein MazF (88). Although *M. tuberculosis* contains homologous toxin-antitoxin gene modules (89, 90) it appears that these modules may play a role in *M. tuberculosis* growth arrest and persistence under adverse conditions, rather than in programmed cell death, as originally suggested (90).

3.2 Mechanism of Resistance

Although resistance to INH alone is common in *M. tuberculosis*, resistance to rifampin alone is rare, and more than 90% of rifampin-resistant isolates are also resistant to INH. Therefore, rifampin resistance has been used as a surrogate marker for multidrug-resistant tuberculosis (91). In *M. tuberculosis*, resistance to rifampin develops in a single step, at a frequency of 10^{-7} to 10^{-8} organisms (92).

As in *E. coli* (93–95), resistance to rifampin in *M. tuberculosis* arises from mutations in *rpoB*, which encodes the β-subunit of RNA polymerase (96). Over 90% of rifampin-resistant clinical isolates contain point mutations clustered in an 81-base pair region between codons 507 and 533 of the *rpoB* gene (97, 98). Although at least 35 distinct *rpoB* mutant allelic variants have been described (97), amino acid

substitutions at one of two positions (Ser_{531} and His_{526}) account for the great majority of mutations conferring clinical resistance to rifampin (96, 98–100). Consistent with the clinical data, selection of spontaneous rifampin resistance *in vitro* in the *M. tuberculosis* laboratory reference strain H37Rv yields *rpoB* mutations only at Ser_{531} and $His_{526,}$ with the Ser_{531} Leu mutation predominating (101). Strains with the point mutations CAC→TAC (His→Tyr) at codon 526 and TCG→TTG (Ser→Leu) at codon 531 account for 30% and 25%, respectively, of rifampin-resistant clinical isolates in the US (99) while the same mutations represent 12% and 47%, respectively, of predominantly foreign rifampin-resistant isolates (96), suggesting that there may be geographic variation in the frequency of occurrence of particular *rpoB* mutations (97). Unlike mutations in codons 531 and 526, which confer high-level resistance to rifampin (MIC > 32 µg/mL) and cross-resistance to all rifamycins (62) mutations in codons 511, 516, and 522 are associated with low- or high-level resistance to rifampin and rifapentine (MIC 2–32 µg/mL), but preservation of susceptibility to rifabutin and the new rifamycin, rifalazil (102–104). Rare mutations in *M. tuberculosis* have also been reported in the 5′ region of the *rpoB* gene, and one such mutation at V176F confers intermediate- to high-level resistance to rifampin (105, 106).

Several fast-growing strains of mycobacteria, including *M. smegmatis*, *M. chelonae*, *M. flavescens*, and *M. vaccae*, are able to inactivate rifampin by ribosylation, leading to inherent resistance to this antibiotic (107, 108). However, this mechanism of rifampin resistance has not been described in *M. tuberculosis*. Nevertheless, a small percentage of rifampin-resistant isolates (<5%) do not contain any mutations in the *rpoB* gene, suggesting additional molecular mechanisms of rifampin resistance in *M. tuberculosis*, such as altered rifampin permeability or mutations in other RNA polymerase subunits (97).

4 Pyrazinamide

The use of pyrazinamide (PZA) in combination with rifampin in modern antituberculosis regimens has permitted shortening the duration of therapy from the previous 9–12 months to the current 6 months (109). Although its bactericidal activity is inferior to that of isoniazid and rifampin (110) the reduction of relapse rates associated with the addition of PZA in 6-month regimens is attributed to the drug's unique ability to target semi-dormant populations of bacilli residing within an acidic environment (111). Consistent with this hypothesis, the drug was shown to be more active against old, non-growing tubercle bacilli than against young, actively replicating organisms (112). Interestingly, despite its established activity *in vivo* (113–116) PZA is inactive against *M. tuberculosis* grown under normal conditions *in vitro* (117) and requires acidification of the medium pH to demonstrate antituberculosis activity (118).

4.1 Mechanism of Action

PZA is an amide derivative of pyrazine-2-carboxylic acid and a nicotinamide analog (see Fig. 1) (119). Despite recognition of its antituberculosis activity more than half a century ago (113), the mechanism of action of PZA remains poorly understood. Because of the strict requirement for an acidic micro-environment, it was originally hypothesized that the site of action of PZA was in the macrophage phagolysosome (120) where intracellular *M. tuberculosis* resides. However, the interior pH of these organelles may be neutral or only slightly acidic (121, 122), well above the range where PZA is active (123). In addition, although older studies suggested otherwise (120, 124), more recent studies have demonstrated that PZA has neither bacteriostatic nor bactericidal activity against intracellular *M. tuberculosis* in human monocyte-derived macrophages (125). An alternative hypothesis is that PZA acts against bacilli residing in acidified compartments of the lung that are present during the early inflammatory stages of infection (111), which is consistent with the clinical observation that the potent sterilizing activity of PZA is limited to the first 2 months of therapy (126–128). Anaerobic and microaerophilic conditions *in vitro* have been shown to enhance the activity of PZA against *M. tuberculosis*, suggesting an alternative explanation for the higher sterilizing activity of PZA against *in vivo* organisms residing within oxygen-deprived granulomas, as compared to bacilli grown under *in vitro* conditions, with ambient oxygen tension (129).

PZA enters *M. tuberculosis* through passive diffusion and via an ATP-dependent transport system (130). The drug accumulates intracellularly because of an inefficient efflux system unique to *M. tuberculosis* (131). Similar to INH, PZA is a pro-drug, which requires activation to its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PZase) (132, 133). The uptake and accumulation of POA in *M. tuberculosis* is enhanced when the extracellular pH is acidic (131). The inhibitory effects of POA accumulation were initially attributed to direct inhibition of the mycobacterial fatty acid synthase I (FAS-I) enzyme (134), which is responsible for *de novo* synthesis of C_{16} fatty acids from acetyl-CoA primers and their elongation to C_{24-26} fatty acyl-CoA derivatives (135, 136). However, subsequent studies showed that, although the PZA analog 5-chloro-pyrazinamide

irreversibly inhibits fatty acid synthesis through inhibition of FAS-I, POA does not directly inhibit purified mycobacterial FAS-I, suggesting that the enzyme is not the immediate target of PZA (137). It has been proposed that the antituberculosis activity of PZA is not attributable to inhibition of a specific cellular target, but rather may reflect disruption of the proton motive force required for essential membrane transport functions by POA at acidic pH (138). These findings could explain the enhanced susceptibility to PZA of old, non-replicating bacilli, which have a relatively low membrane potential (131) and reduced ability to maintain membrane energetics (139) as compared to young, actively replicating organisms (138). Alternatively, the accumulation of POA or other weak organic acids may lower the intracellular pH sufficiently to inactivate FAS-I or other vital enzymes required for cellular metabolism (137).

4.2 Mechanisms of Resistance

It has been known for some time that PZA resistance in *M. tuberculosis* is associated with loss of PZase activity (132). More recently, pyrazinamide resistance has been attributed to mutations in *pncA*, the gene encoding PZase (133). Consistent with these findings, integration of wild-type *pncA* into a pyrazinamide-resistant *pncA* mutant of *M. tuberculosis* is sufficient to restore susceptibility to PZA (140). *M. bovis*, another member of the *M. tuberculosis* complex, is inherently resistant to PZA, most frequently because of a point mutation at codon 169 of the *pncA* gene, which renders the enzyme nonfunctional (141). In contrast, studies of PZA-resistant clinical isolates of *M. tuberculosis* revealed that 72–97% of these strains may contain various missense mutations, insertions, deletions, or termination mutations throughout the *pncA* gene or its promoter (142–144).

A small percentage of isolates with high-level PZA resistance contain no mutations in *pncA* or its promoter, suggesting other potential mechanisms of resistance to the drug (142) including, perhaps, deficient uptake (130), enhanced efflux, or altered *pncA* regulation. Alternately, these findings may reflect the intrinsic problems associated with PZA susceptibility testing, as PZA resistance may be reported erroneously when the culture medium contains excessive bovine serum albumin or a high inoculum of *M. tuberculosis*, as both of these conditions may raise the pH of the medium and falsely elevate the MIC of the drug (112). In fact, reliable methods for susceptibility testing of PZA have only recently been developed, using media with slightly higher pH (6.0–6.2) and higher concentrations of PZA (ranging from 300 μg/mL to as high as 1,200 μg/mL, depending on the culture broth) (145).

5 Ethambutol

Ethambutol (EMB; dextro-2,2′-(ethylenediimino)-di-1-butanol), a synthetic compound structurally similar to D-arabinose (see Fig. 1) (146) was initially reported to have antituberculosis activity in 1961 (147). In addition to its role as a first-line agent against *M. tuberculosis*, EMB is an important component of combination therapy against *M. avium* complex (5) and the drug exhibits activity against other mycobacteria, including *M. kansasii*, *M. xenopi*, and *M. marinum* (148). EMB kills only actively multiplying bacilli (149), although its early bactericidal activity is not as potent as that of INH (150, 151). EMB has poor sterilizing activity, as its addition to a regimen of INH, rifampin, and streptomycin does not improve culture conversion rates after 2 months of therapy (152) and its substitution for PZA increases clinical relapse rates (153). Because of its modest contribution to the standard regimen of INH, rifampin, and PZA, the principal role of EMB is in the empiric treatment of individuals who are deemed at increased risk for harboring INH-resistant or multidrug-resistant *M. tuberculosis*, until drug susceptibility results become available.

5.1 Mechanism of Action

The mechanism of action of EMB remains incompletely understood. EMB has been reported to inhibit numerous mycobacterial cellular pathways, including RNA metabolism (149, 154), transfer of mycolic acids into the cell wall (155), phospholipid synthesis (156, 157), and spermidine biosynthesis (158). However, the primary pathway affected by EMB appears to be that of arabinogalactan biosynthesis (159) through inhibition of cell wall arabinan polymerization (160).

Initial studies showed that treatment of *M. smegmatis* with EMB results in rapid bacterial disaggregation and morphological changes, consistent with alterations in cell wall composition (161). A potential explanation for this phenomenon was provided by the observation that EMB inhibits transfer of mycolic acids to the cell wall in *M. smegmatis* (155), leading to rapid accumulation of trehalose monomycolate, trehalose dimycolate, and free mycolic acids in the culture medium (162). Subsequently, EMB was shown to inhibit arabinogalactan synthesis, as MIC levels of the drug immediately inhibited the transfer of label from D-[14C]glucose into the D-arabinose residue of arabinogalactan in EMB-susceptible *M. smegmatis*, but not in a drug-resistant strain (159). In addition to inhibiting the synthesis of the arabinan component of the mycobacterial cell wall core polymer arabinogalactan, EMB inhibits biosynthesis of the arabinan of

lipoarabinomannan, a lipoglycan noncovalently associated with the cell envelope (163, 164). The observations that the latter effect is delayed relative to the former (160) and that EMB treatment results in rapid accumulation of β-D-arabinofuranosyl-1-monophosphoryldecaprenol (decaprenol phosphoarabinose) (165), an intermediate in arabinan biosynthesis, suggested that the primary site of EMB action is not on *de novo* synthesis of D-arabinose or on its activation, but rather in the final polymerization steps (160).

Using target overexpression by a plasmid vector as a selection tool, Belanger et al. demonstrated that the translationally coupled *embA* and *embB* genes of *M. avium* are both necessary and sufficient to render a naturally susceptible *M. smegmatis* strain resistant to EMB (166). Subsequently, the *embCAB* gene cluster encoding the homologous arabinosyl transferase enzymes EmbA, EmbB, and EmbC was cloned, sequenced, and characterized in *M. tuberculosis* (167). Although it has been proposed that these genes constitute an operon, there is evidence to suggest that the *embB* gene can be expressed from a unique promoter (168), the location of which remains unknown. The Emb proteins are thought to be integral membrane proteins with 12 transmembrane domains and a large carboxyl-terminal globular region of approximately 375 amino acids, with a predicted nocytoplasmic location (167, 169). Genetic and biochemical studies have shown that the EmbA and EmbB proteins are involved in the formation of the proper terminal hexaarabinofuranoside motif during arabinogalactan synthesis (170), while EmbC is involved in lipoarabinomannan synthesis (171). As the majority of EMB-resistant clinical isolates contain mutations in *embB* (see below) (167, 169, 172), the EmbB protein has been proposed as the main target of EMB, although X-ray crystallographic data supporting this interaction are lacking.

5.2 Mechanisms of Resistance

In *M. smegmatis*, high-level resistance to EMB appears to require multiple steps, including overexpression of the Emb proteins, as well as mutations in the conserved region of EmbB, or further increases in protein expression levels (167). Resistance to EMB in *M. tuberculosis* is usually associated with point mutations in the *embCAB* operon, commonly involving amino acid substitutions at codon Met306 of the *embB* gene (167, 169, 172). EmbB mutations have been identified in 47–69% of EMB-resistant isolates of *M. tuberculosis* (167, 172). Mutations in the *embB* gene were reported to be associated with high-level EMB resistance (173), with the mutations Met306Leu or Met306Val yielding a higher MIC (40 μg/mL) than the Met306Ile substitution (20 μg/mL) (172). However, a study of 183 epidemiologically unlinked *M. tuberculosis* isolates collected in St. Petersburg, Russia, detected the presence of *embB* mutations at codon 306 in not only 48% of EMB-resistant isolates, but also in 31% of EMB-susceptible isolates, suggesting that *embB* mutations may not be sufficient to confer resistance to EMB, or the presence of a compensatory mutation that reverses the EMB-resistance phenotype of *embB* mutants (174). Interestingly, the discrepancy in phenotypic and genotypic EMB resistance tests was restricted to strains already resistant to other antituberculosis drugs; specifically, *embB* mutations at codon 306 were noted in 40 of the 69 (60%) EMB-susceptible strains resistant to isoniazid, rifampin, and streptomycin, but in none of the 43 pan-susceptible strains (174).

Nucleotide polymorphisms in the *embC-embA* intergenic region have been reported in association with resistance-associated amino acid replacements in EmbA or EmbB, suggesting that these intergenic mutations represent secondary or compensatory changes (169). Other potential mutations involved in EMB resistance include a Gln379Arg replacement in *M. tuberculosis embR*, a homologue of the synonymous gene encoding a putative transcriptional activator of *embAB* in *M. avium* (166), as well as mutations in *rmlD* and *rmlA2*, which encode proteins involved in rhamnose modification (169). In addition, mutations associated with EMB resistance have been described in *Rv0340* (169), a gene transcribed in the same orientation and upstream of the *iniBAC* operon, which is significantly upregulated following exposure to EMB *in vitro* (175). As many as one quarter of all EMB-resistant *M. tuberculosis* isolates do not harbor mutations in any of the genes described above, suggesting alternative mechanisms of EMB resistance.

6 Aminoglycosides

The discovery of streptomycin (see Fig. 1) in the early 1940s represented the first breakthrough in the chemotherapy of tuberculosis (176). Patients treated with streptomycin and bed rest improved initially compared to those assigned to bed rest alone, but streptomycin monotherapy inevitably led to relapses with streptomycin-resistant *M. tuberculosis* (177). Although relapse rates are comparable when streptomycin is substituted for ethambutol as the fourth drug in addition to INH, rifampin, and PZA, the poor oral absorption of streptomycin, which necessitates parenteral administration, as well as the toxicity profile of the aminoglycosides have favored the use of ethambutol in first-line antituberculosis therapy (178). Other aminoglycosides with significant antimycobacterial activity include kanamycin and amikacin (179). The detailed mechanisms of action of the aminoglycosides will be addressed elsewhere, and this section will cover mechanisms of aminoglycoside resistance identified specifically in *M. tuberculosis*.

As in other bacteria, the mode of action of the aminoglycosides against mycobacterial species is through their binding to the 30S ribosomal subunit, which affects polypeptide synthesis, and ultimately results in inhibition of translation (180). In clinically relevant bacteria, resistance to the aminoglycosides most often results from modification of the aminoglycoside molecule. Although genes encoding aminoglycoside-modifying enzymes have been identified in the chromosome of slow-growing mycobacteria (181, 182) and disruption of aminoglycoside 2′-N-acetyltransferase genes has been correlated with increased aminoglycoside susceptibility in *M. smegmatis* (183), this mechanism of resistance has not been described for *M. tuberculosis* (92). Instead, resistance to streptomycin and the other aminoglycosides in *M. tuberculosis* usually develops by mutation of the ribosome target binding sites. Interestingly, although cross-resistance is observed between amikacin and kanamycin (184), these drugs are not cross-resistant with streptomycin (185), suggesting distinct mechanisms of resistance. Amikacin is a derivative of kanamycin and the two drugs are structurally related, each containing a 2-deoxystreptamine moiety, while streptomycin is structurally distinct, containing a streptidine moiety. High-level resistance to amikacin and kanamycin, with preserved susceptibility to streptomycin, has been reported in *M. abscessus* and *M. chelonae* (186) and in *M. tuberculosis* (187) in association with a point mutation at position 1,400 (corresponding to position 1,408 in *E. coli*) of the *rrs* gene, which encodes 16S rRNA (185, 186). On the other hand, streptomycin resistance in mycobacteria is most commonly associated with mutations in the *rpsL* gene, which encodes the ribosomal protein S12 (188–193). Specifically, a missense mutation resulting in the substitution of an arginine for a lysine at codon 43, as well as point mutations in codon 88, account for the majority of *rpsL* mutations in *M. tuberculosis* (193). As in *E. coli*, streptomycin resistance in *M. tuberculosis* also commonly arises from *rrs* mutations, which are usually clustered in the regions surrounding nucleotides 530 or 912 (188, 189, 194). Unlike most other bacteria, which have multiple copies of the *rrs* gene, *M. tuberculosis* and other slow-growing mycobacteria have a single copy of the gene, making it an easily selected mutation site. Thus, alterations in the drug target arising from reduced association of the 16S rRNA with the S12 ribosomal protein lead to an inability of aminoglycosides to disrupt translation of mycobacterial mRNA, thereby resulting in antibiotic resistance. Mutations in *rpsL* and *rrs*, which occur in about 50% and 20%, respectively, of streptomycin-resistant *M. tuberculosis* clinical isolates, are usually associated with intermediate-resistance (MIC 64–512 μg/mL) or high-level resistance (MIC > 1,000 μg/mL) (92). The mechanisms responsible for streptomycin resistance in other *M. tuberculosis* isolates, particularly those with low-level resistance (MIC 4–32 μg/mL), are unknown but may involve changes in cell envelope permeability and diminished drug uptake (188, 190).

7 Fluoroquinolones

The fluoroquinolones demonstrate excellent activity against several mycobacterial species, including *M. tuberculosis*, *M. kansasii*, and *M. fortuitum*, but not against others, such as *M. avium*, *M. marinum*, *M. chelonae*, and *M. abscessus* (195). In particular, drugs of the fluoroquinolone class are highly active against *M. tuberculosis,* both *in vitro* (196, 197) and in animal models (198–200). In descending order of activity, fluoroquinolones active against *M. tuberculosis* include moxifloxacin, sparfloxacin, levofloxacin, ofloxacin, and ciprofloxacin (201). The 8-methoxy-fluoroquinolone moxifloxacin has bactericidal activity similar to that of INH against *M. tuberculosis*, both *in vitro* and in the murine model of TB (200, 202, 203), as well as early bactericidal activity comparable to INH in patients with pulmonary TB (204–206). Unlike gatifloxacin, which appears to lack sterilizing activity against stationary-phase cultures of *M. tuberculosis* (207), moxifloxacin, when substituted for INH, is able to shorten the duration of therapy needed to effect stable cure in murine TB (208, 209), suggesting that the drug has significant sterilizing activity. Until recently, the fluoroquinolones have been recommended primarily as second-line agents in the treatment of multidrug-resistant tuberculosis (2). However, the use of a fluoroquinolone as the only active agent in a failing regimen for treatment of multidrug-resistant *M. tuberculosis* constitutes the most frequent cause of fluoroquinolone resistance (201). Resistance to fluoroquinolones also may arise extremely rapidly following use of these drugs for other infections (210, 211). Despite the widespread use of fluoroquinolones to treat a variety of bacterial infections, fluoroquinolone resistance is detected in fewer than 2% of *M. tuberculosis* isolates in the United States and Canada (212). Because of its potent bactericidal and sterilizing activities, moxifloxacin is currently under investigation as a first-line agent in the treatment of tuberculosis. The mechanism of action and detailed mechanisms of resistance to this class of drugs will be discussed in another chapter, and this section will highlight specific mutations identified in fluoroquinolone-resistant *M. tuberculosis*.

Fluoroquinolones exert their powerful antibacterial activity by trapping gyrase and topoisomerase IV on DNA as ternary complexes, and blocking the movement of replication forks and transcription complexes (213). Unlike most other bacterial species, *M. tuberculosis* lacks topoisomerase IV, but does contain the genes *gyrA* and *gyrB*, which encode the A and B subunits, respectively, of DNA

gyrase (181). Consequently, fluoroquinolone resistance in *M. tuberculosis* is most commonly associated with mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*, conserved regions involved in the interaction between the drug and DNA gyrase (201). Spontaneous fluoroquinolone resistance develops in laboratory strains of *M. tuberculosis* at frequencies of 2×10^{-6} to 1×10^{-8} (214). The most frequent mutations associated with high-level fluoroquinolone resistance involve substitutions at codons 88, 90, 91, and 94 of the *gyrA* gene (215–217). Mutations in the QRDR of the *gyrB* gene in the absence of *gyrA* mutations have been identified in some laboratory isolates (218, 219) but are extremely rare in fluoroquinolone-resistant *M. tuberculosis* clinical isolates (220). The degree of resistance to fluoroquinolones depends on the specific amino acid substitution in the QRDR, and on the number of resistance mutations present. Therefore, while single mutations in *gyrA* may confer low-level resistance (MIC > 2 μg/mL) (218), high-level resistance to fluoroquinolones usually requires a stepwise process of at least two mutations in *gyrA*, or a combination of mutations in *gyrA* and *gyrB* (216, 218). Mutations in the QRDR of *gyrA* do not occur following exposure of *M. tuberculosis* to low concentrations of fluoroquinolones *in vitro*, and the selection pressure for mutants in *gyrA* increases when *M. tuberculosis* is exposed to high concentrations of fluoroquinolones *in vitro* (219). However, mutations in the QRDR region of *gyrA* are identified in only 42–85% of fluoroquinolone-resistant clinical isolates, suggesting alternative mechanisms of resistance. The *lfrA* gene, which encodes a multidrug efflux pump, has been shown to confer low-level resistance to fluoroquinolones when expressed on multicopy plasmids in *M. smegmatis* (221, 222). Furthermore, expression of MfpA, a member of the pentapeptide repeat family of bacterial proteins (223), which includes McbG in *E. coli* and Qnr in *K. pneumoniae*, confers low-level resistance (4- to 8-fold increase in the MIC) in *M. smegmatis* to ciprofloxacin and sparfloxacin (224). Fluoroquinolone resistance related to MfpA has been attributed to DNA mimicry, as MfpA can directly bind to and inhibit DNA gyrase, thus preventing the formation of the DNA gyrase-DNA complex required for fluoroquinolone binding (225). High-level resistance of *M. smegmatis* to ciprofloxacin (MIC = 64.2 μg/mL) also has been associated with overexpression and chromosomal amplification of the *pstB* gene, which encodes a putative ATPase subunit of the phosphate-specific transport (Pst) system, and disruption of this gene in *M. smegmatis* results in a twofold increase in sensitivity to fluoroquinolones relative to the isogenic wild-type strain (226). Although homologues of *lfrA*, *mfpA*, and *pstB* appear to be present in *M. tuberculosis* (181), mutations or amplifications of these genes have not been identified in fluoroquinolone-resistant clinical isolates.

8 Macrolides

Clinical outcomes of patients with AIDS and disseminated *M. avium* complex have improved substantially since the introduction of the extended-spectrum macrolides, which are now considered the cornerstone of any potent regimen (227–229). However, combination therapy with at least one other antimycobacterial agent, usually ethambutol, is necessary to prevent the emergence of macrolide resistance (230–232). Although clarithromycin and azithromycin are both effective against disseminated *M. avium* complex infection, several studies directly comparing these two drugs when used in combination with ethambutol suggest trends toward more rapid clearance of bacteremia with clarithromycin (230, 231). The mechanism of action of the macrolide antibiotics will be covered elsewhere in this book, and this section will focus on known macrolide resistance mutations occurring in *M. avium* complex.

The macrolides exert their antibacterial effect by binding to the bacterial 50S ribosomal subunit and inhibiting RNA-dependent protein synthesis (233). However, these drugs have limited activity against wild-type *M. tuberculosis* (234). This intrinsic resistance is believed to be associated with expression of the *erm* (37) gene (235, 236), which is induced upon exposure of *M. tuberculosis* to clarithromycin (237). Interestingly, disruption of the *pks12* gene, which encodes a polyketide synthase required for synthesis of the major cell wall lipid dimycocerosyl phthiocerol, results in increased susceptibility of *M. tuberculosis* to clarithromycin relative to its parent strain, but no change in susceptibility to ciprofloxacin or penicillin (238).

In *M. avium*, spontaneous resistance to clarithromycin has been estimated to occur at a rate of 10^{-8} to 10^{-9} organisms (239, 240). Clarithromycin resistance in *M. avium* isolated from patients with pulmonary disease has been associated with point mutations in the generally conserved loop of domain V of 23S rRNA (241) corresponding to position 2058 in *E. coli* 23S rRNA, which confer resistance to erythromycin and the macrolides-lincosamide-streptogramin B antibiotics (242). Similarly, clarithromycin-resistant *M. avium* isolates obtained from patients with AIDS and disseminated *M. avium* infection contained point mutations in the domain V sequences of 23S rRNA at position 2274 (243). Mutations in the *M. avium* 23S rRNA gene are associated with high-level resistance (MIC ≥ 128 μg/mL) (244). As in *M. avium*, clarithromycin resistance in *M. chelonae* and *M. abscessus* has been associated with point mutations in the 23S rRNA peptidyltransferase region at positions 2058 or 2059 in strains with a single chromosomal copy of the rRNA operon (245). However, a few clarithromycin-resistant *M. avium* isolates, particularly with low-level resistance, have been described in which no mutation can be identified in the peptidyltransferase region of the 23S rRNA (240, 246), suggesting alternative mechanisms of drug resistance.

8.1 Cross-Resistance of Antimycobacterial Agents

In general, there is low cross-resistance among most antituberculosis drugs. When present, the degree of cross-resistance depends on the particular mutations and mechanism of drug resistance. Although the most commonly observed INH-resistance mutations (i.e., those involving *katG*) do not generate cross-resistance to other agents, mutations in *inhA* itself, or in its promoter region, confer resistance to the second-line antituberculosis drug ethionamide (36, 38, 247). Mutations in *ethA*, which confer ethionamide resistance, also yield cross-resistance to thiacetazone and thiocarlide (248).

Mutations in the *rpoB* gene of *M. tuberculosis*, particularly in codons Ser531 and His526, have been associated with high-level resistance (MIC > 32 µg/mL) to rifampin and cross-resistance to all the rifamycins. On the other hand, the *rpoB* mutations L511P, D516Y, D516V, or S522L, which are associated with low- to high-level resistance to rifampin and rifapentine, do not significantly alter susceptibility to rifabutin (MIC 0.5 µg/mL) or rifalazil (MIC 0.01–0.04 µg/mL) (104, 249). In one study of 25 rifampin-resistant *M. tuberculosis* isolates (MIC > 2 µg/mL), 3 of these isolates (12%) retained susceptibility to rifabutin (250). Another study of 112 *M. tuberculosis* clinical isolates detected 73% cross-resistance between rifabutin and rifampin (251), suggesting that rifabutin may have a role in the therapy of multidrug-resistant tuberculosis in cases where the isolate retains susceptibility to rifabutin.

Cross-resistance among the aminoglycosides is variable. Thus, cross-resistance is usually seen between the 2-deoxystreptamine aminoglycosides, amikacin and kanamycin (187) but not between these two drugs and the streptidine aminoglycoside streptomycin (185). In addition, cross-resistance may be observed between kanamycin and capreomycin or viomycin (252, 253). Although cross-resistance has not been reported between fluoroquinolones and other classes of antituberculosis agents, mutations associated with individual fluoroquinolone resistance appear to confer cross-resistance to the entire class of drugs (201). Similarly, resistance to clarithromycin or azithromycin in *M. avium* complex is usually associated with class-wide resistance to the macrolides (234, 243).

8.2 Mechanism of Spread of Resistance

Although drug resistance may be spread by plasmids or transposons among many bacterial species, including the fast-growing *M. fortuitum* (262), these mobile genetic elements are not known to cause drug resistance in *M. tuberculosis* (92). As described above, drug resistance in *M. tuberculosis* is caused by mutations in specific chromosomal genes. In general, genetic resistance of *M. tuberculosis* to specific antimycobacterial drugs does not alter the fitness or virulence of the organism (92), suggesting that drug-resistant isolates may spread to previously uninfected individuals and cause disease equivalent to that caused by drug-susceptible isolates. One notable exception to this rule is in the case of certain INH-resistant *M. tuberculosis* isolates with reduced catalase activity, which demonstrate decreased virulence in the guinea pig model of tuberculosis (51). Reduced catalase activity in these isolates correlates well with increased INH resistance, as well as decreased virulence (92). Molecular genetic studies have shown that integration of a functional *katG* gene into the genome of INH-resistant, catalase-defective *M. bovis* restores INH susceptibility as well as virulence in the guinea pig model (39). Consistent with these findings, KatG-deficient *M. tuberculosis* is attenuated relative to a wild-type strain during infection of immunocompetent mice and mouse-derived macrophages, as a result of exposure to the peroxides generated by the phagocyte NADPH oxidase (254). Although *M. tuberculosis* clinical isolates containing the S315T mutation appear to retain full virulence and transmissibility in humans (255), it is unknown if other *katG* mutants, with more greatly reduced catalase activity, are less transmissible or virulent in humans. On the other hand, restoration of virulence may be associated with promoter-up mutations in the *ahpC* gene, which may compensate for loss of catalase activity resulting from mutations in *katG* (71). Full transmissibility and virulence are expected among *M. tuberculosis* strains in which INH resistance is mediated by mutations in genes other than *katG*, such as *inhA* or *ndh* (92).

The efficient spread of drug-resistant isolates certainly may occur from person to person, as evidenced by the ecologically successful W strain. This strain, which is resistant to as many as 11 antimycobacterial drugs, caused a multidrug-resistant outbreak of tuberculosis in New York City and spread across the United States (256). However, the emergence of drug resistance in a particular individual is most often not due to primary infection with a drug-resistant isolate, but rather a result of human error. Thus, a prior history of tuberculosis and antituberculosis therapy has been implicated strongly in the causation of multidrug-resistant *M. tuberculosis* (257). Factors associated with acquisition of drug resistance include incomplete and inadequate treatment, such as the use of a single drug to treat tuberculosis, the addition of a single drug to a failing regimen, and the failure to identify preexisting resistance, as well as inadequate treatment adherence on the part of the patient (258).

8.3 Alternative Agents

M. tuberculosis strains that are resistant to either isoniazid or rifampin may be treated effectively with other first-line drugs. However, strains that are resistant to both drugs, termed "multidrug-resistant" strains, require the use of "second-line drugs", which are generally less effective and more toxic (257). These drugs include ethionamide, capreomycin, cycloserine, and paraaminosalicylic acid. Promising new antituberculosis drugs, such as the nitroimidazole PA-824, are currently being tested in clinical trials. Known mechanisms of action and resistance for each of these drugs will be discussed briefly in this section.

Ethionamide, a synthetic compound structurally related to INH, was shown to have antituberculosis activity in the late 1950s (259). Although less potent than INH, ethionamide also inhibits mycolic acid synthesis (36, 260). Ethionamide is a pro-drug, requiring activation by the monooxygenase EthA (248, 261, 262), which itself is negatively regulated by the transcriptional repressor EthR (261). Similar to INH, ethionamide inhibits mycolic acid synthesis by binding the ACP reductase InhA (36). Using a cell-based activation method, Wang et al. recently showed that the thioamide drugs, ethionamide and prothionamide, form covalent adducts with NAD, which are tight-binding inhibitors of *M. tuberculosis* and *M. leprae* InhA (263). Approximately three-quarters of *M. tuberculosis* isolates with high-level ethionamide resistance (MIC > 50 µg/mL) have mutations in *ethA* or *inhA* (247).

Although often grouped together with the aminoglycosides because of similar activity and toxicities, capreomycin is a macrocyclic polypeptide antibiotic isolated from *Streptomyces capreolus* (179). Like streptomycin and kanamycin, capreomycin inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA (62). In *M. smegmatis*, mutations in *vicA* and *vicB*, which encode components of the 50S and 30S ribosomal subunits, confer resistance to capreomycin and viomycin (252, 253). In *M. tuberculosis*, mutations in the *rrs* gene encoding 16S rRNA have been associated with resistance to capreomycin as well as kanamycin (187, 264).

Cycloserine interrupts peptidoglycan synthesis by inhibiting the enzymes D-alanine racemase (AlrA) and D-alanine:D-alanine ligase (Ddl) (265, 266). Overexpression of *M. tuberculosis* AlrA and Ddl on a multicopy vector results in resistance to D-cycloserine in *M. smegmatis* and *M. bovis* BCG (265, 266) and *M. smegmatis alrA* mutants lacking D-alanine racemase activity display hypersusceptibility to D-cycloserine (267). In *E. coli*, cycloserine resistance has been attributed to mutations in *cycA*, which encodes a permease responsible for uptake of the drug (268) but such a mechanism of resistance has not been described for mycobacteria. In addition, mutations in a gene homologous to that encoding *E. coli* penicillin-binding protein 4 (PBP4) were shown to confer resistance to D-cycloserine, as well as to vancomycin in *M. smegmatis* (269). However, the mechanism of cycloserine resistance in *M. tuberculosis* remains unknown.

Paraaminosalicylic acid (PAS) was introduced in 1945 (270, 271). Although its activity was inferior to that of streptomycin when used alone, the combination of PAS with streptomycin significantly reduced the emergence of streptomycin-resistant organisms (272). The mechanisms of action and resistance to PAS have not been well characterized, but it has been suggested that the drug may inhibit folic acid biosynthesis and uptake of iron (62). Recently, PAS-resistant transposon mutants of *M. bovis* BCG were found to harbor insertions in the *thyA* gene, which encodes the enzyme thimidylate synthesis in the folate biosynthesis pathway (273). In addition, mutations in the *thyA* gene resulting in diminished enzymatic activity were identified in PAS-resistant *M. tuberculosis* clinical isolates, suggesting that PAS may act as a folate antagonist and that *thyA* mutations may mediate clinical PAS resistance (273).

PA-824, a small molecule nitroimidazopyran related to metronidazole, was recently shown to have bactericidal activity against replicating and static *M. tuberculosis* cultures *in vitro*, as well as in murine and guinea pig models of tuberculosis (274). In the mouse model, PA-824 has bactericidal activity comparable to that of INH (275, 276). However, unlike INH, but like metronidazole, the drug also has potent activity against nonreplicating bacilli exposed to microaerophilic conditions (274, 275). In addition, PA-824 is highly active against multidrug-resistant clinical isolates of *M. tuberculosis* (MIC < 1 µg/mL), suggesting no cross-resistance with current antituberculosis drugs (275). Like metronidazole, PA-824 is a pro-drug that requires bioreductive activation of an aromatic nitro group in order to exert an antitubercular effect (274). Although the precise mechanism by which PA-824 exerts its lethal effect is unknown, the drug appears to inhibit the oxidation of hydroxymycolates to ketomycolates, a terminal step in mycolic acid synthesis (274). Similar to INH, resistance to PA-824 is most commonly mediated by mutations that lead to loss of pro-drug activation. Mutations in *fgd1* and *fbiC* result in the loss of a specific glucose-6-phosphate dehydrogenase and its deaza flavin cofactor F_{420}, respectively, which together provide electrons for the reductive activation of PA-824 (277). In addition, resistance to PA-824 has been associated with mutations in *Rv3547*, a gene encoding a conserved hypothetical protein that appears to be involved in PA-824 activation (277). Among laboratory strains, the frequency of resistance to PA-824 is slightly less than that to INH, approximately 9.0×10^{-7} (274).

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Chapter 25
Amphotericin B: Polyene Resistance Mechanisms

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1 Introduction

The polyene antibiotics represent a class of biologically active fungal metabolites isolated from the genus *Streptomyces*, an aerobic actinomycete obtained from soil (1). While more than one hundred polyene antibiotics have been described, amphotericin B and nystatin are the two agents most commonly used to treat fungal and some protozoal infections in humans (see Fig. 1). Amphotericin B is active against most pathogenic fungi in humans, and for over 40 years has been the cornerstone of therapy for critically ill patients with invasive fungal infections. Nystatin is generally used to treat mucosal *Candida* infections.

Resistance to amphotericin B is still rare. Resistance has become more evident recently, because of the increase in the rate of non-albicans *Candida* species and emerging invasive mould infections that have intrinsic or acquired resistance to azoles and polyenes. Non-albicans candidemia now accounts for 30–60% of all candidemias (2, 3). Resistance can be categorized into three main categories: primary or intrinsic, acquired, and clinical resistance. Intrinsic or primary resistance occurs without exposure to anti-fungals. Acquired or secondary resistance develops during treatment, and often occurs as a result of one or several genetic mutations (4). Intrinsic resistance to amphotericin B is rare among pathogenic fungi infecting humans, and acquired resistance during therapy is even less common (5, 6). Although polyene resistance has not been a major clinical problem to date, polyene-resistant yeasts and moulds continue to be reported (9). Identification of a particular yeast or mould to the species level helps to predict possible polyene resistance, and can be extremely important to help guide the choice of antifungal therapy. Clinical resistance, i.e., failure of anti-fungal therapy, is multifactorial, and depends on a variety of factors, such as the immune status of the host, pharmacokinetics of the antifungal agent, and the species of infecting fungus. In many instances, resistance to amphotericin B may not be related to the minimum inhibitory concentration (MIC), but to failure of the antifungal agent to penetrate into infected tissue (7).

It must be emphasized that the true rate of amphotericin B resistance is not known (8, 9). Detection of resistance in fungi can be technically difficult, and current testing methods may not be sensitive enough to detect resistance to amphotericin B (10–12, 13). The minimum inhibitory concentration (MIC) of amphotericin B can vary depending upon the test format, type of media, and the fungal species being tested. The Clinical Laboratory Standards Institute (CLSI) has developed a standardized broth dilution methodology for in vitro susceptibility testing of *Candida* species against amphotericin B, flucytosine, fluconazole, and itraconazole (16). This method cannot always distinguish between amphotericin B-susceptible and amphotericin B-resistant isolates due to the narrow range of MIC values that is generated. Limitations with the current methodologies have precluded the establishment of interpretative MIC breakpoints for amphotericin B for yeasts and moulds. Antibiotic medium-3 and E-test strips have been reported to enhance detection of fungal strains with diminished susceptibility to amphotericin B, because a broader range of MIC values can be generated (17, 10, 18). Prior studies have suggested that minimum fungicidal concentration (MFC) may be a better measure for detecting fungicidal activity in vivo and in vitro (14, 15). An evaluation of different in vitro susceptibility test formats for amphotericin B against *Candida* spp.– i.e., broth microdilution using different media, E-test, and MFC – did not generate results that correlated with therapeutic success or failure (19).

There is a narrow range of MIC values (0.06 ~ 2 µg/mL) for amphotericin B against *Candida* species; therefore, a one-dilution shift in a breakpoint can greatly alter how susceptibility or resistance is reported (20). *Candida* spp. with MIC > 1 µg/mL are considered resistant to amphotericin

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Fig. 1 Structure of some polyenes commonly used in clinical practice

Amphotericin B

Nystatin

Nystatin A_1

Pimaricin

B (16). Time-kill assays show that the time required for fungicidal activity for amphotericin B is species-dependent. In a recent study, the time required for fungicidal activity was fastest for *C. albicans*, and the time increased respectively for the following species: *C. lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis* (21).

1.1 Epidemiology of Polyene Resistance

Amphotericin B resistance is rare, but has been reported in some *Candida* species, as well as *Cryptococcus, Trichosporon, Aspergillus, Scedosporium*, and *Fusarium* species (9). Although amphotericin B-resistant strains of *C. albicans* (defined as an

MIC ≥ 2 μg/mL) have been reported, amphotericin B resistance is more common in non-albicans species (22). Resistance is seen in a small but significant percentage of *Candida* species: *C. lusitaniae* (5–20%), *C. rugosa* (5–20%), *C. krusei* (10–15%), and *C. guilliermondii* (5–10%) (2). Ostrosky-Zeichner et al. reported a 2–3% rate of resistance to amphotericin B (defined as MIC > 1.0 μg/mL) in *C. parapsilosis* and *C. krusei* isolates in a surveillance study of *Candida* blood stream isolates in the USA (23). *C. lusitaniae* and *C. guilliermondii* are known for inherent or rapid acquisition of amphotericin B resistance (2, 24, 25). *C. glabrata* and *C. krusei* are generally susceptible to amphotericin B, but they tend to have higher MICs than *C. albican*s. A small proportion of isolates of both species have been found to be resistant to amphotericin B with MIC ≥ 2 μg/mL (26). Pfaller et al. reported *C. glabrata* with amphotericin B MIC ≥ 2 μg/mL in less than 1% of USA isolates, and in 4.4% of European isolates (27). *Trichosporon* species, for example *T. asahii* (formerly *T. beigelii*), are generally resistant to amphotericin B; isolates may be inhibited, but are not killed by achievable serum levels of amphotericin B (28).

An in vitro susceptibility study of 100 *Aspergillus* species against amphotericin B demonstrated that 67% of the isolates had an MIC ≥ 2 μg/mL, and 90% had an MIC ≤ 4 μg/mL (15). *A. fumigatus*, *A. flavus, A. nidulans, A. niger* are generally susceptible to amphotericin B, but resistance has been reported (9). *A. terreus* (MIC range: 1 to > 4 μg/mL) is inherently resistant to amphotericin B (29, 30). *Scedosporium apiospermum* (MIC range: 1 to >16 μg/mL), *Pseudallescheria boydii* (MIC range: 1 to ≥16 μg/mL), some strains of *Sporothrix schenkii* (MIC range: 0.5 to 4 μg/mL), and some *Fusarium* species (MIC range: 1 to > 4 μg/mL) have variable resistance to amphotericin B (9, 31, 32, 33, 34). *Scedosporium prolificans* (MIC range: 4 > 16 μg/mL) is another emerging infectious dematiaceous mould that is usually resistant to amphotericin B (35, 36). The dimorphic fungi, *Histoplasma*, *Coccidioides*, and *Blastomyces* are generally susceptible to amphotericin B, and have MIC values that range from 0.5 to 1.0 μg/mL. Zygomycetes are typically susceptible to amphotericin B (MIC range: 0.5–2.0 μg/mL) (37, 33, 38, 39).

1.2 Emergence of Polyene Resistance

Emergence of resistance during amphotericin B therapy is an uncommon phenomenon (9). The fungal pathogen may acquire resistance, or the patient may become infected with a different species intrinsically resistant to amphotericin B. Amphotericin B is often used as empiric therapy for neutropenic fever, and yeast isolates from patients undergoing myelosuppressive chemotherapy or hematopoietic stem cell transplantation have been reported to have significantly higher MICs to amphotericin B than colonizing isolates from immunocompetent patients (42, 43). Dannaoui et al. investigated the emergence of antifungal resistance in 200 sequential isolates of *A. fumigatus* from immunocompromised patients on antifungal therapy, and found that resistance was rare (44).

Strains of *C. albicans* acquiring resistance to amphotericin B or amphotericin B plus azoles have been isolated from patients receiving treatment with these antifungals (45, 46). Nolte et al. reported two cases of candidemia in leukemia patients, which were caused by fluconazole and amphotericin B-resistant isolates of *C. albicans*. The patients had received empiric therapy with both of these antifungals (45). A cluster of cases of *C. rugosa* candidemia, reported from Brazil, were refractory to amphotericin B therapy and associated with a high mortality; two patients had received prior therapy with amphotericin B (47).

An association between in vitro decreased susceptibility to amphotericin B in *Candida* species isolated from severely immunocompromised patients with candidemia and subsequent poor clinical outcome has been reported. Bloodstream isolates of *C. albicans* with MIC > 0.8 μg/mL were associated with a high mortality in severely immunocompromised patients (42). Infection with polyene-resistant isolates of *C. lusitaniae* and *C. guilliermondii* has been described in patients who received amphotericin B therapy (46, 48). Cross-resistance to azoles and polyenes has been reported in *Cryptococcus neoformans* in HIV-infected patients following several courses of azoles, or azoles plus amphotericin B (49). For *Aspergillus* spp. and other moulds, there is little data on the ability of MICs to predict clinical outcome. In a study of 29 patients with hematological malignancies, infected with *A. flavus* 41% (12), *A. fumigatus* 28% (8), and *A. terreus* 31% (9), infection with an *Aspergillus* species with MIC ≥ 2 μg/mL was associated with a high mortality rate. All patients infected with *A. terreus* (MIC ≥ 2 μg/mL) died (50).

2 Mechanism of Action of Amphotericin B

Amphotericin B acts mainly at the plasma membrane, and impairs membrane barrier function. Susceptibility to polyenes depends on membrane structure, including sterols and other components such as phospholipids (51). Sterols are essential components of eukaryotic cells, and ergosterol is the principal sterol in the fungal cell membrane. Similar to mammalian cholesterol, ergosterol serves as a bio-regulator of membrane fluidity, and of membrane integrity and permeability. Ergosterol also has a role in active growth phases of fungal cells (52). Amphotericin B and nystatin bind to ergosterol present in the cell membrane of susceptible fungi, and also bind to cholesterol in human cells, but they bind more avidly to ergosterol-containing membranes than to cholesterol-containing membranes (53). Amphotericin

B has toxic effects on mammalian cells. It has been shown that in the presence of serum, amphotericin B binding is not limited to membrane-binding, but also to binding with low-density lipoprotein (LDL) receptors. These toxic effects may be due to its capacity to modify or weaken the structure of LDLs by an oxidative process (54).

Ergosterol and its biosynthetic pathway are the two major targets for polyene antibiotics. The antifungal effects of amphotericin B are believed to be by two primary mechanisms: an increase in permeation by binding sterols in cellular membranes, and a pro-oxidant effect causing oxidative damage in target fungal cells. The type and quality of sterols in fungal cell membranes also influences the level of interaction between the cells and polyenes. The interaction leads to increased cell permeability, and sometimes to cell death. The latter effect does not always follow changes in cell permeability, and is probably affected by a separate process involving oxidative damage (55, 56).

2.1 Interaction with Sterols in the Fungal Cell Membrane

The most widely accepted model for the mechanism of action of amphotericin B was proposed in the early 1970s (57–59) (see Fig. 2). Interaction of amphotericin B with sterols causes the formation of transmembrane pores or channels that cause disruption of normal membrane function. Polyene antibiotics were one of the first model systems used to study transmembrane ionic channel structures. Amphotericin B binds to membrane ergosterol, which results in the production of aqueous pores. These pores consist of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols forming the staves in a barrel-like structure, with a hydrophilic interior and a hydrophobic exterior. The hydrophilic channel has a diameter of approximately 8 Å (60, 59, 58).

The formation of membrane pores or channels causes altered membrane permeability, leakage of potassium ions, and of other vital cytoplasmic components, leading to membrane disruption, and possible fungal cell death. To replace potassium loss, a subsequent transfer of hydrogen ions from the environment follows. The subsequent inflow of protons causes acidification of the fungal cytoplasm, which results in precipitation of cytoplasmic components (61, 62).

Leakage of potassium ions does not always result in fungal cell killing (63). In yeasts, increased permeability to small ions has been observed at low concentrations of amphotericin B (0.02–0.1 μg/mL), and cell lysis and death

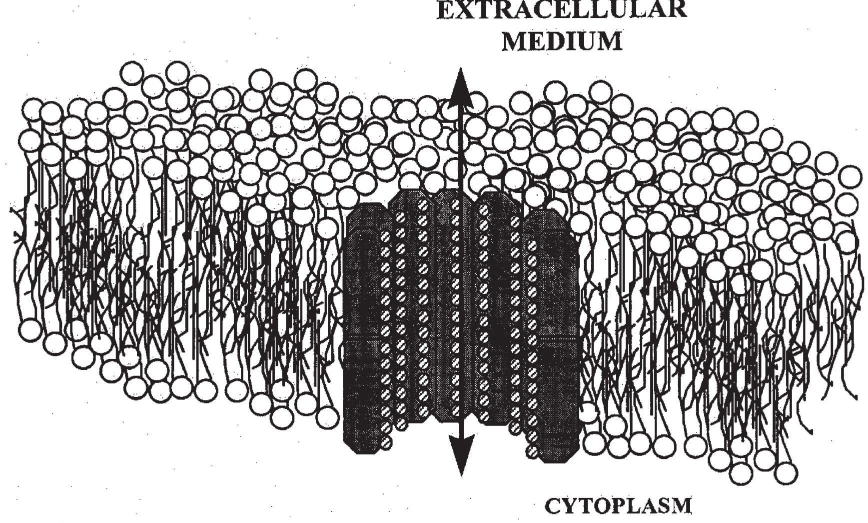

Fig. 2 Schematic representation of the interaction between amphotericin B and cholesterol in a phospholipid bilayer reproduced with permission from Ghannoum *et al.*, reference 81. **A.** Membrane pore. **B.** Molecular orientation in an amphotericin B–cholesterol pore. The short dashed lines represent hydrogen bonds between amphotericin B and cholesterol molecules. The dotted lines between the hydrocarbon chains of phospholipids represent short range van der Waals forces

was observed at higher concentrations of the drug (64, 65). Previous studies on *Saccharomyces cerevisiae* demonstrated that low concentrations of amphotericin B and nystatin and other polyenes caused leakage of potassium ions, and, at high concentrations, caused fungal cell death and red cell hemolysis (66). Different types of channels are formed with selectivity for different ions, and the type and number of channels formed has been shown to be critically dependent upon the concentration of polyene (67, 68).

In *Candida* species, the dose of amphotericin B needed to cause leakage of ions (Na^+, K^+, Cl^-) from the cell membrane is lower than that required to cause cell death, which led to the theory of two separate types of resistance mechanisms (63, 56, 68). There is experimental evidence that amphotericin B has a number of mechanisms of cell disruption. Recent studies on artificial membranes have demonstrated that sterols do promote, but may not be necessary to produce, highly cationic selective amphotericin B channels (69). Osmotic stress has been shown to sensitize sterol-free phospholipid bilayers to the action of amphotericin B, and to enhance the formation of amphotericin B channels in sterol-free egg phosphatidylcholine membranes (70).

2.2 Oxidative Damage to the Fungal Cell Membrane

Membrane permeability changes and membrane disturbances may explain the fungistatic effects of amphotericin B, but does not explain its lethal effects. Evidence from several studies has shown that killing of fungi and lysis of red cells can result from oxidative damage by amphotericin B (71, 72). Amphotericin B-induced oxidative stress on fungal cells may be as important a factor as channel formation in causing cell disruption. Cell membrane damage is due to the formation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, that result in membrane disruption and cell death through membrane lipid peroxidation (73). Defense against oxidative damage is involved in the resistance to amphotericin B (72). Andrews et al. found that antioxidants, e.g., catalase, enhanced the anti-fungal activity of amphotericin B, and postulated that this effect resulted from protection of the amphotericin B molecule from autooxidation, thus enhancing or prolonging the drug's action. In the absence of oxygen, amphotericin B may act as an antioxidant, and therefore as a chain terminator of the peroxidation process, and it may partially protect the fungus against phagocytosis (74).

There is some experimental evidence that amphotericin B may act as an antioxidant. The presence of seven conjugated double bonds in amphotericin B suggests that it is prone to autooxidation. This tendency to autooxidation suggests that amphotericin B could also act like an antioxidant, possibly at low oxygen tensions (75).

3 Mechanisms of Resistance

Resistance to polyenes has developed slowly over time, because the interaction of amphotericin B with the plasma membrane is complex, and multiple changes may be required to prevent disruption of the cell membrane (53, 76). Mechanisms of resistance to polyenes include alterations in membrane sterols, defense mechanisms against oxidative damage, defects in ergosterol biosynthetic genes, factors such as fatty acid composition of the cell membrane, and alterations in sterol to phospholipid ratio. Additionally, the existing ergosterol structure may be reoriented or masked – for example, by sequestration within phagocytes – leading to steric interference between the polyene and ergosterol (76). The growth phase of the fungal cell and changes in cell wall structure are also involved in polyene resistance.

Much of the knowledge of the mechanisms of resistance to polyenes in fungal species has come from studies on mutant isolates of *Saccharomyces cerevisiae*, *Candida*, and *Aspergillus* species generated by exposure to mutagenic agents, or serially passaged in media containing increasing amounts of the polyene (77). The majority of the amphotericin B-resistant yeast characterized so far have quantitative or qualitative alterations in the sterol composition of their cell membranes (78, 79). Efflux mechanisms have not been described to be involved in the development of polyene resistance. The large molecule volume of amphotericin B and its derivatives may inhibit its use as a substrate for efflux pumps in the fungal cell (80).

3.1 Polyene Resistance in Experimentally Induced Mutants and Clinical Isolates

Alterations in the sterol content of the plasma membrane occur in different ways – for example, the total ergosterol content of the fungal cell can be decreased without concomitant changes in the sterol composition. Some or all of the polyene-binding sterols maybe replaced by sterol intermediates, such as fecosterol or episterol, which bind polyenes less well (81).

Genetic alterations in the ergosterol biosynthetic pathway or ERG genes have been shown to decrease sensitivity to polyenes and azoles. A limited number of studies have addressed the genetic basis of polyene resistance. The *ERG3* gene from *S. cerevisiae* has been cloned. The *ERG3* gene

encodes Δ 5, 6 sterol desaturase, which is required for ergosterol biosynthesis. It may not, however, be essential for cell viability (82). Micro-array analysis of experimentally induced *C. albicans* mutants (resistant to amphotericin B and fluconazole) and a wild strain showed that 134 genes were expressed. Cell stress genes and *ERG5*, *ERG6*, *ERG25* were found to be upregulated when differences in the expression of the ERG genes were compared with the wild-type strain. The mutant strains accumulated sterol intermediates such as lanosterol and eburicol, which have a reduced affinity for amphotericin B (83) (see Fig. 3).

Studies in *C. albicans* and *S. cerevisiae* have shown that the *ERG6* gene is not essential for viability, but is very important for the production of ergosterol and for sensitivity to polyenes (84). The *ERG6* gene encodes sterol methyl transferase activity, and ERG6 mutants have altered membrane permeability. Artificially induced ERG11 mutants of *C. albicans* and *S. cerevisiae* have been described to be resistant to amphotericin B, and to accumulate sterol intermediates (85) (see Fig. 3). Young et al. investigated genetic alterations in the ergosterol biosynthetic pathway of *C. lusitaniae*. An ERG6 mutant strain of *C. lusitaniae* was designed to investigate amphotericin B resistance in this species. Amphotericin B-resistant isolates of *C. lusitaniae* were found to have increased levels of *ERG6* transcript, as well as reduced ergosterol content. Further transcript analysis showed that expression of the *ERG3* gene, which encodes C-5 sterol desaturase, was reduced in two of the amphotericin B-resistant isolates. These findings demonstrate that mutation or altered expression of ergosterol biosynthetic genes can result in resistance to amphotericin B in *C. lusitaniae* (24).

Several precursors of ergosterol have been identified as the major sterols in nystatin-resistant mutants of *S. cerevisiae*. A mutant strain resistant to low levels of nystatin was found to contain a 5,6 dihydroergosterol, an immediate precursor of ergosterol (86, 87). *S. cerevisiae* mutants with mutations in the ergosterol synthetic genes, *ERG4*, *ERG6*, and *ERG3*, were shown to lack ergosterol, and were resistant to polyenes (82). Sterols have been shown to be absent in membranes of amphotericin B-resistant *Leishmania donovani* promastigotes (88). Mutant strains of *A. fennelliae* resistant to polyenes had a decreased amount of ergosterol content compared to wild-type strains, and contained metabolic blocks for a dehydrogenation and a reduction step in the biosynthesis of ergosterol (52). In an animal model of *A. terreus* infection, strains with the highest MIC and minimum lethal concentration (MLC) were found to have the lowest ergosterol content (29).

Based on an analysis of sterol composition, some clinical isolates of *C. albicans* may be defective in *ERG2* or *ERG3* genes (94, 89, 90) (see Fig. 3). For example, some *C. albicans* isolates resistant to azoles and polyenes were found to have a low ergosterol content, associated with a defect in the *ERG3* gene (45, 89). Other amphotericin B-resistant *Candida* isolates were unable to form amphotericin B-generated pores in the cell membrane (90). A clinical isolate of *C. glabrata* with decreased susceptibility to polyenes demonstrated lack of ergosterol with a build-up of late sterol intermediates, suggesting a defect in the final step in the ergosterol pathway. Sequencing of *ERG 11*, *ERG 6*, *ERG 5*, and *ERG4* revealed a unique missense mutation in *ERG6*, leading to an amino acid substitution in the corresponding protein (91). Evaluation of a number of polyene-resistant *Candida* species showed that incrementally more resistant isolates possessed principal sterols arising from blockage of the biosynthesis of ergosterol at successively earlier stages. Cultures of *Candida* spp. possessing Δ8-sterols were more resistant to polyenes than those possessing Δ7-sterols, which, in turn, were more resistant than those possessing Δ5,7-sterols (92). In a hematopoietic stem cell transplantation population, polyene resistance was found in 55 *Candida* isolates (*C. albicans*, *C. tropicalis*, and *T. glabrata*) from six neutropenic patients, and resistance in these isolates was associated with loss or reduction of ergosterol in the cell membrane (22).

Clinical isolates and mutant strains of *C. albicans* cross-resistant to azoles and polyene have been shown to accumulate sterol intermediates in the cytoplasmic membrane due to a decrease in 5,6 desaturase activity. The altered membrane sterols pattern may provide a common basis for the dual resistance, by preventing polyene binding and by reducing azole inhibition of ergosterol synthesis (78, 93). Resistance to amphotericin B and azoles in clinical isolates of *Candida* was found to be related to the accumulation of sterol intermediates, 3-β-ergosta-7,22-dienol and 3-β-ergosta-8-dienol, which was associated with a defect in *ERG3* that encodes the Δ5,6 desaturase (45). Kelly et al. compared the susceptibility and sterol pattern of two *Cryptococcus neoformans* isolates (pre- and post-treatment) from an AIDS patient who failed antifungal therapy. These authors observed a correlation between resistance to amphotericin B and the sterol pattern in the cell membrane. The resistant, post-treatment isolate had a defect in the Δ8,7-sterol isomerase, leading to accumulation of ergosta-5, 8,22-dienol, ergosta-8,22-dienol, fecosterol, and ergosta-8-enol. Ergosterol accounted for only 4% of the sterols in the resistant isolates, compared to 75% in the pre-treatment isolates (43).

3.2 Resistance to Oxidation

Defense against oxidative damage is involved in the resistance of *C. albicans* cells to the lethal effects of amphotericin B. Increased levels of intracellular or extracellular catalase, as well as incubation under hypoxic conditions, have been shown to reduce the lethal effects of amphotericin B on *C. albicans* cells and protoplasts (72). Amphotericin

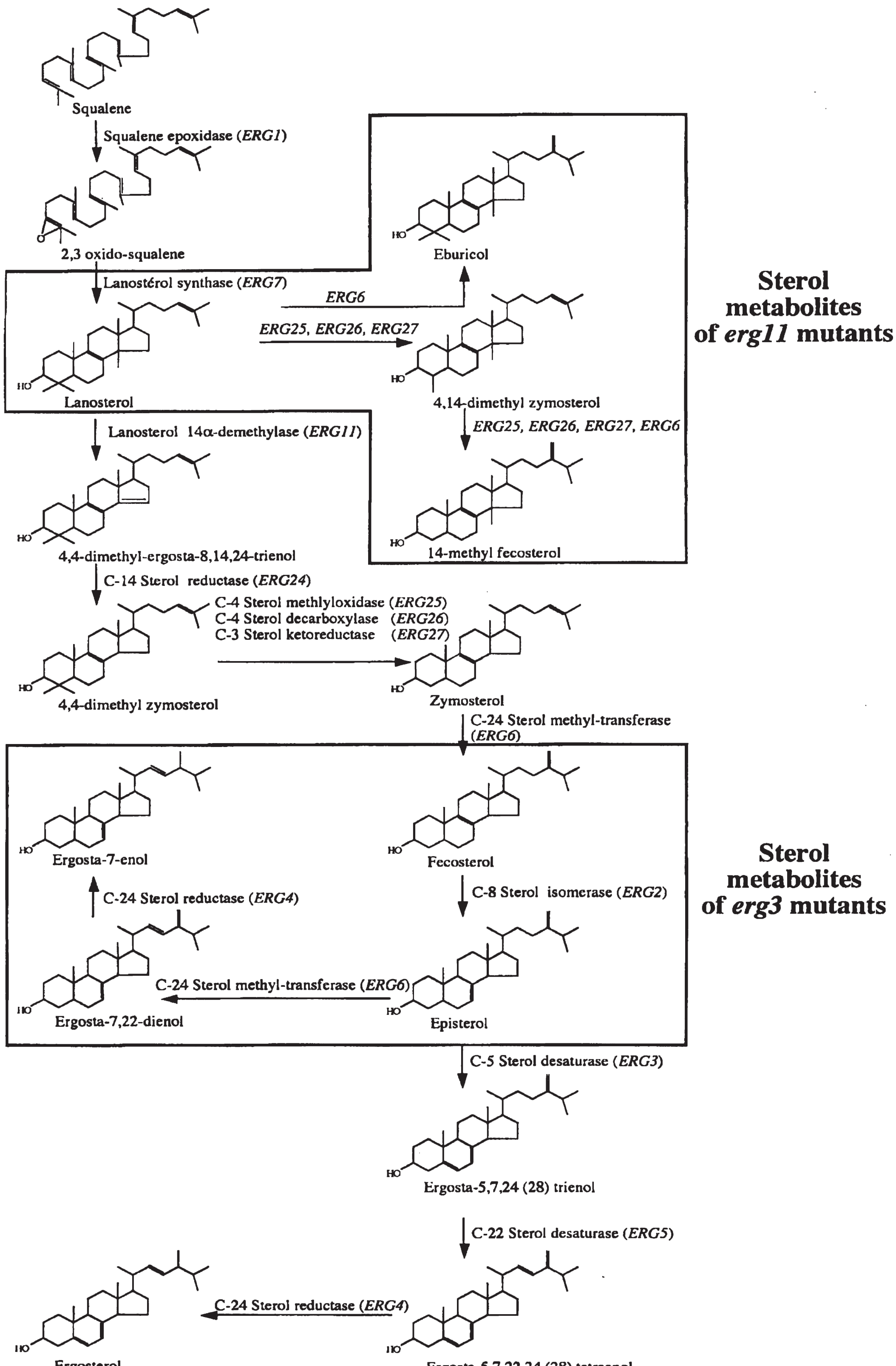

Fig. 3 Schematic of the ergosterol biosynthetic pathway of *C. albicans* reproduced with permission from Sanglard *et al.,* reference 85. The accumulation of ergosterol intermediates (in boxes) related to the disruption in the function of ERG3, and ERG11 are shown

B-induced leakage of potassium was not hindered under hypoxic conditions or in the presence of catalase (56). Further studies on amphotericin B-resistant strains of *C. albicans* demonstrated that these strains were significantly less sensitive to hydrogen peroxide. In the presence of amphotericin B, these resistant strains produced significantly more intracellular and extracellular catalase than controls (72). Catalases are antioxidants, and therefore can remove hydrogen peroxide, a source of hydroxyl radicals, and thus ameliorate oxidative damage. Resistance to amphotericin B may arise from the ability of strains to cope more efficiently with the oxidative stress initiated by amphotericin B through increased catalase activity.

3.3 Biofilm Formation

Candida spp. produce biofilms on biological and inert surfaces. The resistance of *Candida* biofilms to antifungal drugs has been previously documented. The mechanisms by which *Candida* biofilms are resistant are not well understood. One possible resistance mechanism is related to the slow growth rate of biofilm cells (94). Lipid formulations of amphotericin B and echinocandins appear to be more active than triazoles (voriconazole, ravuconazole), fluconazole, and nystatin in experimental *Candida albicans* and *Candida parapsilosis* biofilms (95).

3.4 Fatty Acid Composition

Alteration of sterol content and/or composition is not sufficient to explain polyene resistance. Previous work has shown that the type of sterols and phospholipids in cellular membranes were important in polyene resistance, but did not adequately explain resistance (96). Some polyene-resistant mutants of *C. albicans* have been shown to have altered fatty acid compositions. Pierce et al. measured the phospholipid composition of sensitive and mutant strains of *C. albicans*, and noted a slightly higher proportion of saturated fatty acids in the resistant mutants, compared with the sensitive strains. The proportion of long chain fatty acids was similar (96). Broughton et al. designed, by nitrous acid mutagenesis, amphotericin B-resistant mutants of *C. albicans* that were similar in sterol to the wild type. When the fatty acid composition was examined, there were no significant differences among the major fatty acids compared to the wild type. The authors suggested that an increase in membrane fluidity might confer resistance to amphotericin B. Changes in membrane fluidity were associated with changes in membrane permeability and in cell growth characteristics (97).

3.5 Cell Wall Alterations

Cell wall components may affect the interaction of polyenes with the cytoplasmic membrane. Several authors have observed that some cell wall constituents were involved in the sensitivity or resistance of cells to amphotericin B; for example, low chitin content is associated with increased resistance to amphotericin B in *C. albicans*, *Kluyveromyces* spp., and *Schizosaccharomyces* spp (98, 99). Chitin, an aminopolysaccharide, is an essential structural component of the cell wall, and is usually present in small quantities. Bahmed et al. described two amphotericin B-resistant mutant strains of *Kluyveromyces*. The mutants had an increased amount of chitin in their cell walls. In both mutants, chitinase activity was significantly reduced in comparison with that of the wild-type strain, but no significant change in the chitin synthase enzymes could be detected (99). The precise relationship between amphotericin B resistance and cell wall chitin content remains to be demonstrated.

Hammond et al. demonstrated that polyene resistance in *C. albicans* may be partly determined by binding factors in the cell wall (100). Alterations in the cell wall components of mycelia were shown to lead to resistance in an *A. flavus* mutant. Chemical analysis of the cell wall showed that the level of glucans was higher in resistant mycelia, compared to wild type amphotericin B susceptible strains (51). The precise role of glucans in the cell wall in inhibiting amphotericin B access to ergosterol and in contributing to resistance is poorly understood.

3.6 Yeast Cell Cycle

Ergosterol plays an essential role in the yeast cell cycle. Sterol-starved yeast cells undergo G1 phase arrest, and this can be reversed by adding exogenous ergosterol (84). A study of polyene susceptibility in exponential- and stationary-phase *Candida* cells demonstrated that stationary-phase cells were more resistant than cells in the exponential phase (101). This observation may be associated with reduced chitin synthase activity in the stationary growth phase (98).

4 Conclusions

Polyenes, particularly amphotericin B and its lipid formulations, are drugs of choice for the treatment of a wide range of invasive mycoses. Correlation between polyene resistance in vitro and clinical outcome has been difficult to demonstrate due to host and laboratory factors. With increased use and

availability of different classes of antifungal agents, it is anticipated that there will be an increasing number and variety of fungal species resistant to these agents. Continued efforts to study the mechanisms of antifungal resistance, and the development of experimental systems to study resistance mechanisms, will be important components of a strategy to limit the emergence of polyene and other antifungal drug resistance in the future.

Strategies to overcome polyene resistance would include modifications of existing drugs, development of new classes of antifungal agents, and new treatment strategies, such as combination antifungal therapy. Combination antifungal therapy has been shown to be synergistic in some animal models (102). Combinations of echinocandins with azoles or amphotericin B appear to be the most promising regimens in the clinical setting (103, 104). Optimization of dosing regimens of currently available antifungal drugs for specific infections is important, as is also monitoring of anti-fungal susceptibility patterns and distribution of fungal species (105, 106). Refinement of current in vitro susceptibility testing, establishment of breakpoints, development of molecular tests for detection of resistance, and the establishment of clinical databases to complement information gained from clinical trials are additional important strategies to better understand polyene resistance.

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Chapter 26
Fungal Drug Resistance: Azoles

Jose L. Lopez-Ribot and Thomas F. Patterson

1 Introduction. Azole Antifungal Agents: History, Mode of Action, and Clinical Utility

Azole derivatives represent one of the major groups of antifungal drugs used in clinical practice to treat fungal infections in humans, including skin and vaginal infections in the general population, and more serious life-threatening invasive mycoses in severely immunocompromised patients. Although this new class of antifungal agents was developed in the 1960s and 1970s, the first available azole derivative for the oral treatment of systemic fungal infections, ketoconazole, an imidazole, was released in the early 1980s. A few years later, the introduction of the first-generation triazoles, such as fluconazole and itraconazole, constituted a major advance in the treatment of fungal infections and quickly became the drugs of choice for the treatment of a number of fungal infections, particularly candidiasis (1). The recent introduction of the "new generation" triazoles, including voriconazole, posaconazole, and ravuconazole, at different stages in the development pipeline, represent a welcome addition to the limited arsenal of antifungal agents, mainly due to their increased potency and broader spectrum. Voriconazole and ravuconazole are structurally related to fluconazole, whereas posaconazole bears a close resemblance to itraconazole (1).

The mode of action of azole derivatives is by binding to and inhibiting lanosterol demethylase (Cyp51p or Erg11p), a cytochrome P450 responsible for the 14-α demethylation of lanosterol, thus blocking ergosterol biosynthesis (the major membrane sterol of fungi) and leading to a fungistatic effect in the majority of cases (1, 2). The unhindered nitrogen of the imidazole or triazole ring of azole antifungal agents binds to the heme iron of Erg11p as a sixth ligand, thus inhibiting the enzymatic reaction. The remainder of the azole molecule binds to the apoprotein, in a manner that is dependent upon the individual molecular structure of each azole derivative (2). The exact conformation of the active site differs between fungal species and amongst the many mammalian P450 mono-oxygenases. The precise nature of the interaction between each azole molecule and each kind of P450, therefore, determines the extent of the inhibitory effect of each azole antifungal agent in different fungal species (which means that some fungi could be intrinsically resistant to a given azole derivative). Inhibition of 14α-demethylase by azoles leads to the depletion of ergosterol, which is a major bioregulator of fungal cytoplasm membrane fluidity, and to asymmetry and accumulation of sterol-precursors, including 14α-demethylated sterols, resulting in the formation of a plasma membrane with altered structure and function.

Because of the different characteristics in their activity, pharmacodynamics, pharmacokinetics, and safety profiles, each of these azole agents has found utility in different clinical settings (1, 3). In general, as a class, azole antifungals have a broad spectrum of activity, including activity against *Candida* species, *C. neoformans*, dimorphic fungi, and molds. For example, fluconazole has broad clinical efficacy for mucosal candidiasis (vaginal and oropharyngeal), and has also often been considered as a first choice for the prophylaxis and treatment of invasive candidiasis in neutropenic and non-neutropenic patients. It is also active against *C. neoformans* and some of the causative agents of endemic mycoses. However, fluconazole is not active against *Aspergillus* and other molds, and some *Candida* species (namely *C. krusei* and *C. glabrata*) are intrinsically resistant to fluconazole. Itraconazole displays potent activity against *Candida* and *Aspergillus* spp., dimorphic and dematiaceous fungi, and although it has been used less frequently, the availability of an oral solution and intravenous formulation has recently increased enthusiasm for its application for prevention of mold infections (4). Voriconazole has been shown to be superior to amphotericin B deoxycholate in the primary treatment of invasive aspergillosis, thus representing an important therapeutic advance (5). Posaconazole displays

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potent activity and an expanded spectrum of action, and has the most potential as a treatment strategy for zygomycosis (6).

2 Resistance to Azole Antifungal Agents

2.1 General Considerations and Definitions

Reports on resistance to azole antifungal agents were rare until the late 1980s. However, development of resistance to the current clinically used azole antifungal agents has become an increasing problem. This is particularly true in patients requiring long-term treatment, and in those receiving antifungal prophylaxis (7–9). Thus, azole resistance is frequently described in patients with AIDS and mucosal candidiasis (particularly in the era prior to highly active antiretroviral therapy, HAART), oral candidiasis, and less frequently in invasive infections. Resistance to azole treatment can be stable or transient (10). In addition, there is a growing awareness of the changing epidemiology of fungal infections, with a shift toward species that are intrinsically resistant to the most commonly used antifungal agents (fluconazole) (11, 12). Microbiological resistance is defined as a decrease in antifungal drug susceptibility, which can be measured in vitro by appropriate laboratory methods. This highlights the importance of the development of standardized methods for antifungal drug susceptibility testing in the last decade (13, 14) which are considered milestones in the field of medical mycology. By performing these techniques, a distinction between a susceptible and a resistant fungal isolate can be made according to a threshold drug susceptibility value (i.e. the breakpoint MIC, for Minimal Inhibitory Concentration) which could potentially, and should ideally, predict the success or failure of a given antifungal regimen. However, clinically refractory disease (clinical resistance) may result not only from microbiological resistance, but also from the complexity of host/fungus interactions normal in a debilitated patient. In general, with a few exceptions, it has been difficult to correlate the in vitro and in vivo data (15, 16).

Primary resistance occurs in organisms never exposed to a given drug in that host. This intrinsic resistance is displayed by all, or almost all, isolates of one species to a certain drug, and it will be predictive of clinical failure. Examples are the resistance of *C. krusei* and *A. fumigatus* to fluconazole. In contrast, secondary resistance (also defined as acquired resistance) develops only after exposure of the organism to the drug. An example of secondary resistance is the development of fluconazole resistance in *C. albicans* strains isolated longitudinally from HIV-infected patients with oropharyngeal candidiasis under long-term treatment with this drug (8, 9).

2.2 Molecular Mechanisms of Azole Resistance

At the molecular level, different mechanisms contribute to resistance against azole antifungal agents, reviewed in (17, 18). These mechanisms include modification of the antifungal target (in the case of azoles lanosterol demethylase, the product of the *ERG11* gene), decreased drug accumulation inside the fungal cells due to the overexpression of multidrug drug efflux pumps, and other alterations in sterol biosynthesis. Deficiency in the uptake of some azole derivatives could also contribute to resistance. Most studies have been performed in *C. albicans* due to the unique opportunity to analyze series of matched susceptible and resistant isolates recovered sequentially from the same patient (17, 19–24), but recent studies in other pathogenic fungi such as *C. glabrata*, *A. fumigatus*, and *C. neoformans* seem to support these observations (25–32). In most instances, resistance to azoles is a multifactorial process involving several mechanisms. Cross-resistance within the azole class of antifungal agents is common, and is becoming an important issue (33, 34).

2.2.1 Alterations in the Target Enzyme

Alterations in the target enzyme (lanosterol 14-α-demethylase), including point mutations and overexpression, lead to decreased susceptibilities to azole drugs, which may also lead to cross-resistance to other azole derivatives. Pathogenic fungi can overcome the inhibition of azoles by increasing the content of the target enzyme molecules, either by gene amplification or by overexpressing the corresponding gene (*ERG11*). This results in the need for higher intracellular azole concentration to complex all the enzyme molecules present in the cells. However, this mechanism seems to have a limited impact in resistance to azoles, and does not seem to confer high levels of resistance (17, 18). Point mutations in the gene encoding the target enzyme for azoles (*ERG11*) result in amino acid substitutions leading to decreased affinity for azole derivatives. In these studies, *ERG11* alleles from azole-resistant isolates were sequenced and compared to alleles of matched azole-susceptible isolates. While some *ERG11* alleles contain a single mutation responsible for azole resistance, other *ERG11* alleles were found to contain several mutations with potential additive effects (21, 35–38). Importantly, some of these mutations have been repeatedly identified by different groups in different geographical locations, and these mutations may represent “hot spots” for the development of azole resistance. Remarkably, most of these substitutions are present in domains that are highly conserved in lanosterol demethylases across fungi, suggesting the importance of these residues for function maintenance through evolution. According to molecular

modeling of *C. albicans* lanosterol demethylase, these regions correspond to important functional domains of the enzyme in its interaction with the heme moiety at its active site, and at another region believed to play a role in the entry of the substrate in the substrate pocket (39, 40). Interestingly, some of the new-generation azoles, due to differences in the way they interact with Erg11p, may be more insensitive to alterations in the target enzyme. A recent report indicated that posaconazole was active against *C. albicans* isolates that have mutations in their *ERG11* genes causing resistance to other azole derivatives, and that multiple (up to five) mutations in *ERG11* were required to confer decreased susceptibility to posaconazole (36).

2.2.2 Increased Drug Efflux

A second major mechanism leading to azole resistance is by prevention of accumulation of sufficient effective concentrations of the azole antifungal agent in the fungal cells as a consequence of enhanced drug efflux. This mechanism is mediated by two types of multidrug efflux transporters, the Major Facilitators (encoded by *MDR* genes in *C. albicans*) and those belonging to the ATP-binding cassette superfamily (ABC transporters, encoded by *CDR* genes in *C. albicans*) (17, 18). The major facilitators contain a transmembrane pore, but use proton motive force as their energy source. ABC transporters, which have been associated with drug resistance in a variety of eukaryotic cells, include a membrane pore composed of transmembrane segments and two ATP-binding cassettes on the cytosolic side of the membrane, which provide the energy source for the pump (41–43). Upregulation of the *CDR* genes appears to confer resistance to multiple azoles in *C. albicans*, whereas upregulation of the *MDR1* gene alone leads to fluconazole resistance exclusively (19–22, 24, 44, 45). More recent studies suggest the resistance associated with brief exposures to high-dose azoles is transient (as opposed to stable resistance associated with long-term exposure to low-dose azoles) and associated with alterations in transcriptional regulation of CDR pump expression (10). It is not clear, at this moment, how multidrug transporter genes are regulated in pathogenic fungi, including *C. albicans*, although it is believed that gene upregulation might be caused by alterations *in trans* (involving transcription factors). This particular topic is currently the focus of intensive research in different laboratories (46–49).

2.2.3 Mutations in other Genes in the Ergosterol Biosynthetic Pathway

Altered sterol $\Delta^{(5,6)}$ desaturase is also linked to azole resistance in *C. albicans* clinical isolates. In azole-sensitive strains treated with azoles, 14-methyl-3,6-diol accumulates and leads to a fungistatic effect, whereas in sterol $\Delta^{(5,6)}$ desaturase mutants (due to mutations in the gene *ERG3*), its precursor, 14-methylfecosterol, accumulates, which can support growth of the fungal cell. Interestingly, a consequence of this mechanism is that it causes cross-resistance to amphotericin B, due to the fact that ergosterol is absent from cell membranes (50–52).

2.2.4 Prevalence and Combinations of Molecular Mechanisms of Azole Resistance

The multiplicity of resistance mechanisms to azole antifungals represents a set of biological tools that enables fungal cells to develop resistance using different combinations. However, the prevalence and relative frequency of resistance mechanisms in a large population of azole-resistant isolates has been investigated in only a few studies. In the study by Perea et al. (21), most of the resistant isolates presented a combination of resistance mechanisms, such as upregulation of efflux transporters (encoded by *CDR* and *MDR* genes) and point mutations in the *ERG11* alleles. In 85% of resistant isolates, a major mechanism of resistance was the upregulation of multidrug resistance of both families (ABC-transporters and Major Facilitators). Also, almost 60% of patients presented *C. albicans* isolates harboring point mutations in their *ERG11* genes, leading to enzymes with decreased affinity for fluconazole. Overall, 75% of the azole-resistant isolates showed combined resistance mechanisms. All the isolates that showed cross-resistance against multiple azoles presented increase in *CDR* mRNA. Only one isolate overexpressed *ERG11* genes without concomitant upregulation of *CDR* and *MDR* genes, and only two resistant isolates presented point mutations in *ERG11* genes as mechanism of resistance not associated with upregulation of efflux pumps.

2.2.5 Heterogeneity of Molecular Mechanisms of Resistance

An often overlooked and under-appreciated phenomenon is the fact that different fungal subpopulations may exist, that respond and evolve differently under antifungal drug pressure, providing an additional level of complexity in the molecular mechanisms of azole resistance. Earlier studies on molecular mechanisms of azole resistance in oropharyngeal candidiasis were limited due to the fact that only a single isolate from each episode was available for study, but recovery and analyses of multiple isolates from the same episode in some subsequent studies allowed a comprehensive assessment of the epidemiology of resistance in OPC. It was demonstrated that despite their clonal origin, different subpopulations of *C. albicans* demonstrated distinct resistance

mechanisms, including concomitant presence and absence of functional point mutations in *ERG11* genes, and different patterns of expression of genes encoding multidrug efflux pumps. These observations seem to indicate microevolution of fungal populations under azole antifungal pressure. Overall, these studies point to the complexity of the distribution of the molecular mechanisms of azole drug resistance, and indicate that different fungal subpopulations may coexist at a given time in the same patient and may develop resistance through different mechanisms (53, 54).

2.2.6 Biofilm Resistance

Our perception of microorganisms as unicellular life forms is primarily based on the pure culture model of growth. Nevertheless, in most natural habitats, microorganisms grow as structured biofilm communities on biological or inanimate surfaces, rather than individually in suspension. Cells in these biofilms are embedded within a matrix of extracellular polymeric material, and display an altered phenotype; crucially, they are significantly less susceptible to antimicrobial agents. This is of particular significance, as it is now estimated that a significant proportion of all human microbial infections, including mycoses, involve biofilm formation (55). Several groups have demonstrated that the *Candida* biofilm lifestyle leads to dramatically increased levels of resistance to the most commonly used antifungal agents, particularly azoles, reviewed in (55). Thus, this may be one of the main reasons for the lack of correlation between results of antifungal susceptibility testing, as determined by NCCLS guidelines, and clinical outcome in patients suffering from these types of infections (56). As yet, there appears to be no one specific resistance factor responsible for the increased recalcitrance to azole antifungal agents exhibited by biofilms. Instead, biofilm resistance is a complex multi-factorial phenomenon, which still remains to be fully elucidated and understood. Different mechanisms may be responsible for the intrinsic resistance of *Candida* biofilms. These include: (a) high density of cells within the biofilm; (b) effects of the biofilm matrix; (c) decreased growth rate and nutrient limitation; (d) expression of resistance genes, particularly those encoding efflux pumps; (e) altered membrane sterols; and (f) presence of “persister” cells (55).

3 Genomic and Proteomic Techniques to Study Azole Resistance

The completion or near completion of sequencing different fungal genomes are opening new avenues to employ genomic and proteomic technologies to help identify key genes and proteins involved in resistance to azole antifungal agents. For example, genome-wide analyses using DNA-microarrays represent a powerful tool to analyze differential gene expression in matched susceptible and resistant isolates, to identify clusters of co-regulated genes controlled by common regulatory circuits implicated in azole resistance, and to reveal previously unrecognized molecular mechanisms implicated in azole resistance. This technique was exploited by Cowen and colleagues to study the development of azole resistance in experimental populations of *C. albicans* (57). Results indicated that changes in gene expression in response to inhibitory concentrations of fluconazole were constitutive and persisted in the absence of the drug, and cluster analysis identified three distinct patterns of gene expression underlying adaptation to the drug, which included upregulation of genes encoding multidrug efflux pumps. The Rogers group has used DNA-microarrays and proteomic techniques to analyze *C. albicans* response to azole treatment, as well as genes associated with stepwise acquisition of azole resistance in clinical isolates (58–60). Results indicated that reduced susceptibility to oxidative damage may contribute to azole resistance. Similarly, Karababa et al. (61) compared transcript profiles between *C. albicans* clinical strains with known azole resistance mechanisms and laboratory strains exposed to drugs known to induce genes involved in azole resistance. The two major conclusions from these studies were: (a) in vitro drug-induced gene expression only partially mimics expression profiles observed in clinical isolates, and (b) upregulated genes in resistant strains are both “drug resistance genes” (i.e. *CDR* and *MDR* genes) and genes that could be activated under cell damage conditions.

4 Conclusions

Azoles are an important class of antifungal drugs that have found widespread utility in clinical practice for the treatment of fungal infections. However, with their increasing usage, emergence of resistance has become a problem, especially in patients requiring long-term treatment and those receiving azole prophylaxis. Also, azole use has had a tremendous impact in the epidemiology of fungal infections. At the molecular level, the main molecular mechanisms responsible for azole resistance are alterations in the target enzyme and increased efflux of the drug. Many times, resistance is multifactorial, and combinations of different mechanisms are operative in a high proportion of resistant isolates. Because of their increased potency and broader spectrum, the newer-generation azoles constitute a valuable addition to the antifungal armamentarium, and may have particular utility in the treatment of refractory fungal infections. They also offer new and exciting opportunities for combination therapy.

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Chapter 27
Flucytosine: Site of Action, Mechanism of Resistance and Use in Combination Therapy

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1 Background

A fluorinated pyrimidine, 5-flucytosine (fluorocytosine; 5-FC, Fig. 1), was initially developed as a potential anti-cancer agent but it was not sufficiently effective in the field of cancer chemotherapy (1). Later, 5-FC proved to be active in experimental candidiasis and cryptococcosis in mice (2) and was used to treat human infections (3). In addition to its activity against *Candida* and *Cryptococcus*, 5-FC also has an inhibitory activity against fungi causing chromoblastomycosis (4); however, it is ineffective against infections caused by filamentous fungi. 5-FC has a high prevalence of primary resistance in many fungal species. Due to this primary resistance, 5-FC is used mainly in combination with other antifungals (primarily amphotericin B, AmB) and more recently it has been investigated in combination with other agents including fluconazole (FLU), ketoconazole (KTZ), itraconazole (ITRA), voriconazole (VORI) and echinocandins (e.g., micafungin, MICA and caspofungin, CAS). It is used only rarely as a single agent.

2 Mechanism of Action

Antimycotic activity of 5-FC results from its rapid conversion into 5-fluorouracil (5-FU) by the enzyme cytosine deaminase, within susceptible fungal cells. There are two mechanisms involved by which 5-fluorouracil exerts its antifungal activity (Fig. 2). The first mechanism includes the conversion of 5-fluorouracil through 5-fluorouridine monophosphate (FUMP) and 5-fluorouridine diphosphate (FUDP) into 5-fluorouridine triphosphate (FUTP) (5). FUTP is further incorporated into fungal RNA in place of uridylic acid; this alters the aminoacylation of tRNA, disturbs the amino acid pool and inhibits protein synthesis (5). The second mechanism is the metabolism of 5-FU into 5-fluorodeoxyuridine monophosphate (FdUMP) by uridine monophosphate pyrophosphorylase (5) (Fig. 2). FdUMP is a potent inhibitor of thymidylate synthase, which is a key enzyme involved in DNA synthesis and nuclear division (6). Thus, 5-FC acts by interfering with pyrimidine metabolism and protein synthesis in the fungal cell. These activity result in cell lysis and death.

3 5-Flucytosine Resistance

5-FC monotherapy is effective against infections caused by *C. neoformans*, *Candida* species including *C. glabrata*, and in chromoblastomycosis and phaeohyphomycosis (7). However, its use as a single agent is limited due to its high tendency for rapid development of resistance (8–10). There are no extensive data on correlations of in vitro MIC values with outcome of 5-FC monotherapy in clinical infections. Based on a combination of historical data and results from animal studies (11, 12), the National Committee for Clinical Laboratory Standards, subcommittee on antifungal susceptibility testing (13) proposed interpretive breakpoints. Isolates with a flucytosine MIC of ≤4 µg/mL are considered to be susceptible while those with a 5-FC MIC of > 16 µg/mL are presumed to be resistant, and those in between are considered to be intermediate (14). MIC distributions for collection of yeasts are strongly bimodal, with relatively few isolates having an intermediate 5-FC MIC. These breakpoints are described primarily for the *Candida* species but may well also apply to the *C. neoformans* (14). Hospenthal and Bennett (10) provided a recent review of their experience with 5-FC monotherapy for cryptococcal meningitis. In patients in whom resistance developed, MICs rose from <5 to >320 µg/mL. The presence of resistant strains (about 10% of *C. albicans* isolates) and development of resistance vary frequently during treatment (15). MICs of 5-FC ranges between 1 and 10 µg/mL in vitro against most isolates of *Candida* species. The activity of 5-FC in vitro is greatly

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influenced by the composition of growth medium. Purines and pyrimidines present in the medium act as competitive inhibitors of 5-FC uptake by yeasts and Tris buffers increase the MIC values of this antifungal. Surveys conducted in early 1980s have shown that a large proportion of *Candida* isolates develop partial or complete resistance to 5-FC. Two surveys of *C. albicans* conducted by Stiller et al. (16) and Defever et al. (17) provided estimates of resistance frequencies. The majority of the candidal isolates studied were susceptible (60 and 57%), but significant percentages were partially resistant (36 and 37%) or highly resistant (4 and 6%). Earlier,

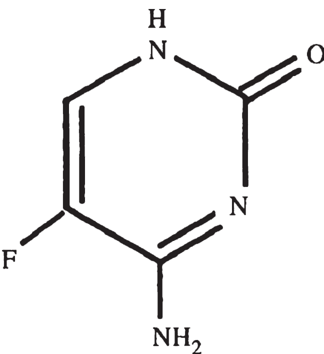

Fig. 1 Chemical structure of 5-flucytosine (5-FC)

Scholer (18) documented 331 5-FC resistant isolates among 3,707 *Candida* strains tested that showed a total prevalence of 8.9%. However, this figure from Scholer's studies showed geographical and species variation in resistance. For example, geographically 23% of 302 *Candida* isolates were resistant to 5-FC in the USA as compared to 7.5% of 3,306 isolates in Europe and 14% of 99 isolates from different parts of the world (18). It has also been found that 5-FC resistance among *Candida* isolates is dependent on the serotype, in that 5-FC resistance is more common in *C. albicans* serotype B than in *C. albicans* serotype A. Prevalence of resistant strains among the B serotype range between 49 and 90% as compared to only 1–11% for type A strains (16, 19).

Additionally, earlier studies have shown that the incidence of resistance to 5-FC varies between candidal species (20). Some *Candida* species represent higher proportions of resistant strains than *C. albicans* do (21, 22). For example, *C. tropicalis*, *C. krusei* and *C. parapsilosis* are some of the common *Candida* species that are frequently more resistant to 5-FC than *C. albicans*. Although one study has found *C. tropicalis* to be more sensitive than *C. albicans* (23). *C. glabrata* have been found to be as sensitive to 5-FC as *C. albicans* (24). The exact incidence of primary 5-FC resistance depends upon numerous variables. Different studies have shown rates ranging between 8 and 44% for *Candida* species. An important explanation for the

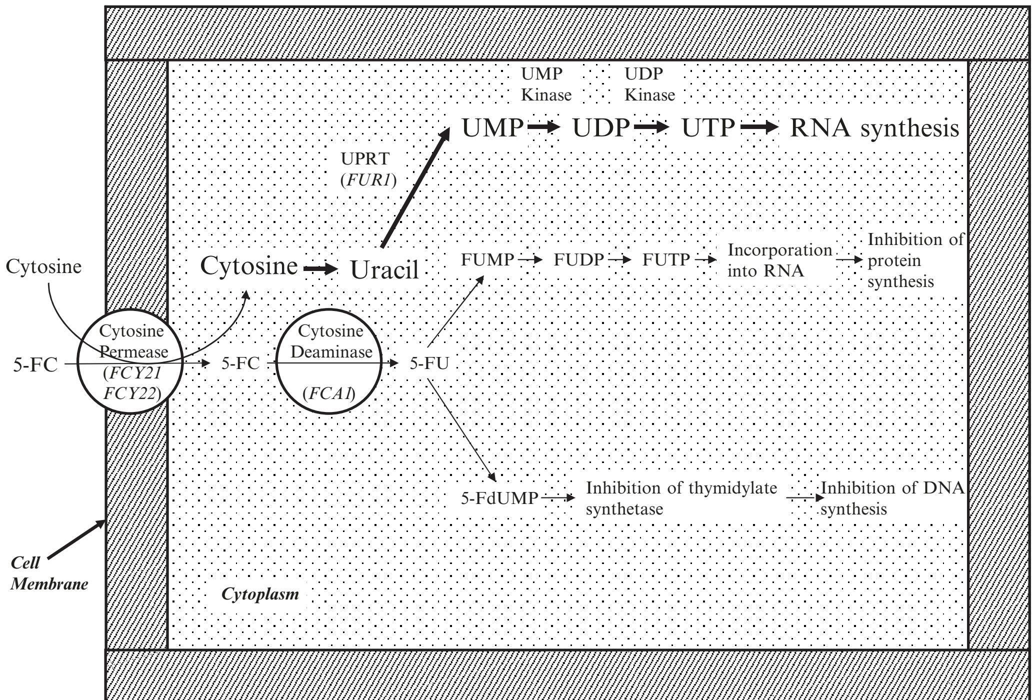

Fig. 2 Intracellular pathway and mechanism of action of 5-flucytosine. *5-FC* 5-flucytosine; *5-FU* 5-fluorouracil; *FUMP* 5-fluorouridine monophosphate; *FUDP* 5-fluorouridine diphosphate; *FUTP* 5-fluorouridine triphosphate; *FdUMP* 5-fluorodeoxy-uridine monophosphate; *UPRT* uracil phosphoribosyltransferase; *UMP* uridine monophosphate; *UDP* uridine diphosphate; *UTP* uridine triphosphate. Genes *FCA1, FUR1, FCY21* and *FCY22* encode for cytosine deaminase, UPRT and two purine-cytosine permeases, respectively

wide variation and discrepencies in the extent of resistance may be attributed to the differences in the susceptibility methods used to evaluate 5-FC MIC (14). It is well documented that a slight variation in the technique used to measure susceptibility may significantly influence the MIC value. Although primary resistance to 5-FC is stated to occur among 10–15% of *C. albicans* isolates and even higher among the other *Candida* species (18), there is a dearth of data for evaluating antifungal susceptibility testing using the standardized National Committee for Clinical Laboratory Standards (NCCLS) antifungal susceptibility testing method (13). Recently, Pfaller et al. (25) re-evaluated the in vitro antifungal activity of 5-FC against 8,803 clinical isolates of *Candida* spp. obtained from more than 200 medical centers all over the world between 1992 and 2001. They determined MICs using broth dilution tests performed according to NCCLS M-27A methodology. Data interpretation was based on the interpretive breakpoints selected by the committee (see above). Their data showed that 5-FC was very active against the vast majority of the 8,803 *Candida* isolates tested, i.e., 95% were susceptible. A total of 99–100% of *C. glabrata*, *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondiim* and *C. kefyr* were susceptible to 5-FC at the NCCLS breakpoints. *C. albicans* and *C. tropicalis* were only slightly less susceptible. In contrast, *C. krusei* was the least susceptible species: 5% susceptible (26). The in vitro activity of 5-FC, combined with previous data that demonstrated prolonged post-antifungal effect (2.5–4 h) and concentration-independent activity (optimized at 4 × MIC), suggest that 5-FC could be used in lower dosage to reduce the risk of host toxicity while maintaining antifungal efficacy (25).

3.1 Epidemiological Factors Responsible for Resistance

A number of factors, both drug and host-related, can contribute to 5-FC resistance in candidal and cryptococcal infections. One such factor is impaired drug absorption/penetration, which can be modulated by the route and vehicle of drug administration. Oral administration of 5-FC can lead to impaired absorption and inadequate serum/tissue drug concentration due to the unique oral environment, where the flushing effect of saliva and the cleansing action of oral musculature tend to reduce the drug concentration to sub-therapeutic levels causing treatment failure (27, 28). Some clinical trials have suggested that low concentrations of 5-FC used for therapy are associated with risk of treatment failure (29). Maximal absorption of certain drugs is achieved by administration of the drug in a particular solution and if this is not done then it can cause low absorption and treatment failure (29).

Properties of the second drug used in combination with 5-FC can also influence antifungal activity. In this regard, absorption of azole antifungal agents is impaired by high gastric pH (observed in some patients with acquired immunodeficiency syndrome), frequent vomiting (common in patients with neutropenia), or the intake of antacids, H2-antagonists, and sucralfate (28). All these factors can affect 5-FC absorption when used in combination with azoles.

The second host factor that can affect drug resistance is the rate of drug metabolism – both for singly used drug and drug combinations. In this regard, properties of the second drug used in combination with 5-FC can also influence its metabolism.

The third host factor influencing drug activity is the immune status of the infected host. A *C. neoformans* strain from cutaneous lesions of a patient with thrombotic thrombocytopenia purpura was tested and was found to be resistant to 5-FC (30). Risk factors for acquired resistance for antifungal drugs in HIV patients increases due to recurring episodes of candidal or cryptococcal infections and due to prolonged exposure to antifungal agents (28).

Antagonism is another factor for 5-FC treatment failure when it is used with AmB or FLU during combination therapy. Numerous studies conducted have reported antagonism-between drugs, in particular, in the case of the combination of AmB and FLU (31, 32) and the combination of 5-FC and azoles (33).

The presence of indwelling medical devices is now recognized to be a major risk factor for the development of candidemia (34) and persistence of the infection despite adequate treatment. The development of *Candida* biofilms coating the lumen of the catheter appears to enable the organism to persist and resist the action of antifungal drugs (34). Al-Fattani and Douglas (35) used a filter disk assay to investigate the penetration of antifungal agents through *Candida* biofilms. FLU permeated all single-species *Candida* biofilms more rapidly than 5-FC. The rates of diffusion of either drug through biofilms of three strains of *C. albicans* were similar. However, the rates of drug diffusion through biofilms of *C. glabrata* or *C. krusei* were faster than those through biofilms of *C. parapsilosis* or *C. tropicalis*. In all cases, after 3–6 h the drug concentration at the distal edge of the biofilm was very high (many times the MIC). Nevertheless, 5-FC/FLU failed to produce the complete killing of biofilm cells. These results indicate that antifungal penetration through biofilms plays a minimal role in drug resistance mechanisms and there may be other mechanisms involved in the drug resistance for *Candida* biofilms (35). Thus, formation of biofilm on indwelling medical devices by *C. albicans* is an important factor that can lead to resist the action of 5-FC.

An unusual interaction and cross-resistance between antifungals occur when they are used simultaneously in

combination studies. For example Noel et al. (36) showed cross-resistance between 5-FC and FLU against 60 *Candida lucitaniae* clinical isolates. Among eight isolates resistant to 5-FC (MIC ≥ 128 μg/mL) and susceptible to FLU (0.5 < MIC < 2 μg/mL), four became 5-FC-FLU cross resistant when both antifungals were used simultaneously. FLU resistance occurred only in the presence of high 5-FC concentrations, and the higher the FLU concentration used, the greater the 5-FC concentration necessary to trigger the cross-resistance (36). These authors suggested that cross resistance to FLU resulted from the competitive inhibition between the two antifungals.

3.2 Prevention and Control of Resistance

Strategies to avoid the emergence of antifungal resistance has not been defined. However, approaches recommended for antibacterials could be used as a guide (37–39). These are: (1) prudent use of antifungals, (2) appropriate dosing of antifungals with special emphasis on avoiding using low dosages, (3) avoiding prolonged use of one antifungal agent, (4) use of appropriate antifungals for combination therapy (in cases where the etiological agent is known), and (5) use of surveillance studies to determine the true frequency of antifungal resistance. Additionally, advances in rapid diagnosis of fungi may be helpful in reducing the use of inappropriate antifungals to treat organisms that are resistant to a particular agent.

4 Mechanism of Resistance

Although 5-FC resistance mechanisms have been investigated and reviewed in depth (40, 41) in the late 1980s and the early 1990s, new data using molecular techniques emerged recently which warrant a review of our current knowledge of 5-FC resistance mechanism. Two mechanisms of 5-FC resistance can be distinguished: (a) Decreased cellular transport or uptake of 5-FC due to the loss of enzymatic activity (loss of permease activity) responsible for conversion to FUMP. The resistance due to decreased uptake is found in *S. cerevisiae* and *C. glabrata*; this mechanism does not seem to be important in *C. albicans* or *Cryptococcus neoformans* (40, 41); (b) Resistance of 5-FC may also result from increased synthesis of pyrimidines, which compete with the fluorinated antimetabolites of 5-FC and thus decrease its anti-mycotic activity (42). Defective uridine monophosphate pyrophosphorylase is the most common type of acquired 5-FC resistance in fungal cells (9).

Available data suggest that blocking the formation of FUMP by loss of cytosine deaminase activity or by loss of uracil phosphoribosyltransferase (UPRTase) activity is sufficient to confer 5-FC resistance. Cytosine deaminase and UPRTase constitute the pyrimidine salvage pathway and are not essential for growth under normal circumstances in which pyrimidines are synthesized de novo. Resistance in the large majority of both clinical and laboratory strains of *C. albicans* and *C. neoformans* is attributable to mutational loss of one of the pyrimidine salvage enzymes (43–45). Decreased UPRTase activity was associated with resistance in a gene dosage-dependent manner in *C. albicans* (45). *FCY/FCY* homozygotes possessed high UPRTase-specific activity (approximately 3 U), whereas *FCY/fcy* heterozygotes possessed less activity (approximately 1.5 U) and *fcy/fcy* homozygotes possessed barely any detectable activity.

Recently, tools of new molecular biology including DNA microarray analysis, have been employed to investigate 5-FC resistance mechanisms. In order to understand the changes in DNA and protein synthesis following the exposure to 5-FC, Zhang et al. (46) used DNA microarray-based approach to study the expression profile of *S. cerevisiae*. A total of 96 genes were identified which were responsive to a 90 min treatment with 25 μg/mL 5-FC. This sub-inhibitory treatment caused about 17% inhibition in growth (46). Genes that are involved in DNA repair, synthesis, and replication represented the highest proportion of induced genes identified which agrees with the known antifungal mode of action of 5-FC i.e., interference with DNA synthesis. Two genes, both involved in the ubiquitin-dependent pathway, were also up-regulated (2.1-fold). It is known that ubiquitin-dependent pathway is related to DNA repair and selective removal of damaged or obsolete proteins (47, 48). These induced genes may be responsive to the faulty protein synthesis caused by 5-FC. It has been proposed that increased DNA repair is often associated with resistance to DNA damaging agents (49). Apart from the known cellular resistance mechanism to 5-FC, which can result from loss or mutation of any of the enzymes involved in 5-FC activation (see above), Zhang et al., inferred from the induced genes after treatment with 5-FC, that the cellular resistance to 5-FC might also result from the increased DNA repair in the cell (46).

A recent study by Pujol et al. (50) revealed that *C. albicans* can be separated into five clades, groups I, II, III, SA, and E. Groups SA and E are highly prevalent in South Africa and Europe, respectively. Pujol et al. (50) generated a dendrogram that included 243 *C. albicans* isolates which were DNA fingerprinted with the complex probe Ca3 and then analyzed for 5-FC susceptibility. Of the 243 *C. albicans* isolates tested, 9 (3.7%) proved to be 5-FC resistant (i.e., MIC ≥ 32 μg/mL). The MIC for one isolate was intermediate (MIC = 16 μg/mL). When these ten isolates were color-coded in the dendrogram, they all clustered exclusively in

group I (50). Ten additional isolates highly resistant to 5-FC (i.e., MIC ≥ 128 μg/mL) identified separately in a collection of 5,208 *C. albicans* isolates (25) were DNA fingerprinted with Ca3 and added to dendrogram of the collection of 243 isolates. These ten isolates were color coded blue and also clustered exclusively in group I. These results indicated that among natural isolates, 5-FC resistance is specific to group I, although resistant strains represented only 4% of group I isolates. MIC_{50} (minimum inhibitory concentration inhibiting 50% of the isolates) tested for group I and non-group I isolates were 1.00 and 0.12 μg/mL, respectively. When an arbitrary MIC threshold for strains with decreased 5-FC susceptibility was used as 0.5 μg/mL, 97% of isolates for which MIC was ≥0.5 μg/mL proved to be in group I, while the remaining 3% were in groups other than group I (50). These studies have demontrated that isolates that are naturally resistant to 5-FC are restricted to group I and that group I isolates are generally less susceptible to 5-FC than non-group I isolates. Isolates of group I represent 47% of isolates in North America (51), 20% of isolates in Europe (51), and 19% of isolates in South Africa (52). Therefore, group I represents a major *C. albicans* clade in all the geographical regions studied so far. These studies for the first time showed the clade specificity of a clinically relevant trait (5-FC resistance) and suggest that intraclade recombination may be common, while interclade recombination is rare (50).

5 5-Flucytosine in Combination with Amphotericin B or Fluconazole

As mentioned above, use of 5-FC as a single agent is limited due to the observation that it has a high tendency for rapid development of resistance (9, 10, 53), and therefore, this agent is used almost always in combination with other antifungal agents. Therefore, review of the literature regarding 5-FC combination therapy is warranted. One of the earliest studies on combination therapy with 5-FC was performed by Bennett et al. (54), who compared AmB alone and in combination with 5-FC in the treatment of cryptococcal meningitis. Subsequent studies also have advocated the use of 5-FC combined with azoles like FLU, ITRA, and echinocandins like CAS. Interactions between different drugs are described variously as synergistic, indifferent, additive, or antagonistic. Assessments of in vitro drug interactions are usually based on the "no interaction" theory, which assumes that drugs in combination do not interact with each other. When the observed effect of the drug combination is more than that predicted from the "no interaction" theory, synergy is claimed. On the other hand, antagonism is claimed when the observed effect is less than that predicted (55).

5.1 5-Flucytosine + Amphotericin B

5.1.1 In Vitro Studies

All the combination studies are summarized in Table 1. The effect of AmB + 5-FC in vitro against yeasts like *Candida* and *Cryptococcus* have been studied in great detail. In one such study, AmB + 5-FC combination was found to have synergistic activity against different *Candida* species in vitro (56). This synergistic action of AmB + 5-FC against yeasts was supported by a separate study, which reported no antagonistic or additive effects in vitro for the combination of AmB and 5-FC (57). Time-kill analysis was used to test six isolates of *C. albicans* and *C. neoformans*. Five antifungal regimens with two combination drug regimens of 5-FC and AmB were tested against each isolate. Single drug regimens included 5-FC (50 μg/mL); low AmB (0.125 μg/mL), and high AmB concentrations (2.4 μg/mL). A fixed concentration of 5-FC was used in combination studies with either low AmB (5-FC 50 μg/mL + AmB 0.125 μg/mL) or high AmB concentrations (5-FC 50 μg/mL + AmB 2.4 μg/mL). There were no differences between combination regimens with respect to either 5-FC pre-exposure or timing, i.e., staggered versus simultaneous administration. In both the low and high concentration combination regimens, the drug interactions were indifferent. Regardless of the AmB concentration, no antagonism or additive effects were observed. The lack of antagonism noted is not surprising since these two antifungals have different modes of action. 5-FC, as shown above, exerts its activity via its inhibition of fungal RNA/DNA synthesis, while AmB inhibits fungal growth by interacting with membrane sterols and causing disruption of cell membrane (58). Since the differing mechanisms of action exhibited by these agents preclude the occurrence of antagonism, their combination may be beneficial for preventing the development of resistance.

5.1.2 In Vivo Studies

Studies with animals have demonstrated that 5-FC plus AmB has significantly improved mycologic activity against meningitis caused by *C. neoformans* (59). Diamond et al. (59) evaluated the antifungal efficacy of AmB colloidal dispersion (ABCD) combined with 5-FC in a murine model of cryptococcal meningitis. The following dosages were tested: ABCD at 0–12.5 mg/kg of body weight given intravenously 3 days/week, and 5-FC at 0–110 mg/kg/day. Meningitis was established in male BALB/c mice by intracerebral injection of *C. neoformans* (59). A 100% survival rate was achieved with ABCD in combination with 5-FC, but ABCD or 5-FC when used alone was not able to prevent the weight loss (59).

Table 1 Combination table

Organism	Disease	Drug combination	Study	Drug effect	References
Candida species	–	5-FC + AmB	In vitro	Synergistic	(56)
C albicans and *C. neoformans*	–	5-FC + AmB	In vitro	Synergistic	(57)
C. neoformans	Murine *Cryptococcal meningitis*	5-FC + AmB	In vivo	Improved	(59)
C. neoformans	Murine *Cryptococcal meningitis*	5-FC + AmB	In vivo	Positive	(60)
C. neoformans	Cryptococcal meningitis	5-FC + AmB	Clinical study	Positive	(54)
Candida isolates	Hepatosplenic candidiasis	5-FC + AmB	Clinical study	Positive	(62)
Candida isolates	Candidal meningitis	5-FC + AmB	Clinical study	Positive	(62, 63)
Candida species	Candidal peritonitis	5-FC + AmB	Clinical study	Beneficial	(64)
Candida species	Candidal cystitis	5-FC + AmB	Clinical study	Beneficial	(65)
C. albicans	Endocarditis and truncus arteriosus communis	5-FC + AmBisome	Clinical study	Beneficial	(67)
C. neoformans	–	5-FC + FLU	In vitro	Mostly synergistic	(68)
Candida species	–	5-FC + FLU	In vitro	Generally antagonistic/ synergism in few cases	(69)
C. tropicalis	Murine disseminated infection	5-FC + FLU	In vivo	Synergistic/antagonistic	(70)
C. glabrata	Murine C. glabrata infection	5-FC + FLU	In vivo	Beneficial	(71)
C. neoformans	Murine Cryptococcal meningitis	5-FC + FLU	In vivo	Synergistic	(72)
C. neoformans	Murine Cryptococcal meningitis	5-FC + FLU	In vivo	Synergistic	(73)
C. neoformans	Cryptococcal meningitis	5-FC + FLU	Clinical study	Improved	(75)
C. neoformans	Pulmonary Cryptococcosis	5-FC + FLU	Clinical study	Beneficial	(76–78)
C. albicans and *C. neoformans*	–	5-FC + AmB + FLU	In vitro	Combined effect depending on conc. of drug	(93)
C. neoformans	Murine Cryptococcal meningitis	5-FC + AmB + FLU	In vivo	No effect	(94)
C. neoformans	Murine Cryptococcal meningitis	5-FC + AmB + FLU	In vivo	Some effect	(100)
C. neoformans	Meningeal encephalitis	5-FC + AmB + FLU	Clinical study	Negative	(97)
C. neoformans	Cryptococcal meningitis	5-FC + AmB + FLU	Clinical study	Some effect	(98)
Candida species	–	AmB or 5-FC + KTZ	In vitro	No effect	(79)
Candida species	–	Inhibitors of sterol biosynthesis (FLU, KTZ etc.) + nucleic acid and protein biosynthesis (5-FC, 5-FU etc)	In vitro	Concentration-dependent antagonism	(33)
C. neoformans	–	ITRA + 5-FC	In vitro	63% synergistic, 31% additive and 6% indifferent but no antagonism	(80)
Aspergillus species	–	AmB + 5-FC and ITRA + 5-FC	In vitro	Antagonistic	(69)
Aspergillus species	–	VORI + 5-FC	In vitro	Antagonistic	(81)
C. neoformans	Murine *Cryptococcal meningitis*	5-FC + AmB and 5-FC + KTZ	In vivo	Additive	(82)
C. neoformans	Rabbit *Cryptococcal meningitis*	5-FC + AmB and 5-FC + KTZ	In vivo	Some effect	(83)
Candida cryptococcus and *Asperillus* species	Murine model of candidiasis, cryptococcosis and aspergillosis	5-FC + AmB and 5-FC + KTZ	In vivo	Synergistic or addtitive in candidiasis, additive in cryptococcosis and slightly additive or indifferent in aspergillosis	(84)
			In vivo	Additive or indifferent in candidiasis, indifferent in cryptococcosis and aspergillosis	
Candida species	Rabbit model of candidiasis	5-FC + KTZ	In vivo	Better fungistatic activity	(85)
C. neoformans	Cryptococcal meningitis	AmB + 5-FC + ITRA	Clinical study	Positive	(87)
Exophiala jeanselmei	Subcutaneous phaeohyphomycosis	AmB + 5-FC + ITRA	Clinical study	Positive	(88)
Candida and Cryptococcus	Candidiasis and Cryptococcosis	ITRA + 5-FC	Clinical study	Positive	(89–92)
Aspergillus species	–	CAS + 5-FC + AmB	In vitro	Synergistic	(81)
		VORI + 5-FC		Antagonistic	
		CAS + VORI + 5-FC		Synergistic	
C. albicans	Oropharyngeal candidiasis	VORI + AmB + 5-FC + CAS	Clinical study	Positive effect after addition of CAS	(99)

Another comparative study using five different antifungals for the treatment of an experimental model of murine cryptococcosis was carried out (60). It was found that for animals treated for 2 weeks, the combinations of AmB and 5-FC was the most useful, leading to 90% negative cultures and prolonging the animal survival time up to 60 days (60).

5.1.3 Clinical Studies

The addition of low-dose, intravenous AmB to 5-FC therapy of cryptococcosis has appeared to decrease the frequency of secondary 5-FC resistance. In addition, the two drugs have an additive or slightly synergistic effect against 5-FC susceptible isolates of Cryptococcus and *Candida*. The combination is probably the treatment of choice in cryptococcal meningitis and offers promise in the therapy of systemic candidiasis (61). Bennett et al. (54) studied the comparison of AmB alone and combined with 5-FC in the treatment of cryptococcal meningitis. Out of 50 patients, 27 were treated with AmB and 24 with the combination. Even though the combination regimen was given for only 6 weeks and AmB for 10 weeks, the combination cured or improved more patients (16 vs. 11), produced fewer failures or relapses (3 vs. 11), more rapid sterilization of the cerebrospinal fluid, CSF ($P < 0.001$) and less nephrotoxicity ($P < 0.05$) than did AmB alone. Adverse reactions to 5-FC occurred in 11 of 34 patients but were not life threatening (54). AmB penetrates the blood-brain barrier poorly, while 5-FC has excellent penetration; this may explain the improved outcome of treating cryptococcal meningitis when these two agents are used in combination as compared to AmB used alone (28).

A number of studies have shown that combination of 5-FC and AmB offers promise in the therapy of systemic candidiasis. The use of a combination of 5-FC and AmB for the treatment of hepatosplenic candidiasis has been reported (62). Furthermore, it has been shown in a retrospective study that patients with candidal meningitis respond well to this combination (62, 63). 5-FC in combination with AmB is also beneficial in patients with candidal peritonitis associated with continuous ambulatory peritoneal dialysis (64). Furthermore, some studies have shown that in the treatment of uncomplicated candidal cystitis, 5-FC has been used alone as well as in combination with AmB (65). 5-FC is absorbed very rapidly and almost completely: 76–89% is bioavailable after it has been administered orally (66) and its peak concentrations are attained in serum and other body fluids within 1–2 h (66). The rapid absorption and penetration of 5-FC into most body fluids including cerebrospinal, peritoneal, and vitreous and into inflamed joints is due to its small size and high water solubility (61). This explains why the combination of 5-FC and AmB has an improved outcome when these two agents are used in combination as compared to AmB used alone.

C. albicans endocarditis occurs mostly in patients with congenital heart disease; open heart surgery is the greatest predisposing factor. Hauser et al. (67) reported that on a child with truncus arteriosus communis and a large *Candida* vegetation within the prosthetic pulmonary valve, causing severe right ventricular outflow tract obstruction, treatment with liposomal AmB (AmBisome) and 5-FC followed by surgery resulted in a favorable outcome. Initially, AmB (1.0 mg/kg/day) was administered which later was changed, due to severe side effects, to AmBisome, a liposomal preparation that allows the use of better-tolerated higher dosages (5 mg/kg body weight). The drug was given over a period of 4 weeks without any side effects in combination with 5-FC. These studies showed that prolonged administration of high doses of AmBisome in combination with 5-FC can be curative and life-saving in such patients (67). The above studies provide convincing non-clinical and clinical data establishing the benefits of combining 5-FC with AmB in the treatment of a variety of yeast infections.

5.2 5-Flucytosine + Fluconazole

5.2.1 In Vitro Studies

Nguyen et al. (68) tested FLU + 5-FC (0.125–128 μg/mL concentration range for each) against 50 clinical strains of *C. neoformans*. Combination of FLU with 5-FC resulted in significant reductions in the geometric mean of FLU MIC (from 5 to 1 μg/mL, $P = 0.001$) and of the 5-FC MIC (from 12 to 0.1 μg/mL, $P = 0.0001$). Synergy was observed in 62% (31/50) of cases, additive interaction in 6% (3/50), while autonomous or indifferent interaction was observed in 24% (12/50) of the isolates. Addition of FLU greatly affected the in vitro inhibitory action of 5-FC; the 5-FC MICs for *Cryptococcus* isolates were markedly decreased to concentrations which were several fold lower than the achievable cerebrospinal fluid 5-FC concentration. However, if the initial FLU MIC for the isolate was ≥8 μg/mL, addition of 5-FC did not greatly enhance the in vitro activity of FLU (68).

Only a limited number of studies have been performed with FLU + 5-FC combination against *Candida* infections. In a recent study, Te Dorsthorst et al. (69) evaluated the in vitro efficacy of FLU + 5-FC combination against 27 *Candida* species including *C. albicans* ($n = 9$), *C. glabrata* ($n = 9$), and *C. krusei* ($n = 9$). Synergism was observed for five and antagonism for four *C. albicans* isolates. For *C. krusei*, synergy was observed for only one isolate, and antagonism for eight isolates. Notably, this combination was antagonistic against all the *C. glabrata* isolates tested (69). These studies showed that the FLU + 5-FC combination is generally antagonistic against *Candida* species.

5.2.2 In Vivo Studies

In vivo studies with 5-FC + FLU combinations have reported both synergistic and antagonistic interactions. Graybill et al. (70) evaluated the in vivo efficacy of combining 5-FC with azoles in a murine model of disseminated *C. tropicalis* infection. Survival and tissue burden of the spleen and kidneys were used to evaluate the efficacy of the antifungal therapy. These studies showed that combining 5-FC with FLU did not increase efficacy against *C. tropicalis* infection (70). In a separate study, Atkinson et al. (71) established an immunosuppressed mouse model of *C. glabrata* infection to evaluate the efficacy of combinations of AmB, 5-FC, and FLU treatments in vivo. Treatment with FLU, 5-FC, AmB, or a combination was begun one day after infection. Kidneys and spleen CFU counts following 5 days of treatment revealed that the FLU + 5-FC combination was superior to these agents alone in reducing the tissue burden in the kidneys for one isolate of *C. glabrata*. High doses of FLU alone produced modest reductions in kidney counts but did not reduce spleen fungal tissue burden. Moreover, there was a poor correlation between in vitro MICs and in vivo results (71).

Nguyen et al. (72) evaluated the efficacy of FLU + 5-FC combination as therapy for cryptococcosis in a murine model of meningitis. Three strains of *C. neoformans* for which the range of FLU MICs was wide – 2 μg/mL (susceptible strain), 8 μg/mL (moderately susceptible strain), and 32 μg/mL (resistant strain) – were used to challenge the mice and establish infection. One day postinfection, the mice were randomized into eight treatment groups: placebo; 5-FC (40 mg/kg of body weight/day); FLU at 3 mg/kg/day (low dosage), 10 mg/kg/day (moderate dosage), or 20 mg/kg/day (high dosage); and combined 5-FC and FLU at low, moderate, or high doses of FLU. These studies showed that: (a) MICs for the isolates correlated with the in vivo efficacy of FLU as assessed by the reduction in cryptococcal brain burden, (b) a dose-response curve (a higher dose of FLU was significantly more efficacious than a lower dose [$P < 0.001$]), and (c) the combination of FLU + 5-FC was superior to therapy with either agent alone ($P < 0.01$) (72). Similar synergistic effects of FLU + 5-FC combination were demonstrated in vivo by Larsen et al. (73).

These studies suggested that combination of FLU + 5-FC can show both synergistic and antagonistic effects which could be the function of species/strains studied. Synergistic effects appear more likely when 5-FC and FLU is combined to treat cryptococcosis.

5.2.3 Clinical Studies

Although one early report described efficacy of FLU + 5-FC combination against *Candida* infection in a clinical setting (74), more recent reports support the use of this combination against cryptococcal infections. To test the efficacy of FLU + 5-FC combination therapy in the clinical setting (75) Mayanja-Kizza et al. performed a randomized trial to compare FLU monotherapy (200 mg/d qd, for 2 months) with combination therapy of FLU (200 mg/d qd, for 2 months) + short-term 5-FC (150 mg/kg/d, for the first 2 weeks). Fifty-eight patients with AIDS-associated cryptococcal meningitis were enrolled, of which 30 patients were randomized to receive combination FLU + 5-FC therapy and 28 were randomized to receive monotherapy with FLU. Patients in both groups who survived for 2 months received FLU as maintenance therapy (200 mg three times per week for 4 months). Within 2 weeks, death was prevented among patients with combination therapy, and within 1 month there was significant decrease in the severity of headache in patients administered combination therapy, compared to those treated with FLU monotherapy ($P = 0.005$). Importantly, after 6 months of treatment, the differences in survival rates among patients receiving combination therapy had increased significantly compared to those receiving monotherapy (survival rates were 32% and 12% for combination and monotherapy, respectively, $P = 0.022$). These investigators suggested that treatment with FLU + 5-FC can be a cost-effective and safe regimen to treat patients with AIDS-associated cryptococcal meningitis. In a separate study, Yamamoto et al. (76) retrospectively analyzed the clinical efficacy of combination therapy of FLU and 5-FC in patients with pulmonary cryptococcosis. The clinical efficacy of the two drugs when combined was good in 90% (9/10) of the patients, suggesting that a combination therapy using FLU and 5-FC is clinically useful in patients with pulmonary cryptococcosis who otherwise show a limited response to monotherapy. Similar studies of successful treatment of cyroptoccosis have been reported by other investigators (77, 78). Taken together, these studies demonstrate that the combination of 5-FC and FLU has some merit in treating cryptococcal infections.

5.3 *Flucytosine in Combination with Other Triazoles*

5.3.1 In Vitro Studies

Most in vitro studies involving combination of 5-FC and ketoconazole suggest an antagonistic or indifferent effect against yeast strains, although this effect was also dependent on the method used to determine the type of interaction as well as the organism being tested. Van der et al. (79) used a method combining automatic turbidimetry and sequential viable count determinations to evaluate the in vitro activity of various antifungal agents alone and in combination against three clinical isolates of *Candida* spp. (two *C. albicans* and one *C. tropicalis*) at two inocula (10^5 and 10^6 CFU/mL). In this study, it was reported that AmB or 5-FC plus ketoconazole

(KTZ) were indifferent against the three tested strains. Siau and Kerridge (33) also showed that the combination of inhibitors of sterol biosynthesis (terbinafine, miconazole, ketoconazole, clotrimazole, econazole, fluconazole, itraconazole, and amorolfine) and inhibitors of nucleic acid or protein biosynthesis (5-fluorocytosine, 5-fluorouracil, rifampicin, and chlortetracycline) exhibited concentration-dependent antagonism against different *Candida* species, in a strain-specific manner. In a separate study using quantitative colony forming units (CFU) and killing curve assays, Barchiesi et al. (80) determined the efficacy of ITRA + 5-FC combination in vitro against 16 strains of *C. neoformans*. These investigators demonstrated strain-dependent interactions including synergy (63%), additivity (31%), and indifference (6%), but no antagonism. Overall, the combination of ITRA + 5-FC was significantly more active than either drug alone against *C. neoformans* in vitro.

Filamentous fungi responded differently to 5-FC + triazole combinations in vitro. Te Dorsthorst et al. (69) used the fractional inhibitory concentration index (FICI) and interaction coefficient alpha (IC_α) methods to determine in vitro interactions between AmB and 5-FC, and ITRA and 5-FC against isolates of *Aspergillus fumigatus*, *A. flavus*, and *A. terreus*. As expected, these investigators observed higher MIC values for all 20 isolates for 5-FC (median MIC = 128 μg/mL) than either AmB (MIC = 0.50 μg/mL) or ITRA (MIC = 0.25 μg/mL) alone. Both the FICI and IC_α methods revealed that interactions tended to vary by species and isolates for combinations of AmB + ITRA and ITRA + 5-FC, with antagonism noted for all three species tested for both combinations. Similar species/strain variation was noted when the authors used IC_α to define drug-drug interactions. For combinations of AmB + ITRA and ITRA + 5-FC, they again found antagonism (median IC_α = −0.04 and −0.05, respectively), while AmB + 5-FC was synergistic against all *Aspergillus* isolates tested (IC_α = 0.65). Although results from the FICI and IC_α methods revealed occasional discrepancies, in general, both methods suggested antagonism for the ITRA + 5-FC combination while AmB + 5-FC was a more potent combination against the tested *Aspergillus* spp. in vitro. Dannaoui et al. (81) recently demonstrated that VORI + 5-FC was antagonistic for 93% of the *Aspergillus* tested.

These studies suggested that the interaction of other triazoles and 5-FC in combination is dependent on the organism tested as well as the method used to determine the interactions.

5.3.2 In Vivo Studies

In contrast to in vitro observations, the results from in vivo studies of 5-FC + triazole interactions appear to be more consistent, and tend to be beneficial. Craven and Graybill (82) tested the efficacy of KTZ, 5-FC, and AmB alone and in two-drug combinations against cryptococcal meningitis in mice injected intracranially with *C. neoformans*. Mortality was assessed, and numbers of cryptococcal cells in brain and liver were counted. By both of these parameters, the combination of 5-FC + KTZ produced results superior to those of either agent alone. The standard combination of AmB and 5-FC also showed an additive effect in this model. However, the combination of AmB and KTZ consistently showed no additive effect. None of the combinations of drugs was antagonistic. These investigators suggested a possible role for therapy with a combination of oral 5-FC and KTZ as part of the treatment for cryptococcal meningitis.

The therapeutic efficacy of KTZ, AmB, 5-FC, and their combinations was tested in a rabbit model of chronic cryptococcal meningitis (83). Serial quantitative cultures of the cerebrospinal fluid after 2 h of treatment indicated that AmB was the best single-drug regimen. KTZ provided little or no additive effect when used in combination with 5-FC or therapeutic doses of AmB for 2 weeks. However, the combination of KTZ plus AmB was at least as effective as AmB plus 5-FC over a 2-week treatment regimen. The addition of KTZ to subtherapeutic dose of AmB significantly increased the killing of cryptococci in cerebrospinal fluid.

In a separate study, Polak et al. (84) used murine models of experimental candidiasis, cryptococcosis and aspergillosis to test the efficacy of various dual combinations of AmB, 5-FC, and KTZ. The life-prolonging effect of the combinations was compared with the effect of each drug administered alone at the same and at double dosage. The 5-FC + KTZ combination was additive or indifferent in the three candidiasis models, but indifferent in cryptococcosis and aspergillosis. The combination of AmB + 5-FC was synergistic or additive in all three candidiasis models, with the most pronounced synergism occurring in the infection caused by a 5-FC-resistant *C. albicans* strain. In the cryptococcosis model, the effect was additive, but only slightly additive or indifferent in the aspergillosis models.

In a study performed by Hughes et al. (85) KTZ and 5-FC were administered alone and in combination for 10 days to rabbits with four *Candida* isolates growing in subcutaneously implanted semipermeable chambers. The peak concentrations of KTZ in the serum and in the chamber were 20.3 and 3.8 mg/L, respectively, and the concentrations of 5-FC were 47.7 and 37.3 mg/L, respectively. The two drugs combined resulted in better fungistatic activity than either drug alone against all four isolates tested.

5.3.3 Clinical Studies

KTZ has been shown to penetrate into the brain tissue of mice and cerebrospinal fluid of humans and to improve the course of human coccidioidal meningitis. Some clinical case reports have described treatment of fungal infections with a combination of 5-FC and KTZ (86).

Chotmongkol et al. (87) compared AmB (0.3 mg/kg/d) plus 5-FC (150 mg/kg/d) plus ITRA (400 mg/d) (study group) with AmB plus 5-FC (control group) in an open-randomized trial (50 patients/group) in the treatment of cryptococcal meningitis in AIDS patients. There were significant differences between the study group and the control group in treatment success (100% vs. 90%; $P = 0.03$). The role of ITRA in combination therapy with 5-FC was also demonstrated by Clancy et al. (88). These investigators reported a case of subcutaneous phaeohyphomycosis occurring in a bone marrow transplant recipient receiving high doses of immunosuppressive agents, in whom widespread subcutaneous infection due to *Exophiala jeanselmei* was not eradicated by repeated resections and therapy with AmB and 5-FC. The infection was eventually cured after addition of ITRA to the therapeutic regimen. Results of in vitro testing of the isolate for susceptibility to a combination of AmB, 5-FC, and ITRA confirmed the potential role of combination antifungal therapy in the setting of refractory infection.

Several other studies, including double-blind clinical trials have demonstrated the utility of ITRA + 5-FC combination against candidiasis and cryptococcosis (89–92). Therefore, ITRA + 5-FC combination represents a viable alternative to FLU + 5-FC therapy, especially among patients intolerant to FLU, or those infected with FLU-resistant fungi.

5.4 Triple Combination: 5-Fluorocytosine + Amphotericin B + Fluconazole

5.4.1 In Vitro Studies

Ghannoum et al. (93) studied three-drug regimens (AmB, FLU, and 5-FC) against three isolates each of *C. albicans* and *C. neoformans*. Using a microdilution plate technique, two-drug combinations against both *C. albicans* and *C. neoformans* were tested. Results of the two-drug combinations against both *C. albicans* and *C. neoformans* showed that inhibition with AmB + FLU was greater than inhibition by either drug alone. At low concentrations of AmB, addition of 5-FC enhanced the growth inhibitory effect against *C. albicans*, but antagonism was noted at higher concentrations of AmB. Data for the three drug pairs (AmB + FLU; AmB + 5-FC; FLU + 5-FC), were presented as contour plots, which showed distinct upwards or downwards contour plots for *C. neoformans* and *C. albicans*. Results of the three-drug combinations for *C. neoformans* showed inhibition with AmB at varying concentrations of FLU and a single fixed dose of 5-FC. In the presence of 5-FC, the combined effects of AmB and FLU on the growth of *C. neoformans* remained indifferent; when the AmB concentration was greater than approximately 1–1.2 µg/mL, addition of 5-FC had no further effect on growth. These investigators suggested that the effects of a drug combination on in vitro fungal growth depends on the ratios and concentrations of the drugs used, as well as the fungal strains tested, apart from other differences related to variations in study design, pathogens, drug conditions, and regimens.

5.4.2 In Vivo Studies

The main objective of studies using AmB, FLU, or 5-FC in combination or alone were to determine the greatest antifungal effects of these combinations. Larsen et al. (94) evaluated the antifungal activities of AmB, FLU, and 5-FC, alone or in combination, in a murine model of cryptococcal meningitis. Meningitis was established in male BALB/c mice weighing 23–25 g by intracerebral injection of *C. neoformans*. Treatment was started on day 2. AmB was tested at 0.3–1.3 mg/kg of body weight/day by slow intravenous injection. FLU at 10–40 mg/kg/day and 5-FC at 20–105 mg/kg/day were administered in the sole source of drinking water. Ninety five percent of the mice treated with AmB at 0.5 mg/kg survived to the end of the experiment, regardless of the FLU or 5-FC dose used. The greatest activity was seen with AmB plus FLU with or without 5-FC. In this study, the addition of 5-FC did not increase the antifungal activity of AmB or FLU (94). In another study, the effects of FLU singly or in combination with 5-FC or AmB was determined in the treatment of cryptococcal meningoencephalitis in an intracranial murine model. Hossain et al. (95) developed a reproducible intracranial murine model of cryptococcosis. Mice (Balb/c, 5–7 weeks old) were challenged intracranially and treated with intermediate (30 mg/kg) or high (90 mg/kg) dose FLU, and AmB (0.75 mg/kg), administered singly or in combination with 5-FC (100 mg/kg). In single treatment, FLU (30 mg/kg) was more efficacious than AmB or 5-FC. Combination treatment led to significantly increased anticryptococcal activity, which was highest for high dose FLU and 5-FC (90 mg/kg and 100 mg/kg, respectively) as compared to combinations of AmB and 5-FC as well as of AmB with intermediate-dose of FLU (95). These studies suggest that FLU combined with either AmB or 5-FC may have some utility in the treatment of cryptococcal meningitis.

5.4.3 Clinical Studies

Clinical studies using triple combination therapy with 5-FC are very limited in number. Chotmongkol and Jitpimolmard (96) were among the first investigators to describe the use of triple combination of AmB + 5-FC + ITRA to treat cryptococcal meningitis. However, use of the triple combination

may not always be beneficial, as described in a subsequent case report (97) on a lethal meningeal encephalitis due to *C. neoformans* in a 14-year-old girl without serious immunodeficiency inclusive HIV-infection. The patient was treated with a triple combination of AmB + 5-FC + FLU. After 18 days the cerebrospinal fluid was sterile. However, considerable lesions of the brain were detected, and the patient died from the *Cryptococcus* infection on day 74 of the antimycotic therapy.

Recently, study involving triple combination with AmB, 5-FC and FLU was done in 64 patients with a first episode of HIV-associated cryptococcal meningitis (98). Initial treatment with AmB (0.7 mg/kg daily); AmB plus 5-FC (100 mg/kg daily); AmB plus FLU (400 mg daily); or triple therapy with AmB, 5-FC and FLU was done. It was found that clearance of cryptococci from the CSF was significantly faster with AmB plus 5-FC than with AmB alone (p = 0.0006), AmB plus FLU (p = 0.02), or triple therapy (p = 0.02). This study confirmed that AmB plus 5-FC has a greater fungicidal activity compared with AmB alone or AmB plus FLU or the triple combination and that 5-FC can be used safely in the hospital setting (98). There are always concerns of combining AmB and FLU due to their related effects on fungal membrane ergosterol. This study showed some additive effects for AmB + FLU combination against *C. neoformans*. At the doses given, triple therapy was only as effective as AmB + FLU, and was significantly less fungicidal than AmB plus 5-FC (98). Therefore, further clinical studies are needed to assess the benefits of using triple combination therapy against cryptococcal meningitis.

6 5-Flucytosine in Combination with New Antifungals

The efficacy of 5-FC has also been studied in combination with other antifungal agents including the newer triazoles (e.g., voriconazole, VORI) and echinocandins (e.g., micafungin, MICA; caspofungin, CAS). It is still early to reach any conclusions regarding the use of 5-FC with new agents. The following sections described the major trends observed in these studies.

Dannaoui et al. (81) used microdilution broth checkerboard techniques based on the National Committee for Clinical Laboratory Standards methodology to study double and triple antifungal combinations against clinical isolates of *A. fumigatus* and *A. terreus*. The influences of the end-point definition (partial or complete inhibition) and the mode of reading (visually or spectrophotometrically) were determined. Interactions between antifungal drugs were also evaluated by agar diffusion tests. Combinations of caspofungin (CAS) with either AmB or voriconazole (VORI) were additive for all the isolates, and antagonism was not observed. The interaction between CAS and 5-FC was synergistic for 62% of the isolates. The triple combination of CAS with 5-FC and AmB was synergistic for all the isolates tested. Although VORI + 5-FC was antagonistic the triple combination of CAS + VORI + 5-FC was mostly synergistic. Complex interactions were obtained with this triple combination for some isolates, with synergy or antagonism depending on the concentrations of CAS and VORI. The results of these in vitro tests suggest that the activity of 5-FC as part of a double combination with CAS and as part of a triple combination with CAS and AmB against *Aspergillus* spp. warrants further investigations. In an earlier study, Garbino et al. (99) demonstrated that a patient with oropharyngeal candidiasis who failed to respond to VORI, followed by AmB + 5-FC therapy responded favorably to CAS (70 mg loading, followed by 50 mg/d), with complete resolution of signs and symptoms. Further studies are needed to evaluate the in vivo efficacies of these combinations.

7 Conclusions

In summary, the emergence of new data demonstrating the current low level of yeast resistance to 5-FC and the favorable antifungal activity of 5-FC in combination with other antifungal agents should renew the interest in this drug. Additionally, in-depth studies investigating the utility of combining 5-FC with the new antifungal agents (e.g., VORI and the echinocandins) could lead to improved dosing practices and a “rebirth” of this agent as a useful adjunct in the treatment of serious fungal infections.

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Chapter 28
Echinocandins: Exploring Susceptibility and Resistance

Cameron M. Douglas

1 Introduction and Background

1.1 *Fungal Cell Walls and 1,3-β-D-Glucan Synthesis*

The cell wall of most human fungal pathogens consists primarily of an interwoven mesh of glucans, mannoproteins, and chitin which is essential for maintaining cell shape and rigidity. In *Candida albicans* and *Saccharomyces cerevisiae*, branched fibrils of 1,3-β-D glucan in the inner layer form a network which acts as a scaffold for other macromolecules (1, 2). Short 1-6-β-D-glucan chains form a bridge between linear 1,3-β-D glucan and cell wall proteins that coat the external surface of the cell wall. The majority of these proteins are heavily mannosylated through O- and N-glycosidic linkages. Most cell wall proteins are attached to 1-6-β-D-glucan through a glycosyl phospatidylinositol remnant that is processed as the proteins are covalently linked to the growing wall structure. Chitin (a homopolymer of N-acetylglucosamine) can be found both underneath the network of 1,3-β-D glucan and as a linker between glucans. In other pathogenic fungi, including *Aspergillus fumigatus* and *Cryptococcus neoformans*, many of the same polysaccharides and mannoproteins are found in the cell wall, but the organization appears to be quite different (3, 4). Polymers with other linkages between glucose units [viz. α–1,3 glucan, β-(1,3)/(1,4) glucan)], or unique sugars [viz. galactomannan (GM)] also exist in some fungi (5). There are many human fungal pathogens whose cell wall architecture is poorly understood.

When synthesis of a functional cell wall is reduced or eliminated, either by gene disruption or by inhibition with selected antibiotics, cell growth is often adversely affected, leading to lysis and death. This strategy of interfering with cell wall synthesis or assembly to inhibit fungal growth has its roots in nature, where some organisms produce secondary metabolites to provide themselves with an advantage in a specific ecological niche. The most effective targets for these secondary metabolites are processes that are both important for growth and/or viability, and conserved across many different species and genera. It may be for these reasons that inhibitors of 1,3-β-D-glucan synthesis are one of the most oft-found classes of natural products that target the fungal cell wall (6).

The enzyme 1,3-β-D glucan synthase (GS) has been extensively studied in *S. cerevisiae* (7). This membrane-associated complex [UDP-glucose:1,3-β-D glucan 3-β-D glucosyltransferase (EC 2.4.1.34)] uses UDP-glucose as substrate to synthesize a polysaccharide product estimated to be of 60–80 glucose residues in length. Maximal activity was obtained in reaction mixtures buffered to pH 8.0 containing GTP, glycerol, bovine serum albumin, and millimolar concentrations of UDP-glucose. The reaction product was confirmed as authentic 1,3-β-D glucan by virtue of its solubility in alkali, insolubility in water or dilute acid, susceptibility to digestion with purified 1,3-β-D glucanase yet insensitivity to α- and β-amylase, complete resistance to periodate oxidation (which cleaves 1,2 dihydroxy linkages absent in linear 1,3-β-D glucan), and conversion to glucose upon complete acid hydrolysis.

Early work established that *S. cerevisiae* GS is localized to the plasma membrane (8). Extraction of crude, GS-containing microsomes with salt and detergent yielded a soluble fraction and a membrane-associated fraction, neither of which had enzymatic activity on its own, but which could be reconstituted to yield an active complex. Within each fraction, the proteinaceous component(s) required for restoration of activity could be inactivated by heat or trypsin digestion, but protection from the heat inactivation could be conferred to the membrane-associated fractions by UDP-glucose, and to the soluble fractions by GTP-γ-S (9). Subsequent work has confirmed that GS is minimally a heterodimer; one protein is a regulatory, GTP-binding subunit, and the second is a large integral membrane protein that presumably acts as a pore to allow the newly synthesized 1,3-β-D-glucan to be exported to the cell surface. The protein containing the catalytic center has not been definitively

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identified, although some researchers have suggested that the integral membrane subunit has motifs consistent with binding of UDP-glucose (7).

Many of the themes uncovered in these and other early studies are echoed in characterization of enzymatic activity from other organisms. 1,3-β-D glucan synthase has been studied in filamentous ascomycetes such as *Neurospora crassa*, *Aspergillus nidulans*, and *Aspergillus fumigatus*, in nonfilamentous ascomycetes such as *Schizosaccharomyces pombe*, in deuteromycetes such as dimorphic fungus *Candida albicans*, in basidiomycetes such as *Cryptococcus neoformans*, and even in oomycetes such as *Phytophthora* spp. Among these diverse sources, all enzymes are membrane-associated complexes that use UDP-glucose as a substrate (the K_M is typically 1–5 mM in crude enzyme preparations) and produce a linear polysaccharide (7).

The GS enzyme complex has not been purified to homogeneity. Awald et al. (10) provided a key step forward by applying product entrapment, a technique first described for enriching *S. cerevisiae* chitin synthase (11). Since GS remains associated with the 1,3-β-D glucan reaction product, which is insoluble in water, the GS complex can be harvested from reactions by centrifugation followed by washing and solubilization with detergent. This method has been used to obtain enriched GS preparations from *C. albicans* (61) and *A. fumigatus* (13) – it may be applicable to a number of human fungal pathogens, and should enhance progress in the biochemical evaluation of glucan synthesis.

1.2 Inhibitors of GS

There are three general structural classes that define known natural product inhibitors of 1,3-β-D glucan synthesis (7). The first class, the lipopeptides, includes the echinocandins, the aerothricin lipopeptidolactones, and the arborcandins. The second class of inhibitors is the glycolipid papulacandins, which consist of a modified disaccharide linked to two fatty acyl chains. The third and most recently discovered class, the terpenoids, are represented by enfumafungin, ascosteroside, arundifungin, ergokonin A, and the sterol sulfate Sch601324. Several reviews (14–16) provide insight into the diversity of compounds that have been discovered in the lipopeptide and papulacandin classes; at the moment, the acidic terpenoid group consists of only a few members. All of the known classes of GS inhibitors are not competitive with UDP-glucose, the substrate of enzymatic activity. Cells exposed to GS inhibitors lyse, and metabolic labeling confirmed that whole-cell incorporation of radiolabeled glucose into an acid-insoluble, alkali-soluble cell wall polysaccharide was primarily affected (17–19). Moreover, lysis was reduced when *C. albicans* was provided with osmotic support (1 M sorbitol). Of the three classes of GS inhibitors, the echinocandins have been studied most extensively, ultimately leading to semi-synthetic derivatives which are used to treat human fungal disease. The papulacandins and terpenoids will be discussed briefly and the remainder of this chapter will be devoted to the echinocandins.

The papulacandins are a family of modified disaccharides with fatty acyl side chains that possess reasonable antifungal activity against most species of *Candida*, but little activity against *C. neoformans* or filamentous fungi. Both papulacandin A and B exhibited in vivo efficacy against an induced disseminated *C. albicans* infection in mice, albeit at high doses (180 and 80 mg/kg, respectively) and when dosed subcutaneously rather than orally (20). Observations of growing *C. albicans* cells exposed to papulacandin were consistent with an effect on cell wall synthesis, since buds viewed under the microscope appeared to burst, and quiescent cells were much less susceptible to lysis. Metabolic labeling of cells incubated with papulacandins revealed preferential inhibition of incorporation of radiolabeled glucose into the cell wall polysaccharides. When the wall components were fractionated, the alkali-insoluble fraction, which is enriched in glucan rather than mannan, was specifically affected (18). More recent work with papulacandin B and some new members of the papulacandin family has demonstrated direct, noncompetitive inhibition of microsomal GS activity (21, 22).

The terpene glycosides are a distinct class of GS inhibitors (19, 23). Like the papulacandins, these compounds preferentially inhibit incorporation of radiolabeled glucose into glucan in the whole-cell labeling experiments, and directly interfere with microsomal GS activity. The spectrum of antifungal activity includes many *Candida* species, with little to no activity against *C. neoformans* or bacteria. The effect on *Aspergillus* species was distinct – hyphae exposed to these compounds were highly branched, shortened and stunted, with bipolar or vesicular tips, swollen germ tubes, and frequent balloon-like cells (24). Activity of the terpene glycosides against *C. albicans* in liquid microbroth dilution assays was significantly reduced in the presence of osmotic support. Further evidence to show that these compounds are bona fide GS inhibitors comes from the analysis of an *S. cerevisiae* mutant with reduced susceptibility to echinocandins (19). Both whole cells and microsomal GS derived from this spontaneous mutant exhibit cross-resistance to echinocandins, papulacandins, and at least four terpene glycosides. In a mouse model of disseminated candidiasis, one of the sterol glycosides (enfumafungin) had moderate activity when dosed intraperitoneally.

1.3 Echinocandins

The echinocandins are cyclic hexapeptides with an amide-linked fatty acyl side chain (15). Included in this group are the aculeacins, the echinocandins, the pneumocandins, and

the mulunodocandins. One of the more striking features of compounds in this class is the potent activity of several natural product echinocandins in animal models of fungal disease; induced infections with *C. albicans* (25) and even *Pneumocystis carinii* (26) are treated effectively with several underivatized echinocandins at doses near 1 mg/kg. This result prompted significant interest in developing an echinocandin for therapy of human fungal infections. Medicinal chemistry efforts at Merck, Eli Lilly, and Fujisawa have led to the semisynthetic echinocandins caspofungin, anidulafungin, and micafungin, respectively (27). One of these (caspofungin) is currently licensed in many countries for treatment of a number of serious fungal infections, including esophageal candidiasis, candidemia, and other *Candida* infections (including intra-abdominal abscesses, peritonitis and pleural space infections), and invasive aspergillosis in patients who are refractory or intolerant to other therapies (28–31). Caspofungin is also indicated for empirical therapy of suspected fungal infections in patients with persistent fever and neutropenia (32).

1.4 Antifungal Spectrum of Echinocandins

Since the enzymatic synthesis of 1,3-β-D glucan is a conserved function across many fungal genera, inhibition of its synthesis should have consequences for many, if not all, fungi. The echinocandins have uniformly potent activity against *C. albicans* and other *Candida* species, including *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (33, 34). In addition to *S. cerevisiae*, yeasts such as *Schizosaccharomyces pombe* and *Yarrowia lipolytica* are killed when exposed to echinocandins (35). The general effect on filamentous fungi in vitro is more subtle and difficult to characterize (36). With *A. fumigatus* and other *Aspergillus* spp, spores grown in liquid culture in the presence of an echinocandin produce hyphae whose size and shape are dramatically different from those of hyphae from untreated cultures. There is some initial diminution of cell mass, which is not sustained upon prolonged incubation. Some other filamentous fungi exhibit a similar response to echinocandins – for example, black moulds such as *Alternaria* spp., and hyalohyphomycetes such as *Scedosporium apiospermum*, are susceptible to caspofungin in liquid culture (37). In contrast, the in vitro growth of *Rhizopus oryzae* and other zygomycetes seems unaffected by caspofungin (38). Among the dimorphic fungi, echinocandin susceptibility is influenced by the growth form (39); micafungin exhibited potent activity against the mycelial forms of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis* (MIC range, 0.0078–0.0625 μg/mL), while it was very weakly active against their yeast-like forms (MIC range, 32 to >64 μg/mL) (40). Several dermatophytes which have been tested (*Trichophyton rubrum*, *Microsporum canis*, etc.) resemble *Aspergillus* spp. in their response to caspofungin, with diminished growth and misshapen hyphae (41). Finally, the human fungal pathogen *Cryptococcus neoformans* is unresponsive to echinocandins, both in liquid culture and in animal models of cryptococcocis (33, 34). However, the lack of in vitro susceptibility can be reversed by the addition of the calcineurin inhibitor FK506 or a nonimmunosuppresive analog (42). Recent studies with *A. fumigatus* suggest a similar synergism between inhibition of calcineurin and GS was seen with this organism (43, 44). While there are some clues about the molecular mechanism(s) behind the GS-calcineurin connection, this remains an active area of research.

1.5 Genetics of 1,3-β-D-Glucan Synthase

The biochemical characterization of GS from *S. cerevisiae* established in the 1980s that the enzyme was a complex with a minimum of two subunits, one of which was an integral membrane protein. Despite significant effort, until the mid-1990s partial purification of the enzyme complex had not been reported. Through reverse genetics, one group used amino acid sequence data, derived from a protein found in a semipurified enzyme preparation, to clone a gene encoding a large (ca. 215 kDa) polypeptide with multiple transmembrane helices (12). Less than a year earlier, the same gene had been identified by another group in a screen for *S. cerevisiae* mutants that were hypersensitive to Calcofluor White, which binds to chitin microfibrils and thereby disrupts cell wall integrity (45). The convergence of these two strategies seemed to support the notion that this protein could encode the membrane-associated subunit of the 1,3-β-D glucan synthase enzyme complex. However, the gene had also been identified by researchers who were using yeast as a model system to explore the mode of action of the immunosuppressant FK506 (46), and by a group studying the Ca^{2+} binding protein calcineurin (47). Initially, there seemed to be no obvious link between cell wall synthesis, calcineurin, and compounds which suppress the mammalian immune response. The answer lay in a second, highly related gene, whose gene product was also found in the product-entrapped *S. cerevisiae* GS complex (48). Expression of this second gene was highly dependent on calcineurin; loss of calcineurin function through either gene disruption, or direct interaction with a complex of FK506 and its yeast binding protein led to a dramatic depletion of mRNA encoding the alternate GS subunit. The GS complex is functional as long as it contains at least one of these integral membrane proteins, but loss of both is lethal. A third approach based on echinocandin selection also led to identification of these genes (49); this will be discussed later in this chapter. In keeping with accepted standards for genetic nomenclature, the designation

FKS (for *FK*560 hypersensitive) will be used when referring to these genes, since this abbreviation was used to describe the first allele in this gene family (50).

The *S. cerevisiae FKS1* and *FKS2* genes encode alternate versions of the large, integral membrane subunit of GS (48). Overall homology between the proteins is 88% and topological modeling predicts between 12 and 16 transmembrane helices. Fks1p and Fks2p are nearly identical within a 582-amino acid central hydrophilic domain, predicted to be on the cytoplasmic face of the plasma membrane. Little is known about the structure–function relationships for this family of proteins, although some point mutations that abolish function or lead to altered cell wall composition have been described (51). Despite the presence of a motif associated in some glycosyltransferases with UDP-glucose binding (52), there is no direct evidence that Fks proteins contain the 1,3-β-D glucan synthase catalytic center.

Since the cell wall is a dynamic structure whose synthesis and hydrolysis must be tightly coordinated to allow for cell growth, the activity of GS must be tightly regulated. The GTP-binding, regulatory component of the enzyme complex, was identified several years ago as Rho1p (53, 54). Regulation of GS activity through Rho1p provides an intriguing interplay between a MAP kinase-dependent cascade that transmits signals of osmotic stress to transcriptional activators controlling key cell-wall related enzymes, and the Fks proteins, one of the principle effectors (55). Mutations have been identified in *RHO1* that affect one function without any apparent change in the other (56, 57). The GS-associated form of Rho1p must be prenylated to be effective, presumably to assure localization to the periphery of the plasma membrane (58); Rho1p-associated proteins such as Lrg1p help to control switching between the GTP- and GDP-bound forms (59).

What relevance do the S. *cerevisiae FKS* genes have for the echinocandin susceptibility of human fungal pathogens? First, the *FKS* and *RHO1* genes are conserved across numerous fungal genera, including many associated with human disease. A high degree of homology amongst members of the *FKS* gene family has aided cloning of paralogs from *C. albicans* (60, 61), *C. neoformans* (62), *A. fumigatus* (13), *Coccidiodes posadasii* (63), *P. carinii* (64), and several other fungi (7). Conservation of *FKS* extends to the plant kingdom as well, where an *FKS* homolog is associated with the synthesis of plant 1,3-β-D glucan (callose) in cotton and barley (65, 66). Likewise, *RHO1* genes have been identified and characterized in *C. albicans* (67), *C. neoformans* (68), and *A. fumigatus* (13), among others. Second, genetic disruption experiments have established that *FKS* is required for viability in these organisms, since efforts to create strains lacking a functional *FKS* gene were unsuccessful. This provides additional evidence that the most prevalent fungal pathogens must have 1,3-β-D glucan in their cell walls to survive. Third, characterization of several S. *cerevisiae* and C. *albicans* spontaneous mutants selected in the laboratory for reduced echinocandin susceptibility confirmed that the mutations mapped to an *FKS* gene (49, 60). This last point will be discussed in more detail later in this chapter.

2 Measuring Susceptibility to Echinocandins

As pointed out above, the consequences of echinocandin inhibition varies among different fungal genera. Synthesis of a cell wall containing 1,3-β-D-glucan is a common trait for nearly all fungi, but the abundance, localization, and importance of cell wall glucan for growth or viability of each organism will affect on how it responds to GS inhibition. Even the question of whether or not echinocandins are fungicidal or fungistatic is hard to answer – growing *C. albicans* cells lyse when exposed to GS inhibitors (69), but what about *A. fumigatus*? Within a germling, there are sites of active cell wall remodeling and regions that are relatively quiescent, which make it difficult to define the overall effect. Traditional liquid microbroth dilution susceptibility testing may be an acceptable in vitro method for measuring the consequences of echinocandin exposure for some fungi but not for others. Because of this, a number of techniques have been applied or developed to determine fungal susceptibility to this class of compounds.

2.1 *Yeasts*

The choice of growth medium for liquid microbroth dilution assays has a significant impact on MIC values for *C. albicans* (70). An evaluation of the influences of methodological variables on susceptibility to caspofungin identified medium and glucose concentration as important factors – MIC values determined in RPMI 1640 at pH 5 were generally higher than those determined in AM3 medium, and there was a trend towards higher values in 2% glucose compared to 0.2% glucose (71). The choice of inhibition endpoint also influenced the MIC values, and spectrophotometric endpoints of 50 or 80% inhibition relative to growth in the absence of drug were comparable. In general, the 50% endpoint gave more consistent MIC values than the 80% endpoint (72). Several groups have explored the use of tetrazolium dyes to measure drug endpoints in microdilution assays (73–75). Metabolic reduction leads to a color change in these dyes, which include XTT, MTT, and Alamar Blue, and the change can be read either visually or spectrophotometrically. A multicenter comparison of caspofungin MIC values determined for 100 isolates of *Candida* spp. using either an Alamar Blue-based visual endpoint or the endpoint defined in the NCCLS

M27-A2 document suggested there is good agreement between the two methods (76).

Agar-based assays have also been employed for measuring susceptibility to echinocandins (77). Compounds applied to the surface of an agar plate diffuse and form zones of inhibition in the lawn of cell growth; the diameter of the zone reflects the degree of susceptibility. In a study using caspofungin and 94 yeast isolates belonging to three different genera, results from the disc diffusion assay were in good agreement with the data from liquid MIC assays, i.e., lower MIC values generally correlated with larger zones of inhibition (78). This concept has been extended to create the E-test wherein a plastic strip embedded with a continuous gradient of test compound and marked to indicate concentration intervals is placed on the surface of an agar plate spread with the test organism. An elliptical zone of inhibition appears following incubation (79). The endpoint is defined as the point of intersection between the zone and the E-test strip. When caspofungin liquid MIC values and E-test results for 93 *Candida* isolates were compared directly, the correlation coefficients indicated that there was statistically significant and reproducible agreement between the test results (80).

Echinocandins are generally accepted as having fungicidal activity against *C. albicans* and other yeasts. Scanning (SEM) and transmission (TEM) electron micrographs of *C. albicans* cells exposed to echinocandins depict highly distorted, wrinkled, and collapsed cells (SEM) or an initial substantial thinning of the bud cell wall followed by complete lysis of the cell cytoplasmic structure (TEM) (81–83). The loss of *C. albicans* viability upon exposure to echinocandins has also been captured in time-kill studies and dye staining combined with flow cytometry. In the time-kill analysis, viable colony-forming units from actively growing cultures of *C. albicans*, *C. glabrata*, and *C. tropicalis* decreased when incubated with either micafungin (84) or caspofungin (85). The magnitude of killing was dose- and organism-dependent, but with all isolates it took more than 6 h to reach the maximal effect. These kinetics are in contrast to the fungicidal profile of the polyene AmB, which produces a rapid kill (<1 h) for most yeast. The issue of echinocandin kill kinetics for *C. albicans* was explored further in a study of the effects of anidulafungin on uptake of the vital dye propidium iodide (PI). A 5-min pulse treatment followed by a 3-h incubation in the absence of drug caused cells to take up PI, which is excluded from viable cells, and lose their ability to grow (86). This intriguing result suggests that echinocandins may associate quickly with the 1,3-β-D glucan synthase target, but it takes time before the cell wall becomes sufficiently depleted in glucan for cells to die because of lysis. Whether the GS enzyme complex was irreversibly inhibited during the 5-min exposure to anidulafungin, or bound drug was not removed by the washing procedure used by the authors, remains to be established.

2.2 Moulds

Growth of *A. fumigatus* and several other filamentous fungi is significantly inhibited by echinocandins. Under the microscope, hyphae from echinocandin-treated cultures show an aberrant morphology, with swollen, highly branched germ tubes and evidence of lysis at the growing tips (74, 87). The extensive mycelium observed in drug-free cultures is replaced in the presence of echinocandin by small, punctuate clumps which adhere to the bottom of the well in microtiter dishes. Although the initial studies describing the susceptibility of *Aspergillus* spp. to echinocandins categorized the response as “inactive” (25), it has since been recognized that the morphological consequences are a general and significant consequence of the interaction between many moulds and members of this drug class. These effects were dose- and time-dependent, with an endpoint that could be observed either by a naked eye or under the microscope (74, 87). Potency among a collection of echinocandins could be ranked based on the minimal concentration that caused this morphological change, and the term “minimal effective concentration” or MEC was proposed to define this alternate endpoint (87). The efficacy of several echinocandins against *Aspergillus* spp. in both animal models of infection and patients confirms that these drugs are active against these organisms (31, 88, 89).

Dyes that measure cell metabolism or viability have been valuable tools for defining the activity of echinocandins against *A. fumigatus*. Chiou et al. (90) used the metabolic dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to illustrate that micafungin damages hyphae of *A. fumigatus*; the effect was confirmed in electron micrographs of micafungin-treated hyphae. The in vitro combination of micafungin with another cell-wall active antifungal (nikkomycin Z, which inhibits synthesis of the polysaccharide chitin) provides a synergistic effect. Other studies have used the fluorescent dyes 5,(6)-carboxyfluorescein diacetate (CFDA) and *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC), which stain live and dead cells, respectively, to further characterize the antifungal activity of both micafungin (91) and caspofungin (74). The pattern of fluorescent staining was very informative – a majority of the cells at the tips and branch points of treated germlings stained with the dead-cell dye (DiBAC) but failed to stain with the viable cell dye (CFDA). External to some hyphal tips was a plume of debris which stained brightly with DiBAC. In contrast, more than three quarters of the subapical cells within *A. fumigatus* germlings, where little active cell wall synthesis takes place, had a staining pattern consistent with viability. These results represented the first demonstration that an echinocandin can cause lysis and death of cells at the sites of active cell wall remodeling in a filamentous fungus. Subapical cells which are not actively synthesizing new 1,3-β-D glucan

remain viable during the period of echinocandin exposure in vitro. Efforts to measure the effect of an echinocandin on *A. fumigatus* colony forming units over time have only captured the overall viability of individual hyphal clumps, and any differential activity on growing versus quiescent cells within germlings has been missed.

Agar-based assays to determine the susceptibility of filamentous fungi to echinocandins have also been described (92). The E-test and other methods that measure zones of inhibition in a lawn of fungal growth have characterized the inhibition as incomplete, since hazy growth is apparent within the zones (93). This is consistent with the presence of swollen, malformed hyphae detected in liquid cultures grown in the presence of an echinocandin. Inhibition of radial growth is another convenient technique to demonstrate activity – an inoculum of spores is applied to the surface of an agar plate containing a test compound, and the rate of growth is measured and compared to that observed on a drug-free plate (94). Some filamentous fungi which are considered relatively insensitive to an echinocandin based on liquid microbroth dilution results may appear susceptible when evaluated in the radial growth assay.

A direct measure of whole-cell inhibition of 1,3-β-D glucan synthesis based on the specific fluorochrome aniline blue has also been developed (95). Organisms grown in the presence or absence of echinocandin are lysed, and a crude cell wall fraction is incubated with the dye, which is fluorescent only when bound to 1,3-β-D glucan (13). Other cell-wall-associated polysaccharides including chitin, mannan, and 1,6-β-D glucan do not bind to aniline blue in a manner that leads to fluorescence. For fungi whose growth is not inhibited by exposure to echinocandins, this assay is particularly useful for distinguishing between an organism whose GS target enzyme is insensitive to echinocandin and one that can survive and grow with a cell wall depleted of 1,3-β-D glucan. The zygomycete *Rhizopus oryzae* has a high MIC for caspofungin (38) with no significant inhibition of radial growth, despite a reasonable IC_{50} against microsomal GS (96). Results from aniline blue staining suggest that the whole-cell synthesis of 1,3-β-D glucan is reduced by caspofungin. Most likely, this fungus can make a functional cell wall in vitro even when 1,3-β-D glucan levels are reduced. In spite of the poor in vitro susceptibility, caspofungin prolonged survival and reduced fungal kidney burden in diabetic mice infected with *R. oryzae* (96).

Given the variety of methods for measuring susceptibility to echinocandins, which is the best way to identify and quantify resistance? In the next section, several efforts to identify and characterize laboratory mutants that exhibit echinocandin resistance will be reviewed. The studies include work with both natural product and semisynthetic GS inhibitors, and with both *S. cerevisiae* and the human fungal pathogens *C. albicans* and *A. fumigatus*.

3 Exploring Resistance in the Laboratory

3.1 Direct Mutant Selection Using Inhibitors

Most natural product inhibitors of fungal 1,3-β-D glucan synthesis have only weak activity against *S. cerevisiae*, which hampered early efforts to study the molecular mechanism of inhibition in this genetically tractable organism (97). Mutants selected using aculeacin A were altered in the cell surface hydrophobicity, but there was no apparent effect on cell wall composition or GS activity (98). Amine-substituted derivatives of pneumocandin B_0 (such as L-733560) were significantly more potent, and selection in *S. cerevisiae* yielded rare spontaneous mutants with reduced susceptibility (97, 99). Apart from changes in whole-cell echinocandin susceptibility, the mutants had higher 50% inhibition concentration (IC_{50}) values when L-733560 was titrated against microsomal 1,3-β-D glucan synthase. In earlier biochemical studies of GS, Cabib and coworkers had used detergent and salt to separate microsomal enzyme into soluble and insoluble fractions (8). Crude enzymatic activity from the *S. cerevisiae* mutants was similarly fractionated into two components which lacked activity alone but could be reconstituted into an active enzyme complex. By mixing soluble and insoluble fractions from the wild-type and mutant strains, it was established that the mutation responsible for reduced echinocandin susceptibility was in the membrane-associated subunit of the 1,3-β-D glucan synthase complex. A parallel approach using the nonechinocandin papulacandin B identified mutants in both *S. cerevisiae* and *Schizosaccharomyces pombe* with similar properties (100).

The gene which restored partial susceptibility to L-733560 was identified through a genomic library screen in the *S. cerevisiae* mutant. This gene was initially called *ETG1* (for *e*chinocandin *t*arget *g*ene *1*), but it was soon discovered that *ETG1* was identical to *FKS1* (49), which had been studied by a number of groups, most often through analysis of phenotypes conferred by a variety of *fks1* mutations. As mentioned earlier, *FKS1* and the highly homologous gene *FKS2* are integral membrane proteins that serve a partially redundant function in the GS complex. Fks1p predominates in cells growing with fermentable sugars as the carbon source, and *FKS1* expression is cell-cycle regulated (48). In contrast, the expression of *FKS2* is regulated by calcineurin, and the Fks2 protein appears mainly when the cells sporulate, reach stationary phase, or grow in media containing nonfermentable sugars (101). Loss of either *FKS1* or *FKS2* is not lethal to *S. cerevisiae*, but *fks1Δ fks2Δ* strains are inviable. Cells with a loss-of-function mutation in *fks1* are dependent on *FKS2*; in this manner, they become hypersensitive to the immunosuppressants (FK506 and cyclosporin A) that inhibit calcineurin.

A number of mutations in *FKS1* have been reported, and the phenotypes they impart allow them to be grouped into

three classes. The first class, typified by the *fks1-1*, *cnd1-8*, and *cwh53-1* alleles, includes loss-of-function mutations in *FKS1* (46, 47, 102, 103). Cells bearing these alleles grow more slowly than *FKS1* cells and are hypersensitive to FK506 and cyclosporin A. Other phenotypes described for alleles of this class, which are consistent with a defect in production of 1,3-β-D-glucan, include rescue of slow growth with osmotic stabilizers, reduced 1,3-β-D-glucan content in cell walls, and lower specific activity of crude GS activity. The second class of *fks1* mutations is defined by the *etg1-1*, *etg1-3*, and *pbr1-8* alleles (97, 99, 100), each of which confers specific resistance to inhibitors of GS. These mutants have normal growth rates and apparent wild-type levels of GS activity, but that activity is resistant to echinocandins. Finally, the *etg1-4* mutation (99) confers whole-cell and enzyme resistance to echinocandins, with a modest effect on GS specific activity, but cells with this mutation are hypersensitive to the chitin synthase inhibitor nikkomycin Z ($NikZ^{HS}$). All of these mutations, including the echinocandin resistance alleles, were isolated under conditions where Fks1p predominates and *FKS2* is expressed weakly. The fungicidal activity of echinocandins against both wild-type *S. cerevisiae* and *fks1* null mutants indicates that Fks2p is also a likely target of these compounds, but no echinocandin-resistant *fks2* mutants have been described.

In a similar screen to the one described above, *S. cerevisiae* was mutagenized with ethyl methanesulfonate and colonies were selected for growth in the presence of the pneumocandin L-733560 (104). Several strains with defects that were outside of the *FKS* genes were identified, and the mutations mapped to a gene that was closely linked to the *MAT* locus. The wild-type version of this gene (*GNS1*) encoded a 40-kDa protein with five predicted transmembrane helices and two leucine zipper motifs. Disruption of *GNS1* had pleiotropic effects which recapitulated those of the original mutants – the *gns1Δ* strains grew slowly, had mating and sporulation defects, and were significantly reduced in glucan synthase specific activity. Although *gns1Δ* whole cells were resistant to L-733560, GS enzymatic activity derived from them was not. Years later, it was discovered that *GNS1* is in fact allelic to *ELO2*, which is involved in fatty acid elongation and sphingolipid biosynthesis (105). Loss-of-function mutations in *GNS1*/*ELO2* cause accumulation of intermediates in the sphingolipid biosynthetic pathway, and one of these (phytosphingosine) has been shown to directly inhibit the activity of 1,3-β-D-glucan synthase. All of the phenotypes associated with the *gns1Δ* defect can be explained by an overabundance of phytosphingosine. The intriguing link between sphingolipids and GS will be discussed in detail later in this chapter.

Arborcandin C is a structurally distinct cyclic lipopeptide GS inhibitor (106) which was also used to select spontaneous *S. cerevisiae* mutants. With greater than 100-fold increase in arborcandin MIC values and an equivalent shift in GS IC_{50} values, the mutations in these isolates were likely to be associated with a subunit of the GS enzyme complex (107). Genomic libraries constructed from the mutants were used to transform a wild-type strain, and the *FKS1* gene was identified as the mutant allele. DNA-sequence analysis and selected subcloning experiments established that specific substitutions in Fks1p (Lys for Asn at amino acid 470, or Ser in place of Leu at residue 642) conferred the resistance phenotype. In a topological model of Fks1p, Asn 470 is within a transmembrane helix and Leu 642 is predicted to be within a loop on the cytoplasmic face of the protein. The most interesting feature of these mutants is the apparent lack of cross-resistance to an echinocandin (pneumocandin A_0) or a papulacandin (F-10748-C_1).

Direct selection for echinocandin resistance in *C. albicans* has yielded three classes of mutants. The first class, identified from a pool of UV-induced mutants, is resistant to aculeacin A and appeared to have alterations in cellular lipids (108). The second class is exemplified by the *C. albicans* strain CA-2, which was isolated from mutagenized cultures challenged with the echinocandin cilofungin (109, 110). 1,3-β-D-glucan synthase activity derived from strain CA-2 was insensitive to echinocandin inhibition, but the strain was of limited virulence in a mouse model of disseminated candidiasis. The last class of *C. albicans* mutants maintains full virulence in animal models of infection and exhibits both whole-cell and GS enzyme resistance to echinocandins and papulacandins, but retains wild-type susceptibility to other antifungal agents. Members of this last class have been characterized in some detail (111, 112).

The resistance phenotypes of both pneumocandin and cilofungin-insensitive mutants (*C. albicans* strains CAI4-R1 and LP3-1, respectively) are consistent with mutations in the echinocandin target gene *CaFKS1*. IC_{50} values for a variety of GS inhibitors are elevated in both mutants, without an apparent effect on specific activity or the K_M for the UDP glucose substrate. Unlike the aculeacin mutants described earlier, the LP3-1 strain had a normal neutral lipid and phospholipid profile compared with its parent strain (112). When the microsomal GS enzyme complex of strain LP3-1 was extracted with salt and detergent and separated into an insoluble, membrane-associated fraction and a soluble fraction, reconstitution "mix and match" experiments with similar fractions from a wild-type *C. albicans* strain confirmed that the resistant subunit was membrane-associated. The most compelling evidence that *CaFKS1* is the site of the mutations and the gene encoding the echinocandin target in GS came from a genetic analysis of strain CAI4-R1 and three other spontaneous mutants (60, 111). First, it was shown using spheroplast fusion experiments that strain CAI4-R1 is heterozygous at the resistance locus; the segregation pattern was consistent with a single semidominant echinocandin resistance allele and a single wild-type allele. Next, a portion of the *CaFKS1* gene was cloned and a plasmid bearing a *URA3*-disrupted version of the gene was constructed. Transformation of strain CAI4-R1 with the plasmid would

result in the disruption of one of the two *CaFKS1* alleles, and a prediction linking the phenotype of echinocandin resistance to the *CaFKS1* gene could be tested. If the mutation responsible for reduced susceptibility to echinocandins was in another gene, then loss of one *CaFKS1* allele would have no impact on the resistance phenotype. However, if the mutation was at the *CaFKS1* locus, then the disruptants would either be fully susceptible to echinocandins (when the resistant *CaFKS1* allele was disrupted) or fully resistant (when the wild-type *CaFKS1* allele was disrupted). The susceptibility profile of the transformants was entirely consistent with a *Cafks1* mutation in one of the two alleles. This genetic analysis was extended to include other spontaneous mutants; mutations in either or both *CaFKS1* alleles were found among this collection.

Perlin and coworkers have described results from a direct selection for spontaneous *A. fumigatus* mutants exhibiting reduced in vitro susceptibility to caspofungin (113). The mutants described by them have a biphasic response curve, with breakthrough growth at drug concentrations greater than 0.5 μg/mL but susceptibility to caspofungin at concentrations ≥ 16 μg/mL. The mutants were stable and retained virulence in a murine model of pulmonary aspergillosis. Unlike the *C. albicans* spontaneous mutants described above, GS activity from the *A. fumigatus* isolates was not insensitive to caspofungin, and there were no mutations in either the *AfFKS1* coding sequence or the upstream regulatory elements. Transcriptional profiling of one of these strains (RG101) identified nearly 100 transcripts that were differentially expressed when the mutant was grown with or without caspofungin (114). The genes encompassed many aspects of fungal metabolism including the cell integrity pathway, and RNA and protein processing. Ninety percent of the genes shared homology with other fungi, including other species of *Aspergillus* and *S. cerevisiae*. Further characterization of these *A. fumigatus* mutants is in progress.

3.2 Genetic Screens in S. cerevisiae

Beyond direct selection for drug-resistant mutants, several groups have used *S. cerevisiae* genetics to explore pathways and genes which may play a role in echinocandin susceptibility and resistance. Multicopy genomic libraries, promoters that confer regulated gene expression, and collections of deletion mutants covering all nonessential genes in the *S. cerevisiae* genome are tools that have been applied to address these questions.

The 1,3-β-D glucanase laminarinase digests the cell wall of intact *S. cerevisiae* cells and liberates spheroplasts, which lyse in the absence of osmotic support. Lai et al. (115) transformed a laminarinase-sensitive strain of *S. cerevisiae* with a multicopy genomic library and screened for clones that grew on unsupported medium. Five DNA sequences were identified, and three were chosen for characterization based on alterations in the profile of whole-cell glucan synthesis. The first gene, *PBS2*, is a MAP kinase which may serve as a positive regulator of glucan biosynthesis and is activated by high osmolarity. Multiple copies of *PBS2* lead to elevated GS specific activity; deletion of *PBS2* results in a 45% decrease. There is also independent evidence that *PBS2* plays a role in controlling levels of 1,6-β-D-glucan. A second gene (*MHP1*) encodes a microtubule interacting protein, and although the role is not clearly defined, Mhp1p may affect the movement of cell wall biosynthetic enzymes or precursors to the plasma membrane. The third gene discovered in this screen was *LRE1*. This 586 amino acid neutral hydrophilic protein may confer laminarinase resistance, because it represses chitinase expression when overexpressed – failure to degrade chitin will result in a modified cell wall structure with decreased sensitivity to 1,3-β-D glucanase (116). The authors did not explore what role *PBS2*, *MPC1*, or *LRE1* might play in susceptibility to echinocandins.

The *GAL1* promoter provides carbon-source-regulated expression in S. *cerevisiae*, such that *GAL1*–controlled genes are overexpressed when cells are grown in galactose and repressed in the presence of glucose. A *GAL1*-cDNA library was transformed into a caspofungin-susceptible strain and transformants were scored for growth on galactose-containing medium supplemented with caspofungin (117). This strategy yielded the *SBE2* gene, whose protein product is associated with the Golgi apparatus. When *SBE2* was overexpressed, cells were less susceptible to caspofungin and had higher levels of 1,3-β-D glucan synthase activity. A previous report had linked *SBE2* and a related gene (*SBE22*) to cell integrity and wall structure, and the authors proposed that the Sbe2 and Sbe22 proteins may be involved in transport of cell wall components from the Golgi to the plasma membrane (118). Disruption of the *SBE2* gene conferred hypersensitivity to caspofungin.

A collection of *S. cerevisiae* deletion mutants comprising the complete set of nonessential genes is available to the research community, and was used in two separate studies to identify mutants that exhibited differential sensitivity to caspofungin (119, 120). Although the criteria for selecting hypersensitive and resistant mutants were somewhat different, several genes were common to both screens. A listing of deleted genes that conferred enhanced susceptibility to caspofungin, and their function, includes: *FKS1*, glucan synthase; *CHS3/4/7*, required for chitin synthase III-dependent chitin deposition; *MNN10*, protein mannosylation; *SMI1*, regulation of GS thru protein kinase C (*PKC*)-dependent cell integrity signaling cascade; *NPL3*, nuclear export of poly A + mRNA; and *ILM1*, unknown function. Two genes that conferred resistance to caspofungin when deleted were *SLG1*, which encodes a sensor for the

PKC-SLT2 cell integrity pathway, and YNL080C, which plays an undefined role in respiratory growth. The authors suggest that loss-of-function mutations in the latter set of genes may indicate potential mechanisms for clinical resistance, since most of these genes have homologs in human fungal pathogens.

Because of a clear association between the *FKS* family of genes and echinocandin susceptibility, efforts to map domains of the S. *cerevisiae* Fks proteins and identify interacting proteins have yielded important details about their structure and function. The differential sensitivity of Fks1p and Fks2p for the cyclic lipopeptidelactone GS inhibitor aerothricin1 was exploited to identify amino acids that may be important for drug sensitivity (121). A series of *FKS1*/*FKS2* chimeras was created, and a 74-residue region predicted to be on the extracellular face of Fks2p contained the key amino acid(s) responsible for the differential sensitivity. Only one substitution in Fks1p (K1336I) produced a strain with whole-cell and GS sensitivity equivalent to that of strains bearing the native, aerothricin1-susceptible *FKS2* gene. However, the K1336I modification in Fks1p did not cause a dramatic shift in the sensitivity to echinocandin B, so an association between similar mutations in *C. albicans* and clinical use of echinocandins seems unlikely. In another study, a temperature-sensitive allele of *FKS1* (fks1154; K877N A899S Q977P) was combined with deletion of *FKS2* and used to identify multicopy suppressors at the nonpermissive temperature (56). Robust growth at 37° C could be restored by multiple copies of the genes *WSC1*, *WSC3*, *MTL1*, *ROM2*, *LRE1*, *ZDS1*, and *MSB1*. All but *WSC3* and *ZDS1* are positive activators of the Pkc1p-MAPK pathway. However, the authors point out that direct activation by Rho1p is required to control GS, and that Fks1p and Pkc1p are stimulated independently by upstream activators. An independent but more extensive collection of *FKS* temperature-sensitive mutants was used to define structure–function relationship in this protein, and four domains were proposed (51). The regions of Fks1p are implicated in the activation of the GS complex, localizing GS to the site of polarized growth, catalysis, and anchoring the protein in the plasma membrane. None of the domains proposed in this model were associated with echinocandin susceptibility.

3.3 Genomic Profiling

Insights into potential mechanisms for reduced echinocandin susceptibility have also come from the application of some recently developed tools in genomic profiling. In one of the more intelligent approaches, Lesage et al. (119) utilized a synthetic genetic array to find genes whose disruption is synthetically lethal with an *fks1Δ* allele. Many of the gene products were involved in cell wall synthesis and regulation – proteins that play a role in the synthesis or transport of 1,6-β-D glucan, chitin, or mannan were heavily represented – but the pathways for polarity establishment, secretion, and stress response were also significantly connected to GS. This reflects the coordination of polarized growth and cell wall assembly in the mitotic cell cycle. When a microarray analysis of caspofungin-dependent transcriptional changes was conducted, genes with significant increases ($n = 137$) and decreases ($n = 55$) in expression were identified (122). There was overlap with some genes identified in previous screens, but others, such as the gene encoding the Golgi protein Sbe2p, were not induced. Some transcriptional responses were specific to caspofungin and some genes were induced in cells exposed to any one of several antifungal agents with distinct mechanisms (i.e., amphotericin B, ketoconazole, or 5-flucytosine). A related study (123) looked at transcripts from both an *FKS1* wild-type strain and an *fks1Δ* strain. Among 22 genes overexpressed in the strain lacking a functional *fks1* gene, five encoded cell-surface-localized GPI-anchored proteins. Prior analysis had established that one of the members of this family (*CWP1*) is induced in *fks1Δ* cells (124). There appears to be a substantial cell wall salvage pathway that is induced in response to perturbations in wall composition or structure (125, 126). The *S. cerevisiae PKC1* signal transduction pathway is clearly a key component for transmitting information about osmotic stress to sets of responsive genes. It is not surprising, therefore, that exposure to caspofungin activates this pathway. At sub-MIC doses, microarray profiling illustrated that caspofungin activates the MAP kinase Slt2p through phosphorylation; when *SLT2* was deleted, the cells are hypersensitive to caspofungin relative to an *SLT2* wild-type strain (127). The integral membrane protein Wsc1p is a member of a family of cell wall damage sensors, and genetic data implicate Wsc1p as the critical protein for initiating the *PKC1* signaling cascade. Deletion of *WSC1* but not other *WSC* genes blunted the response to caspofungin, suggesting that Wsc1p may be required for sensing echinocandin-induced cell wall damage. While it is likely that exposure to echinocandins increases expression of many cell-wall-related genes, it is not clear that these changes can rescue human fungal pathogens exposed to clinically effective concentrations of caspofungin or the other echinocandins.

3.4 Proteomics

There is only one publication describing the effects of echinocandin treatment on the proteome (128). *C. albicans* was

exposed to mulunocandin or its structural analog HMR3720 at concentrations below the MIC for up to 2 h, and both total protein and microsomal protein fractions were prepared. The proteins were separated on 2D gels and in-gel digested before peptides were analyzed by mass spectrometry. Databases with information about the *C. albicans* proteome were queried in an effort to match spots to specific proteins. Although few proteins could be identified unequivocally, a clustering algorithm assessing the pattern of protein abundance relative to untreated cells could differentiate an echinocandin signature from that produced by exposure to the azoles, fluconazole or itraconazole.

3.5 Biochemical Approaches

Photoaffinity probes have been used in an alternate strategy to characterize subunits of 1,3-β-D glucan synthase (129–131). Partially purified enzyme from either *S. cerevisiae*, *N. crassa*, or *A. fumigatus* has been photolabeled with either 5-azido-[^{32}P]-UDP-glucose or 8-azido-GTP. Rho1p was labeled by 8-azido-GTP when a chromatographically purified GS preparation was used (54). With 5-azido-[^{32}P]-UDP-glucose, a protein of 165 kDa from N. crassa was initially identified (130), but subsequent experiments have identified the N. crassa Fks1p as a target, which supports the hypothesis that Fks1p may be the catalytic subunit of the GS enzyme complex (63). *A. fumigatus* proteins of 31, 50, and 115 kDa have been photolabeled by the UDP-glucose photoaffinity probe (129). There have been no subsequent reports to clarify the role of these polypeptides in GS of *A. fumigatus*. Affinity labeling using photoactivatable echinocandin derivatives has met with mixed success, and may be dependent on the specific analog used as a probe. Members of the antifungal research group at Merck (132) tried this approach by incubating microsomal or partially purified GS from *S. cerevisiae* with a photoactivatable analog of L-733,560 (o-azido '560). Samples were irradiated and the photoproducts were separated by SDS PAGE and probed by Western blotting, using antiserum raised against thyroglobulin-conjugated L-733560. No cross-linked proteins were detected on the blots. In contrast, an echinocandin photoaffinity probe based on the anidulafungin core was used by Radding et al. (133) to identify *C. albicans* target proteins in a solubilized GS preparation. The major photoproducts were proteins of 40 and 18 kDa, and labeling was competed by unlabeled, active echinocandins. Owing to its low labeling efficiency and abundance, the 18 kDa protein could not be characterized by classical biochemical methods, but the 40 kDa protein was successfully purified. A strategy of proteolytic and chemical digestion followed by amino acid sequence analysis provided a partial sequence; since the *C. albicans* genome sequence was not available at the time, the authors looked for matches among the complete set of *S. cerevisiae* predicted proteins. One protein of unknown function with 76% identity to the *C. albicans* peptide sequence was identified; genes encoding this protein, and a second highly homologous open reading frame were detected in the *S. cerevisiae* genome. For several years, these proteins and their potential association with 1,3-β-D glucan synthesis remained unknown. A recent large-scale analysis of protein complexes in *S. cerevisiae* identified an association between these two gene products and the protein Pkh1p (134). Pkh1p is an *S. cerevisiae* homolog of the mammalian 3-phosphoinositide-dependent protein kinase; one of the phosphorylation targets of Pkh1p is the Pkc1p-MAP kinase pathway that regulates cell wall maintenance and integrity (135). Pkh1p and a closely related protein Pkh2p encode a partially overlapping function, and cells require one or the other for viability. The kinase activity of Pkh1/2p is itself controlled by two proteins (Pil1p and Lsp1p) that work together as negative regulators in a pathway controlled by sphingolipids. Remarkably, the 40 kDa protein identified by azido-echinocandin photolabeling is Lsp1p (136). There are no data to establish that photolabeling of Lsp1p reflects a true, mechanistic drug–protein interaction, or that Pil1p and/or Lsp1p interact directly with the GS complex. Neither *PIL1* nor *LSP1* is essential in *S. cerevisiae*, and none of the screens for genes that play a role in echinocandin resistance, or proteins that interact with the GS complex or Fks1p, identified either Lsp1p or Pil1p.

Several lines of evidence suggest a relationship among sphingolipid metabolism, the echinocandins, and GS. First, the *GNS1/ELO2* gene encodes the enzyme required for the synthesis of very-long-chain fatty acids, which are precursors in the synthesis of mature sphingolipids (137); loss-of-function mutations in *GNS1* conferred resistance to L-733560 (104). Second, the Lsp1 protein described above requires sphingolipids for maximal phosphorylation by either Pkh1p or Pkh2p, and phytosphingosine (PHS) is the most effective long-chain base in stimulating kinase activity (135). A study by Abe et al. provided what may be a key piece to the puzzle – this group demonstrated that PHS is a direct, noncompetitive inhibitor of *S. cerevisiae* 1,3-β-D glucan synthase (105). Mutations that lead to accumulation of intracellular PHS, such as deletion of *GNS1*, reduce growth directly through an effect on GS; this effect is reversed when suppressing mutations that restore PHS to wild-type levels are introduced.

3.6 Animal Models

There are many mechanisms which could alter fungal susceptibility to caspofungin in vitro but have little relevance to how an organism responds to caspofungin therapy in vivo. Mutations affecting the growth rate or the yeast to hyphal transition have been identified while screening for echinocandin resistance in the laboratory; in vivo, such changes impart reduced fitness, producing mutants that are unable

to colonize a host or cause disease (111). Animal models of infection have been developed to study a wide range of human fungal diseases, and they can also reflect the state of the host (immunocompetent vs. immunosuppressed) or different routes of infection (pulmonary vs. disseminated disease). These models can be especially useful for evaluating whether or not lab mutants with reduced echinocandin susceptibility might have clinical relevance. Alternatively, when a fungal isolate is recovered from a patient who failed antifungal therapy, measuring susceptibility in an animal model is a critical validation for any suspicion of drug resistance based only on results from in vitro susceptibility testing. MIC results often do not provide an unambiguous delineation between sensitive and resistant isolates, particularly for echinocandins, where trailing endpoints or paradoxical responses (discussed later in this chapter) have been described. Finally, selecting for mutants in infected animals under echinocandin drug pressure should provide an important corollary to selections in vitro so that the strains obtained under both selections can be compared and contrasted.

C. albicans strains CA-2, M-2, and LP3-1 are echinocandin-resistant mutants that were selected in vitro from mutagenized cultures (109, 112, 138). Each of these isolates has been evaluated for virulence and/or drug susceptibility in animal models of infection. Strain CA-2 is distinct from wild-type *C. albicans* not only in its resistance to echinocandins but also because it is unable to form filaments under in vitro conditions that promote hyphal morphogenesis (110). Growth in the yeast phase is unaffected by the mutation(s). In murine models of disseminated candidiasis, strain CA-2 was significantly less pathogenic than wild-type *C. albicans*. Surprisingly, this mutant was capable of causing infection in a rat model of experimental vaginitis, and hyphal filaments were observed in vaginal scrapings from these animals. The relationship between reduced susceptibility to echinocandins in vitro and differences in pathogenicity for strain CA-2 is unclear – this mutant may contain a single mutation responsible for all phenotypes, or multiple, independent mutations responsible for each distinct phenotype. The whole-cell echinocandin resistance of strain CA-2 most likely reflects a change in enzyme susceptibility, since microsomal GS from CA-2 is not inhibited by the echinocandin L-733560 (111). Strain M-2 was also isolated based on resistance to cilofungin; although the GS activity derived from this mutant is insensitive to echinocandin inhibition, the strain grows poorly in vitro and is at least 30-fold less virulent in mice than its parent strain. A parasexual genetic analysis of the echinocandin resistance trait in strain M-2, based on spheroplast fusion and subsequent reduction in ploidy by heat shock, suggested that the mutation conferred either dominant or semidominant resistance (111). Another cilofungin-resistant mutant (strain LP3-1) is likely to be a *CaFKS1* mutant, based on biochemical analysis of GS and the specificity of resistance for echinocandins and papulacandin (112). Strain LP3-1 is fully virulent in a murine model of disseminated candidiasis and susceptible to both amphotericin B and fluconazole in this disease model. The in vivo efficacy of echinocandins against the LP3-1 mutant was not reported.

C. albicans CAI4-R1, NR2, NR3, and NR4 are spontaneous *Cafks1* mutants with echinocandin-resistant GS activity (60, 111). The mutations confer reduced whole-cell susceptibility to echinocandins without any significant effect on virulence, and each strain is still susceptible to amphotericin B. In mice infected with these *Cafks1* mutants, kidney burdens could be lowered with echinocandin therapy, but the dose required to achieve 90% reduction in burden (ED_{90}) for each mutant was significantly higher than the amount required for the wild-type parent. The ED_{90} values suggested the mutants fell into two classes – strains CAI4-R1, NR2, and NR4 required between five to tenfold more L-733560 to achieve 90% efficacy, while strain NR3 required over 100-fold more drug. This difference reflects the presence or absence of a wild-type allele at the *CaFKS1* locus; all of the strains with modest elevations in ED_{90} were heterozygous mutants. Only strain NR3, with mutations in both *Cafks1* alleles, required much more L-733560 to reach 90% efficacy. Most important, the results from liquid MIC performed under NCCLS conditions did not distinguish between these heterozygous and homozygous *Cafks1* mutants.

The analysis of these mutants has significant implications for the clinic. Liquid MIC assays performed in clinical microbiology labs provide information about drug susceptibility that can be used to choose a course of therapy, and breakpoints are values that define a clinical isolate as either susceptible or resistant. With these *CaFKS1* mutants and the existing NCCLS testing methodology, any breakpoint that would identify the homozygous mutant as echinocandin resistant would also flag the heterozygous mutants. Yet, each *Cafks1* heterozygote can be effectively treated in infected mice with doses of L-733560 less than 1 mg/kg, which is well under the maximum tolerated dose for this echinocandin. Unfortunately, the most effective assays to discriminate between these heterozygous and homozygous *Cafks1* mutants (echinocandin inhibition of purified GS, or titration in a mouse model of disseminated candidiasis) are not practical for clinical microbiology labs. A thorough evaluation of different methods for measuring in vitro susceptibility, incorporating both laboratory and clinical mutants with altered echinocandin susceptibility, will be required before meaningful progress can be made towards establishing breakpoints for this class of antifungals.

Several semisynthetic echinocandins are active against *A. fumigatus* in animal models of infection (88, 89, 139–141). The original natural product echinocandins had little or no activity against *Aspergillus* spp. but select substitutions have dramatically improved the potency against these filamentous fungi. In vivo efficacy has been demonstrated in models of

pulmonary or disseminated aspergillosis, both alone and in combination with other antifungals. As with the endpoints for measuring the in vitro activity of echinocandins against *A. fumigatus*, there is a strong debate about how to accurately determine drug efficacy in animal models. With the exception of prolongation of survival, most assays are designed to quantify organ burden: counting colony-forming units (CFU) in tissue homogenates, measuring serum galactomannan (142), β-glucan (143), or chitin (144), or using quantitative PCR (145) are methods developed to provide information about fungal mass and the severity of infection. The developmental program of *A. fumigatus* morphogenesis during infection (from spore to germling to mycelium) makes it difficult to rely on tissue CFU measurements to monitor the progression of disease – these values probably under-represent the tissue load at any given time (146). Measuring the level of cell-wall-associated polysaccharides shed from organisms at the site of infection into serum may be accurate under some circumstances, but the assays suffer from inherent challenges that stem from differences in local environments inside the infected tissues and the influence of antifungal therapy on release of cell wall material. Quantifying fungal DNA in tissues has been used to monitor the progression of aspergillosis in infected animals, and efficacy of caspofungin in reducing organ burden has been demonstrated by using this technique. Concerns over whether or not fungal DNA is degraded after cells die, such that the qPCR signal truly equates with viability, still need to be addressed before this technique is widely accepted. In one model of invasive aspergillosis, the qPCR values from lungs of infected rats suggested a paradoxical response to caspofungin, with higher burdens in animals dosed with 4 mg caspofungin/kg/day than in rats given 1 mg/kg/day (147). It is not known whether this result is an artifact of the qPCR method or a consequence of evaluating caspofungin in this particular model of disease. The most thorough evaluation of *A. fumigatus* infection and antifungal efficacy has been described by Walsh and colleagues – in their rabbit model of pulmonary aspergillosis, burden is estimated using CFU, galactomannan (GM) levels, and qPCR values, and severity of infection is monitored by CT scan, lung infarct score, lung weight, and survival (148). For most antifungals, these parameters provide a consistent, comprehensive view of efficacy, but with the echinocandins, the CFU and GM values suggest that burden is actually higher in the caspofungin-treated animals than in the vehicle-treated animals (148, 149). The reason(s) for this discrepancy are not well understood. Since there has been only one description to date of an *A. fumigatus* mutant with reduced caspofungin susceptibility, and the in vivo susceptibility of that isolate has not been reported (113), it remains to be seen which models and which endpoints will provide the most meaningful insight into the echinocandin resistance in *Aspergillus* species.

Other fungi besides members of the *Candida* and *Aspergillus* genera have been evaluated for echinocandin susceptibility in animal models. *P. carinii* is a major cause of morbidity and mortality in HIV-infected patients, and caspofungin is efficacious in a rat model of *P. carinii* pneumonia (150). There are no in vitro susceptibility correlates for evaluating the activity of echinocandins against *P. carinii* because this organism has not been grown in culture. Another human respiratory pathogenic fungus (*C. immitis*) produces disseminated disease when injected into mice, and the infection can be effectively treated with caspofungin, which prolongs survival and reduces CFU in several different target organs (39). The MIC values for two different *C. immitis* isolates were not predictive of the observed efficacy in vivo, but MEC results provided a better correlation. *H. capsulatum* is a dimorphic fungus that has been evaluated for caspofungin susceptibility in murine models of disease with mixed results. In one study, caspofungin was effective in both immunocompetent and athymic mice infected intravenously with modest numbers of yeast cells of a strain whose MIC was 0.25 µg/mL (151); in another study, caspofungin had only a slight effect on survival and fungal burden in a pulmonary model of histoplasmosis (152). The strain used in these immunocompetent mice had an MIC of 8 µg/mL. While there are several differences (strains, route of infection, MIC evaluation of yeast vs. mycelial-phase cells) that could account for the disparate conclusions about susceptibility, the value of in vivo assessment to gauge the response of *H. capsulatum* to echinocandins is clear. Results from in vivo studies with other filamentous fungal pathogens (*R. oryzae*, *Scedosporium prolificans*, *Fusarium solani*) have been reported (96, 153, 154), and the data suggest the antifungal spectrum of caspofungin is broader than predicted by the in vitro susceptibility testing (38).

4 Potential for Cross-Resistance

When a new antibiotic with a novel mechanism of action is introduced into clinical practice, there is usually a widespread expectation that the new agent will be effective against organisms resistant to the existing classes of drugs. Prior to the approval of the first echinocandin in 2001, polyenes and azoles were the predominant antifungal agents used to treat systemic disease. Azole- and polyene-resistant mutants have been identified in both laboratory and clinical settings, and the underlying ergosterol-specific mechanism characterized for several azole-resistant strains suggests there is little likelihood for cross-resistance to echinocandins (155). Studies focused on resistance mechanisms, susceptibility profiles in vitro and in animal models of infection, and clinical outcomes

in patients will be described here in an effort to understand the potential for cross-resistance between echinocandins and other classes of antifungal agents.

Drug efflux is a documented mechanism for reduced susceptibility to azoles implicated in clinical resistance. Genes encoding the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p are induced in vitro when *C. albicans* is exposed to several different azoles. Schuetzer-Muehlbauer et al. (156) evaluated three clinical isolates with elevated MIC values for fluconazole, ketoconazole, and itraconazole – all the three mutants overexpress both *CDR1* and *CDR2* mRNA. The susceptibility of these strains to caspofungin was unchanged in liquid microbroth dilution assays, but the MIC values were slightly higher (≤4-fold) when an agar plate assay was used. Functional expression of the *C. albicans* *CDR2* gene in *S. cerevisiae* conferred reduced susceptibility to caspofungin, and constitutive expression of *CDR2* in *C. albicans* permitted growth at a level of caspofungin twofold higher than the concentration sufficient to inhibit the wild-type parent. A role for efflux in echinocandin resistance implies that these drugs are internalized within the susceptible fungi, which has been suggested in a recent report (157). This paper proposes that a saturable high-affinity facilitated diffusion transporter may be responsible for caspofungin uptake. No specific transporter has been identified, and the question of whether or not echinocandins must be internalized to exert their antifungal activity is still unresolved.

In vitro susceptibility testing of panels of clinical *Candida* species has been used to gauge the prevalence of cross-resistance between azoles and echinocandins. From a collection of nearly 4,000 isolates, 157 *Candida* spp. were categorized as resistant to fluconazole (158). Ninety-nine percent of these strains had caspofungin MIC values less than or equal to 1 μg/mL, which is generally accepted as a level consistent with susceptibility. A study of 2,000 bloodstream isolates from the US demonstrated that three different echinocandins (anidulafungin, micafungin, and caspofungin) are effective against most isolates in the panel, with no correlation between azole resistance and elevated echinocandin MIC values (159). Smaller collections of clinical *Candida* spp., chosen because of poor susceptibility to azoles, were also echinocandin-sensitive (160–163). Finally, the relationship between specific mechanisms of azole resistance and caspofungin susceptibility was explored (164). *C. albicans* clinical isolates used in this study were obtained by direct swab from 12 HIV patients with recurrent oropharyngeal candidiasis, and the resistance mechanisms in these mutants included specific substitutions in the gene encoding the target of the azoles (lanosterol demethylase; *ERG11*), elevated expression of *ERG11*, or induction of efflux pumps in the *CDR* or multidrug resistant (*MDR*) families. None of the mutants, including those that overexpressed *CDR* efflux pumps, were resistant to caspofungin.

A critical counterpart for in vitro analyses is the determination of echinocandin efficacy against drug-resistant fungi in animal models of infection. In one study, two clinical isolates from AIDS patients with oropharyngeal candidiasis were evaluated in mice – these mutants were resistant to fluconazole in vitro and overexpressed the mRNA for either *CDR1* or *MDR1* (165). Induced disseminated infections were treated once a day for 5 days with either micafungin or AmB, and activity was measured by prolongation of survival or reduction in kidney burden at day 6 postinfection. The drugs provided significant efficacy against both the mutants. In another study, a strain of *C. tropicalis* with high MIC values for both fluconazole and AmB was used to challenge immunosuppressed mice and determine the efficacy of micafungin, fluconazole, and AmB against the infection (166). Only micafungin (at doses between 2 and 10 mg/kg) was able to reduce fungal loads in lungs, livers, and kidneys to below the limit of detection – mice treated with AmB or fluconazole still had substantial burdens at the end of therapy. A third study assessed AmB-sensitive and resistant *A. fumigatus* isolates for susceptibility to anidulafungin or AmB in a murine model of invasive aspergillosis (141). Drugs or vehicle were administered to infected mice daily for 10 days; anidulafungin was effective at prolonging survival and reducing lung and kidney burden irrespective of the AmB-susceptibility phenotype, but only the AmB-susceptible strain responded to AmB therapy. These reports highlight the value of animal models in understanding the potential for cross-resistance between echinocandins and other antifungal agents; a broader assessment, using mutants representing different mechanisms of resistance and a variety of different drugs, should provide the most meaningful preclinical guidance for choosing a second drug when confronted with clinical resistance.

Is there any information from the clinic about the potential for echinocandin cross-resistance to azoles or polyenes? There are a number of anecdotal examples of patients who responded to echinocandin therapy following an unsuccessful course of treatment with an azole (167–169) or AmB (170–174), but these cases are complex, and many factors could influence the initial failure and ultimate success of each therapeutic regimen. A retrospective analysis based on four Merck-sponsored trials of esophageal candidiasis (175) identified two different cohorts of patients with relevant information: 15 patients had infections refractory to fluconazole, and 17 patients were infected with *Candida* species whose fluconazole MIC values were high. Among the 14 refractory patients whose infections did not respond despite ≥1 week of fluconazole therapy, 7 of 11 responded to caspofungin, and 2 of 4 responded to AmB. For the 17 patients infected with *Candida* species with reduced susceptibility to fluconazole, 79% (11 of 14) responded to caspofungin and 67% (2 of 3) responded to AmB. Different species of *Candida* (*C. albicans*, *C. guillermondii*, and

C. krusei) with different degrees of reduced fluconazole susceptibility (susceptible – dose dependent versus resistant) were equally represented among the groups. Although the number of patients in this retrospective analysis is small, either caspofungin or AmB was generally efficacious in treating esophageal candidiasis when patients either failed to respond to fluconazole or harbored *Candida* species with reduced fluconazole susceptibility. Taken together, results from the studies of mechanism, susceptibility, and clinical use suggest that reduced azole susceptibility is not linked to echinocandin resistance.

5 Perspective Beyond the Laboratory

A goal for most laboratory studies of susceptibility is to provide an underpinning for recognizing and understanding when clinical failures can be attributed to a resistant pathogen. Since patients with systemic fungal infections nearly always have underlying diseases with high morbidity and mortality, and microbiological responses are not always clear cut, poor outcomes do not mean that resistance was to blame. When an organism is isolated from a patient who did not respond to therapy, it is difficult to define which criteria from laboratory studies are most relevant for defining reduced susceptibility. Experience to date with the echinocandins suggests that *Candida* isolates with high MIC values can often be isolated from patients who responded well to therapy – this phenotype alone was not associated with a poor outcome (176). Expanded clinical use will drive the need for alternative strategies to measure echinocandin susceptibility and sharpen the correlation between values determined using these techniques and clinical response.

5.1 Paradoxical Effect

The antibacterial compounds of the penicillin family, like the echinocandins, target cell wall synthesis. Some strains of bacteria exhibit an unusual in vitro paradoxical response to penicillin, wherein cells are unable to grow at low-to-moderate concentrations of drug, but "breakthrough" growth appears at higher penicillin levels (177). This phenotype has also been referred to as the "Eagle effect," in reference to one of the first authors to describe it (178). A paradoxical effect has recently been described for caspofungin. In one report, Wiederhold et al. (147) evaluated the pharmacodynamics of caspofungin in a murine model of pulmonary aspergillosis. Immunosuppressed mice were infected with *A. fumigatus* AF293 and caspofungin was administered starting 12 h after infection at different doses and intervals – efficacy was determined by monitoring survival over time and measuring lung burden (using qPCR and histopathology) at 4 days postinfection. The qPCR results suggested that a dose of 1 mg/kg caspofungin was superior to both 0.25 mg/kg and 4 mg/kg. The trend towards higher burdens at the 4 mg/kg dose compared to 1 mg/kg was independent of how the dose was fractionated (i.e., four times a day versus once a day versus once every other day). However, neither survival nor histopathology recapitulated this apparent paradoxical caspofungin response. Survival among mice receiving caspofungin therapy ranged from a low of 50% to a high of 85%, with no significant trend towards increased mortality at the highest dose. The degree of hyphal swelling and fragmentation of *A. fumigatus* observed in stained lung sections was similar among mice receiving either the intermediate or high dose of caspofungin. The authors also presented evidence of a paradoxical effect in vitro, based on an XTT dye-based viability assay of both *A. fumigatus* AF293 and a *C. albicans* reference strain.

A more detailed exploration of the in vitro paradoxical effect of caspofungin was recently published (179). Of the 24 *C. albicans* clinical isolates evaluated, three exhibited breakthrough, viable growth at drug concentrations well above the MIC. The paradoxical effect was distinct from true resistance – cultures obtained from the high-concentration wells were susceptible to caspofungin but showed an identical paradoxical response when re-evaluated. The authors report that the effect was observed in different media and testing conditions, although there was no paradoxical effect when susceptibility was measured using an agar diffusion assay. For these three isolates, there was no apparent paradoxical response to the echinocandins anidulafungin and micafungin. Finally, a mini-paradoxical effect (incomplete killing at higher caspofungin concentrations) was observed for more than half of the isolates tested.

There are obvious clinical implications if the paradoxical response to echinocandins reflects a true in vivo phenomenon that occurs at a measurable frequency in fungi associated with human mycoses. The dose range for effective therapy would need to be defined, as would the pharmacokinetic parameter(s) most closely associated with a positive microbiological response. To date, results from animal models of infection have suggested that the highest peak concentration in plasma, or the area under the concentration curve, could be the key potential drivers for maximal echinocandin efficacy, but it has been difficult to correlate plasma levels of drug with outcome. Since the overall safety profile for echinocandins is good, and the response rate is disappointing for some severe infections (i.e., the 45% favorable response with caspofungin as salvage therapy for invasive aspergillosis (31)), there has been a call among many clinical mycologists to push higher doses of the echinocandins in selected patient populations. This would clearly be a dangerous strategy if the target organisms are less susceptible at the elevated drug doses. Future studies of the paradoxical effect, particularly with *C. albicans*, must expand into animal models of infection

and utilize multiple efficacy endpoints to ensure that meaningful conclusions can be made about the pharmacodynamic properties of each echinocandin.

5.2 Clinical Isolates

There have been few reports of resistance to CANCIDAS® since the drug was approved in 2001. Fungal strains with high caspofungin MIC values have been isolated from patients who failed therapy, but the reduced susceptibility in liquid microbroth dilution assays alone is not sufficient to confirm the clinical resistance. Since the results from clinical trials with caspofungin provided little correlation between MIC values and outcome (176), supporting data from other assays (animal models of infection, GS enzyme inhibition) are necessary to provide a critical confirmation that the resistance phenotype identified in a liquid microbroth dilution assay is meaningful.

A paper by Hernandez et al. (180) describes an AIDS patient with esophageal candidiasis who responded initially to caspofungin therapy, but failed when the infection returned. Earlier therapy with fluconazole and AmB had also failed. Three *C. albicans* isolates were recovered from the oral cavity of this patient – isolate 1 was obtained prior to caspofungin administration, isolate 2 was obtained when the infection returned (22 days after completion of the first successful treatment with caspofungin, but before the second unsuccessful course), and isolate 3 was collected after the second course of caspofungin therapy was deemed a clinical failure. Genotypic analysis of the three isolates suggested that they were indistinguishable; the patient either had a persistent infection, or was re-infected with the same isolate after the first successful course of caspofungin therapy. The MIC values (determined by NCCLS method M-27A) were 0.25, 0.25, and >64 µg/mL for isolates 1, 2, and 3, respectively. The authors measured the response of these strains to caspofungin in a murine model of disseminated infection. Caspofungin was administered IP once daily at different doses beginning 1 day after infection for a total of 7 days – kidney burden was assessed to determine efficacy. A separate group was given fluconazole (5 mg/kg twice per day by oral gavage), and there was no reduction in burden. Both isolates 1 and 2 responded to caspofungin therapy, with significant reductions in kidney CFU at nearly all doses. In contrast, isolate 3 was less responsive, requiring the highest dose of caspofungin (1 mg/kg/day) before kidney burdens were significantly reduced.

Insights into the key issues regarding echinocandin susceptibility and resistance may be gained from further evaluation of these clinical isolates. The mechanism responsible for reduced susceptibility to both fluconazole (all isolates) and caspofungin (isolate 3) has to be determined. Since the "baseline" isolate for caspofungin therapy was already insusceptible to fluconazole, it is likely that the underlying mechanisms conferring drug resistance to the two drugs are distinct. Rather than a single mutation leading to azole-echinocandin cross-resistance, this *C. albicans* strain may have acquired two or more independent mutations, at different times, that act independently by conferring reduced susceptibility to only one drug. Characterization of the mechanism of resistance will also provide a counterpoint to the in vitro studies described to date – do any of the *FKS* mutations, salvage pathways, or gene expression changes identified in laboratory-based studies of resistance have any relevance in this clinical setting? A simple comparison of caspofungin IC_{50} values for GS derived from isolates 1, 2, and 3 could point to an obvious, target-based explanation for the reduced caspofungin susceptibility of isolate 3. Lastly, an understanding of why acquired resistance developed in this patient, but not in similar cases, might aid in the development of treatment guidelines which could minimize the risk of acquired echinocandin resistance. Factors such as the sequence and duration of antifungal therapy, the presence of a sustained, heavy esophageal burden, or the inherent mutability of isolate 1 may all have contributed to the emergence of the resistant mutant in this patient – appropriate animal models should be developed to explore the influence of each of these factors in resistance development.

6 Outlook

The echinocandins represent the first new class of antifungal drugs available for treatment of human systemic fungal infections in the past decade. Caspofungin was approved in 2001 to treat invasive aspergillosis in patients who are refractory to or intolerant of other therapies (29); since then, indications for esophageal candidiasis, candidemia, other *Candida* infections, and empirical therapy of suspected fungal infections in patients with persistent fever and neutropenia have been approved. As caspofungin is prescribed more often, and new 1,3-β-D glucan synthesis inhibitors are approved for clinical use, it is likely that more echinocandin-resistant mutants will be isolated from patients. There are a few key points to keep in mind when considering the future of echinocandin susceptibility. First, there is a finite, measurable frequency for the emergence of spontaneous echinocandin resistance in a population of cells under selective pressure. Although the mechanism of echinocandin inhibition has not been clearly defined, it is likely that these compounds bind to a component of the GS complex (perhaps the Fks protein), and mutations that perturb the interaction between echinocandins and their putative binding site should confer resistance to their inhibitory effect. For *C. albicans*, a frequency of 1×10^{-8} mutations per cell per generation was obtained in liquid culture using the pneumocandin L-733560 (111); in a resistance induction

study, the MIC value of a wild-type *C. albicans* strain was unchanged following 40 passages in the presence of a subinhibitory concentration of caspofungin (34). Both of these laboratory studies suggest that spontaneous mutations conferring resistance to echinocandins are rare, and the experience with caspofungin in clinical practice to date is consistent with this notion. Second, the potential for cross-resistance among the echinocandins has not been explored. There are no published reports describing micafungin- or anidulafungin-resistant mutants, so the incidence or mechanism of resistance for these agents is unknown. As with caspofungin, mutations in the *FKS* genes are a likely means for resistance development, but it is critical to understand if one set of substitutions in the Fks protein will confer pan-echinocandin resistance, or if each compound will require unique amino acid changes. Third, the lack of correlation between echinocandin susceptibility testing results and clinical outcome suggests that microbiologists and physicians will face a continuing challenge in their efforts to distinguish clinically relevant resistance from phenotypes (such as the paradoxical effect or trailing MIC endpoints) that may not be predictive of microbiological failures. Results obtained with laboratory-derived *C. albicans Cafks1* mutants illustrate the difficulty – the echinocandin MIC values for the heterozygous and homozygous *Cafks1* mutants were equally high, but the heterozygous mutants were much more susceptible to therapy in a mouse model of disseminated infection. If a standardized liquid- or agar-based test could not be used to provide meaningful susceptibility results, and future clinical experience identifies specific, select changes in *CaFKS1* or another gene that are associated with reduced echinocandin susceptibility, perhaps molecular diagnostic tools could be used to quickly and accurately reveal these mutations. Lastly, treatment of infections caused by rare moulds and other less common fungal pathogens represents an unmet medical need, and the potential therapeutic role for the echinocandins is poorly understood. More work needs to be done not only to characterize the structure and synthesis of cell walls in these organisms, but also to clarify the susceptibility both in vitro and in animal models of disease.

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Chapter 29
Antifungal Targets, Mechanisms of Action, and Resistance in *Candida albicans*

Robert A. Akins and Jack D. Sobel

1 Introduction

Antifungal resistance at the gene level has been studied in *Candida albicans* for about a decade now. Cloning of *C. albicans* genes by homology to resistance genes in *Saccharomyces cerevisiae*, heterologous expression of *C. albicans* genes in *S. cerevisiae*, regulated expression in *C. albicans*, and microarray-based expression analysis, have allowed rapid progress in identifying and studying the five major *C. albicans* genes involved in resistance to clinically used antifungals: ABC transporter genes CDR1 and CDR2, major facilitator efflux gene MDR1, and ergosterol biosynthesis genes ERG11 and ERG3. Analysis of these genes indicates that resistance involves alterations to the enzyme targeted by fluconazole (FLZ), encoded by ERG11, and upregulation of P-glycoprotein type ABC transporters and major facilitators (MFs) that efflux azoles and terbinafine. Potential alterations to ERG3 or its regulation have been understudied in *C. albicans*. Resistant isolates from clinical samples, especially in oropharyngeal candidiasis (OPC), typically display stepwise mutations in more than one of these genes. Key mutations hyperactivate transcriptional activators of CDR1 or MDR1. However, it is clear from in vivo and in vitro studies that mutations of these major genes do not completely account for the evolution of high-level azole resistance in some clinical isolates. More work is needed to identify other genes that contribute to resistance in *C. albicans*. Very little is understood about reversible, adaptive resistance of *C. albicans*, despite its potential clinical significance. Most clinical failures to control non-OPC infections occur with in vitro susceptible strains. There has been important discovery of tolerance mechanisms to azoles. Heterologous studies in *S. cerevisiae* on regulation of target genes have been less useful because of differences in regulation in *C. albicans*. Nevertheless, recent progress has been made in identifying genes that regulate CDR1, MDR1, and ERG genes.

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There is no shortage of reviews on the subject of antifungal resistance in *Candida* (62, 153, 205, 220, 278, 301, 464, 465, 475, 547, 548, 575). To the extent that this chapter is useful, it stands on the shoulders of these reviewers. We particularly recommend Sanglard and Bille's book chapter (465) for its broad scope and meticulousness, Lamb and Kelly's clear description of the toxic sterol model for azole inhibition (278), an excellent compendium and analysis of ERG11 mutations from the folks and affiliates at the Janssen Research Foundation (322), and a focused analysis and modeling of efflux mechanisms of resistance (427). Finally, many observations in this chapter derive from and extend those in our earlier review (3). The focus of this chapter is *C. albicans*, but in areas in which *Candida* research is underdeveloped we resort to available information on *S. cerevisiae* or *C. glabrata*.

2 Ergosterol Biosynthesis Genes and Antifungal Resistance

The ergosterol biosynthetic pathway converts acetic acid to ergosterol, using largely the same enzymes as in the mammalian biosynthesis of cholesterol (Table 1). The ergosterol pathway is really two pathways. Reactions up to farnesyl pyrophosphate (ERG20) are essential and constitute the isoprenoid pathway, generating isoprenoids, carotenoids, prenyl groups for membrane attachments, tRNA modifications, etc. The bulk of the intermediates flow on via Erg9p, to ergosterol. In *S. cerevisiae*, genes at or above ERG20 are essential for growth, and cannot be rescued by supplementing with ergosterol, whereas most of the downstream genes are not essential for viability; for example, null mutants at ERG11 can be grown in the presence of ergosterol.

Enzymes in the ergosterol pathway are the targets of many antifungal agents, including the most widely used clinically, the azoles, and terbinafine. However, there are untapped targets in the pathway, including some which are less homologous or not homologous to human enzymes. The identity of the *C. albicans* genes based on homology to the *S. cerevisiae*

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Table 1 Ergosterol biosynthetic pathway: enzymes, gene homologies, inhibitors, and resistance mechanisms within the pathway

Product	Gene	Activity	Ca/Sc ID(sim)	Ca/Hs ID(sim)	Inhibitor	Resistance	Hypersusceptible
Acetyl-CoA							
Acetoacetyl-CoA	ERG10	Acetyl-CoA C-acetyltransferase	63(74)	42(56)			
HMG-CoA	ERG13(HCS1)	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase	67(80)	49(66)			
Mevalonate	HMG1,HMG2	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase	46(59)	38(52)	statins		
Mevalonate phosphate	ERG12	Mevalonate kinase	39(53)	31(49)			
Mevalonate pyrophosphate	ERG8	Phosphomevalonate kinase	40(56)	No ortholog			
Isopentyl pyrophosphate	ERG19	Mevalonate pyrophosphate decarboxylase	56(65)	43(55)			
Dimethylallyl phosphate	IDI1	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase	52(67)	50(68)			
Geranyl phosphate	ERG20	Dimethylallyltranstransferase	73(84)	45(63)			
Farnesyl pyrophosphate	”	Geranyltranstransferase					
Squalene	ERG9	Squalene synthase	56(70)	46(62)	zaragozic acid(Z)	↑ScERG9 (Z,A,T)	↑ScERG9 (AmB)
2,3-Oxidosqualene	ERG1	Squalene epoxidase	52(68)	37(53)	terbinafine(T)	↑ERG1	
Lanosterol	ERG7	Lanosterol synthase	62(74)	39(57)			
4,4,-Dimethyl-8,14,24-trienol	ERG11	C14-lanosterol demethylase	64(78)	39(53)	azoles(A)	erg11p, ↑ERG11, Δerg11(?)	
4,4-Dimethyl zymosterol	ERG24	C14-sterol reductase	57(70)	32(48)	Morpholines (M)	Scerg24p, Δerg24(A), ↑ERG24	
	ERG25	C4-sterol methyloxidase	58(69)	39(53)	PF1163A		
4-Methyl zymosterol	ERG26	C4-sterol decarboxylase	69(80)	32(52)			
Zymosterol	ERG27	C3-sterol ketoreductase	60(74)	23(42)			
Fecosterol	ERG6	C-24 sterol methyl-transferase	65(77)	None		Δerg6(AmB); -↑ERG6(A)	Δerg6(T,M)
	ERG28	erg scaffold	57(74)	38(65)			
Episterol	ERG2	C8-sterol isomerase	58(69)	58(69)	Morpholines		Δerg2(T,A)
Ergosta-5,7,24(28) trienol	ERG3	C5-sterol desaturase	51(61)	45(60)		Δerg3(A, AmB)	↑ERG3
Ergosta-5,7,22,24(28) tetraenol	ERG5	C22-sterol desaturase	71(82)	None	Azoles?		
Ergosterol	ERG4	C24-sterol reductase	58(71)	25(40)			

Sequential products are listed vertically, next to the enzyme responsible for that product. ERG28 may act as a scaffold for genes acting upstream. % identity (% similarity) of the *C. albicans* gene (Ca) to the *S. cerevisiae* (Sc) or *Homo sapiens* (Hs) homolog were derived from Blast analysis on the NCBI website. Mutation symbolism: ergXp: point mutations in the ErgX protein; Δergx: disruption of ERGX (both alleles); ↑ERGX: overexpression of ERGX. Sc prefix indicates data is only available for *S. cerevisiae*. Drugs to which mutations confer resistance or hypersusceptibility are in *parentheses* if different than the inhibitor targeting that enzyme. Evidence and references for these are in the text

genes is unambiguous and likely orthologous, ranging from 39 to 73% identical and 53–84% similar over the length of the entire open reading frame (ORF), with the exception of ERG8. Fungal ERG8, encoding phosphomelvelonate kinase, has no ortholog in man, or in multicellular animals (200). CaERG8 does have orthologs in bacteria, and perhaps in plants. Human phosphomelvelonate kinase has orthologs down to *C. elegans*. This lack of a human ortholog and the fact that ERG8 in *S. cerevisiae* is essential (541) make pErg8 an ideal target for antifungals. However, CaERG8 is not essential (447); it is not yet known whether this is a fundamental difference between species or whether another kinase in *C. albicans* suffices in the absence of CaErg8p. CaERG5 and CaERG6 have no homologs in man since they catalyze reactions found in ergosterol but not in cholesterol.

There are a variety of azoles (Fig. 1) that all act by binding and inhibiting Erg11p, a cytochrome P450 enzyme lanosterol 14α-demethylase (Fig. 2). Some of the newer azoles are active on FLZ-resistant *Candida* species (530). Azole inhibition does not block the pathway at lanosterol, athough lanosterol concentrations do increase. Instead, lanosterol, with its 14-methyl group intact, is acted on by downstream enzymes to generate 14-methylated intermediates. The model, based on observations in *S. cerevisiae*, is that one of these intermediates, 14-methylergosta-8,2(28)-dien-3,6-diol, is toxic and is responsible for growth inhibition. The main evidence for this is that mutation or deletion of the gene encoding Erg3p, which forms this toxic sterol from14-methyl fecosterol, confers resistance to azoles (239, 571). In this model (278) it is not the lack of ergosterol that is inhibitory, but the accumulation of the toxic intermediate. They suggest that the hydroxyl group on the 6th carbon disrupts hydrophobic interactions of the intermediate with plasma membrane phospholipids, permeablizing the membrane.

The toxic diol model is not universally accepted for *C. albicans*. Early observations showed that viable ERG11 mutants accumulated significant amounts of the diol (35, 491). However, this may be a quantitative difference, since

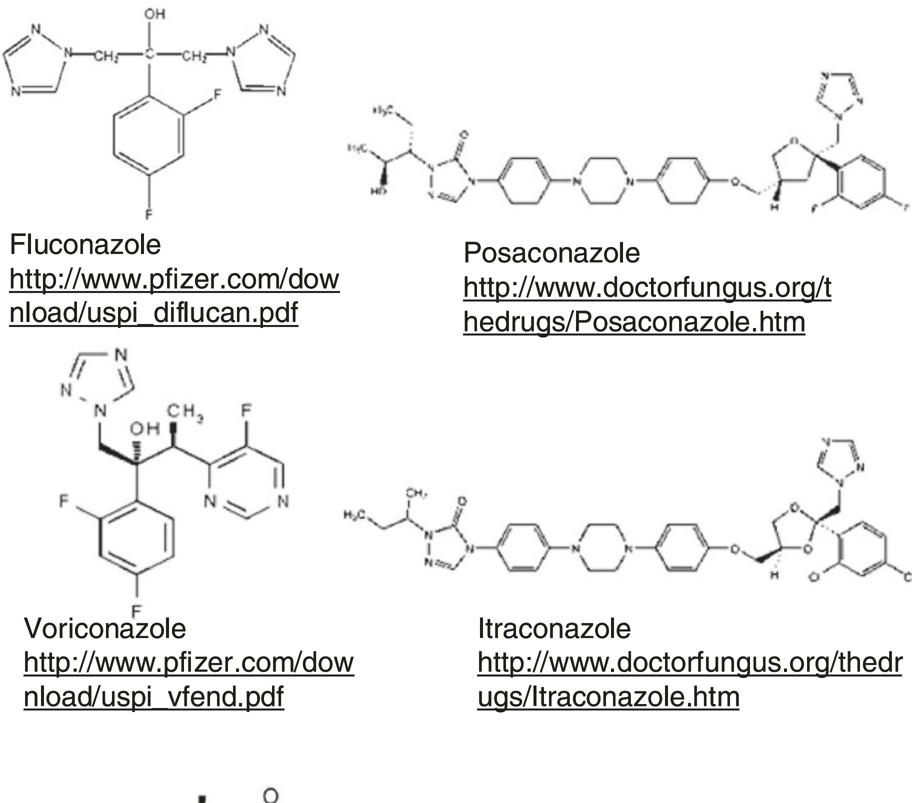


Fig. 1 Structures of select azoles in clinical use or trials

lanosterol

14C

ERGII, C14-lanosterol demethylase

4,4,-dimethyl-8,14,24-trienol

Fig. 2 C-14 lanosterol demethylase encoded by ERG11

the ERG11 mutants show reduced growth. Furthermore, the identity of the diol in these papers is inconsistent with an earlier analysis (32). The ERG11 mutant is not characterized; in other work, CaERG11 mutants have had other defects, for example in respiration (492). Because of these problems, and since CaERG3 mutations behave like ScERG3 mutations, discussed below, the toxic sterol model probably applies to *C. albicans*, although it may have to accumulate to a higher concentration for complete inhibition than in *S. cerevisiae*.

ERG11. Resistance due to point mutations in the target gene ERG11 is complex and does not seem to be the dominant mechanism among resistant clinical isolates. At least 12 point mutations among more than 80 polymorphisms in ERG11 confer or are associated with azole resistance (Fig. 3). It is likely that this inventory is not yet saturated. Figure 3 includes only mutations that are backed by indicated supporting evidence that they are responsible for resistance, such as: heterologous expression in *S. cerevisiae* of wild-type *C. albicans* gene after site-directed mutagenesis; in vitro enzyme assays extracts from *S. cerevisiae* transformed with *C. albicans* gene that was altered by site-directed mutagenesis; overexpression in *S. cerevisiae* of cloned genes from resistant isolates of *C. albicans*; in vitro assays of enzymatic activity of extracts of resistant clinical isolates. The mere presence of polymorphisms, even if seen only in resistant isolates, is not sufficient.

How do these mutations fit into a tentative structural model of the enzyme (278, 322)? Although this integral membrane protein has not been crystallized, homology models based on the crystal structure of CYP51 of *Mycobacterium tuberculosis* have been constructed (136, 215, 216, 312, 585). It is clear that azoles bind near the heme group in the enzyme, and mutations that interfere with this interaction confer resistance to azoles. These mutations preferentially affect the short-chained azoles, FLZ, and voriconazole, but are less effective at blocking posaconazole and itraconazole owing to the additional stabilizing interactions of their long chains with residues along the inner channel 2. Conversely, mutations along channel 2 may confer resistance only to the latter azoles by precluding their binding. A newer triazole, FX0685 (Fig. 1), has been shown to be effective against resistant species, including *Aspergillus fumigatus*, and against strains and enzymes with a limited number of ERG11 resistance mutations, both in vitro and in vivo, although it is still susceptible to efflux by overexpressed Cdr1p (530).

Figure 3 does not include polymorphisms that have been seen in susceptible isolates, listed in (322) and (71), since these are less likely to cause resistance. Nevertheless, some of these play a complex role in resistance when together in critical combinations, or may increase resistance levels conferred by some resistance mutations alone (469). Conversely, mutant S405K alone confers moderate resistance, but is susceptible in combination with some other polymorphisms (322). These observations suggest that more independent isolates need to be screened for polymorphisms and mutations, and more of these need to be functionally tested, ideally by site-directed mutagenesis and expression in *C. albicans*. The variety of mutations and locations in the ORF complicate their molecular screening as a means of predicting clinical resistance.

It is still not entirely clear that an increased level of Erg11p alone is sufficient to confer high-level azole resistance in *C. albicans*. Overexpression of ScERG11 in *S. cerevisiae* on a centromeric plasmid, driven by the GAL1 promoter, conferred galactose-dependent high-level azole resistance (257). Overexpression of Erg11p in *C. glabrata* is associated with chromosomal duplication (323, 549). Interestingly, the duplication results in an eightfold, not a twofold, increase in mRNA, suggesting that ERG11 expression is normally limited by a repressor which is titrated out by the duplication. Resistance via chromosome duplication has not been demonstrated in *C. albicans*, although it does result from chromosome loss (404, 456). Tandem gene duplications have not been demonstrated to be a resistance mechanism in *C. albicans*. Overexpression of ERG11 is seen in some resistant *C. albicans* clinical isolates (442); however, when examined, the level of expression poorly correlated with resistance (71). Upregulation of ERG11 transcription in clinical isolates has recently been shown to result from hyperactivating mutations in its transcriptional activator encoded by UPC2 (313, 385, 501, 576). However, since this mutation upregulates many other genes, resistance due to this mutation cannot be ascribed solely to its effect on ERG11. Recent analysis of a clinical isolate of *C. tropicalis* isolate suggested that its FLZ resistance resulted from a combination of a fivefold overexpression and a point mutation in ERG11, not from increased efflux; this mutant also had an elevated level of respiration and of ergosterol (550).

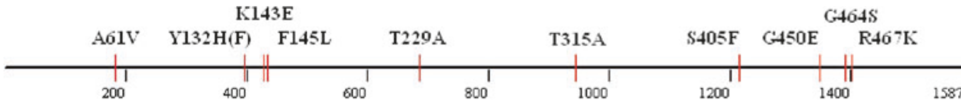


Fig. 3 Resistance mutations in ERG11. Data is compiled from (3, 29, 398). Other mutations were reported [436], but their link to resistance is uncertain since they only appeared in resistant isolates that had other known resistance mutations and/or upregulation of CDR2. These include A107T, G448V, V452A, V509M, Y257H, and G307S. Mutations identified in (589) only documented changes in resistant isolates without analyzing other genes; potential mutations not seen in susceptible isolates include A114S D116E, K128T, Y257H, F449Y, I471T, and Q474K

We have developed a reliable multicopy shuttle plasmid for *C. albicans* to address this issue. The selective marker in this plasmid is the wild-type gene encoding inosine monophosphate (IMP) dehydrogenase; only when overexpressed can this gene confer resistance to the IMP analog mycophenolic acid (MPA), forcing amplification of the plasmid to about 40 copies per cell. Wild-type genes cloned into the plasmid, behind vector-derived or native promoters, overexpress their product (119). Overexpression of a fusion product consisting of mostly CaErg11p in *C. albicans* confers moderate FLZ resistance (119), and overexpression of the native protein driven by its own promoter confers significant resistance to azoles, but still only 16–32 μg/mL (unpublished observations). This is expected, since overproduction of target should allow continued demethylation by enzyme that is not bound by azole. In these transformants, normal to near-normal levels of ergosterol accumulate even in 64 μg/mL FLZ.

What is the effect of disruption of ERG11? Recently, disruption of both alleles of ERG11 in *C. albicans* was reported. The disruptant is aerobically viable, and is resistant to high concentrations of FLZ (474). In contrast, in *S. cerevisiae*, this deletion, or inactivating point mutations, are lethal unless supplemented with ergosterol and fatty acid under anaerobic conditions, or unless there is a second mutation in ERG3. A number of ScERG11 mutants identified as viable and azole resistance turned out to have secondary mutations in ERG3 (35, 150, 227, 239, 571). On one level, the disruption in *C. albicans* is gratifying, since it removes the target of inhibition, so the cell is resistant. However, this poses a problem for the model for azole mechanism of action, and/or calls into question the genotype of the ERG11 disruptant strain (474). Deletion of the gene should introduce the same growth-inhibited phenotype as wild-type cells inhibited by FLZ, because it should generate an inhibitory concentration of 14-methylergosta-8,24(28)-dien-3,6-diol. That is, the disruptant should be viable but capable of very slow growth at best even in the absence of azole. It is possible that the *C. albicans* ERG11 disruptant has other mutations that are responsible for the phenotype (474), despite characterizations of it ERG3 genes.

These results seem inconsistent with a second study, which used a different approach to shut down expression of ERG11. ERG11 was underexpressed by disrupting one allele and placing the second under control of the tetracycline repressor. Under these conditions, growth was reduced by 90% (447). While the authors are not clear whether complete inactivation of expression was cidal, these observations suggest that expression is essential for growth. However, the tet repressor results are called into question by the observation that tetracycline is a potent synergen of some antifungals (384); thus, the observed growth inhibition may have resulted from this synergy rather than further inhibition of ERG11 expression. Until these issues are resolved, it is premature to conclude that ERG11 is not essential for growth in wild-type *C. albicans*, or that its deletion confers resistance to azoles.

If it is eventually resolved that *C. albicans* can grow aerobically without ERG11 and without second mutations in ERG11, we suggest that removing ERG11 is different than inhibiting it with azoles. The difference is that azoles also inhibit the “other” P450 enzyme in the pathway, encoded by ERG5 (Fig. 4). Indeed, azoles inhibit in vitro activity of ScErg5p, a Δ22 desaturase P450 enzyme with almost the same efficacy as Erg11p (206). In this model, azoles contribute to accumulation of toxic sterol in two ways: by blocking demethylation of lanosterol and by constricting the pathway below Erg3p, at Erg5p. Deletion of ERG11 does not completely inhibit growth, in part because active Erg5p hypothetically reduces the pool of 14-methylergosta-8,24(28)-dien-3,6-diol. Consistently, Sanglard’s group showed that the ERG11 disruptant accumulates reduced amounts of intermediates at and downstream of 14-methyl fecosterol (474). In contrast, wild-type cells inhibited by azoles accumulate significant amounts of these downstream derivatives (239) and presumably have induced levels of Erg3p (509). *C. albicans* may be better at this than *S. cerevisiae* because it may upregulate ERG5 more in response to loss of ERG11.

ERG3 encodes the Δ5,6 desaturase (Fig. 5), and is responsible for converting tolerated 14-methyl intermediates, which accumulate because of azole inhibition of 14C-lanosterol demethylase, into the toxic sterol 14-methylergosta-8,24(28)-dien-3,6-diol (239, 571). Therefore, ERG3 inactivation should and does confer azole resistance. Wild-type strains exposed to azoles typically accumulate eburicol, obtusifoliol, and the toxic sterol, whereas ERG3 mutants accumulate mostly ergosta-7,22-dienol in the absence of azole, instead

Fig. 4 22-C sterol desaturase encoded by ERG5

Fig. 6 Squalene epoxidase encoded by ERG1, inhibited by terbinafine

Fig. 5 5,6-C sterol desaturase encoded by ERG3

of ergosterol, and mostly 14a-methylfecosterol after azole exposure, both in *S. cerevisiae* and *C. albicans* (153, 278, 341, 474, 546, 575). Deletions of both alleles of CaERG3 conferred high-level azole resistance (474), suggesting that diol formation by Erg3p is inhibitory in *C. albicans* as in *S. cerevisiae*. Another consequence is a twofold increase in expression of ERG11. No changes were seen in expression of CDR1/2 or MDR1 (341).

Despite this high level of in vitro azole resistance, in vivo resistance was not affected by ERG3 deletion. Kidney burden in a mouse candidiasis model was reduced by FLZ to about the same extent in the mutant as the wild type. Overall virulence of the mutant was reduced, even though in vitro growth rate in the mutant was not changed (341). This intriguing difference between in vitro and in vivo resistance could have many explanations; a simple hypothesis is that 14a-methylfecosterol-substituted membranes in the mutant exposed to azole further reduce its virulence, giving the net result of FLZ susceptibility.

There are species-specific differences in the effects of ERG3 mutations. In contrast to results in *C. albicans* and *S. cerevisiae*, deletion of ERG3 in *C. glabrata* did not confer azole resistance (150). Most spontaneous azole-resistant mutations in *S. cerevisiae* occur in the ERG3 gene and are recessive. These would go undetected in the diploid *C. albicans*. However, if lineages exist or arise that are heterozygous for inactivating mutations in ERG3, then ERG3 mutations could be or become a common mechanism of resistance, restricted to these lineages. It is also reasonable to expect that mutations that repress transcription of ERG3 could confer resistance in clinical isolates, independent of lineage. Sequencing and expression analysis of this gene in clinical isolates therefore need more attention. Upc2p, a global transcriptional activator of many ERG genes, coordinately upregulates ERG3 (121, 611).

ERG1 is an essential gene encoding squalene epoxidase; its inhibition by terbinafine results in ergosterol depletion and squalene accumulation (Fig. 6). Terbinafine is fungistatic for most *Candida* species, but fungicidal for filamentous fungi (213, 457, 458). Terbinafine, like azoles, is fungicidal for *C. albicans* if combined with calcineurin inhibitors (388, 470). Strains that are resistant to azoles are normally not cross-resistant to terbinafine (194), unless resistance is based on overexpression of CDR1.

Single point mutations in ScERG1 confer resistance to terbinafine, but this is not reported for *C. albicans* isolates. The *S. cerevisiae* mutants maintain ergosterol in their membranes despite squalene accumulation, suggesting that normal cells are inhibited by the depletion of ergosterol rather than by accumulation of squalene (250, 285). The *S. cerevisiae* database (http://db.yeastgenome.org/cgi-bin/locus.pl?locus = erg1) indicates that deletion of ERG1 is lethal unless maintained under anaerobic conditions to allow uptake of exogenous ergosterol. More analysis of CaERG1 is warranted, to determine whether *C. albicans* follows the example of *S. cerevisiae*.

Expression of ERG1 and other ERG genes is increased about fivefold upon exposure to terbinafine in susceptible strains of *C. albicans* or *S. cerevisiae* (190, 286), and it is coordinately regulated by Upc2p (121, 611). Consistently, screening of our *C. albicans* overexpression library resulted in the isolation of ERG1 overexpressant that was highly resistant to terbinafine, without cross-resistance to azoles (unpublished data). Similar results were reported in *A. fumigatus* (298). These data are inconsistent with global expression profiling of genes responding to terbinafine, which did not show ERG1 upregulation (603). Parenthetically, overexpression of a napthalene-degrading enzyme, salicylate

1-monooxygenase, conferred terbinafine resistance in *A. nidulans*, presumably by enhancing its degradation (158); if this inactivation is verified, it represents an unusual, if not unique, resistance srategy in fungi that is commonplace in bacteria.

Genes other than the target ERG1 can affect susceptibility to terbinafine. Overexpression of *C. albicans* genes, efflux genes CDR1, CDR2, or MDR1 in *S. cerevisiae* results in resistance to terbinafine and azoles (471, 481, 482). Consistently, clinical strains that overexpress CDR1 are more resistant to terbinafine (458). Disruption of CDR1 in *C. albicans* confers hypersusceptibilty to terbinafine and azoles (473). Disruption of MDR1 in C. *dubliniensis* does not alter susceptibility to terbinafine, even though its overexpression in *S. cerevisiae* results in resistance (582). Mutations in ERG2 or ERG11 result in hypersusceptibility to terbinafine, azoles, inhibitors of sphingolipid synthesis, and other agents (354). Disruption of ERG1 is likely to be lethal, as it is in *S. cerevisiae*, except under conditions that allow uptake of exogenous ergosterol. However, transposon-mediated disruption of ERG1 was achieved in *C. glabrata*, resulting in increased resistance to both terbinafine and azoles (540). Disruption of ERG3 does not alter terbinafine susceptibility, even though it confers azole resistance (474). Disruption of CYB5, encoding a cytochrome component of ERG3, results in hypersusceptibility to terbinafine and azoles (448). Disruption of CDC35, encoding adenylate cyclase, or CAP1, encoding cyclase-associated protein, confers hypersusceptibility to terbinafine and azoles, suggesting a role for cAMP signaling involving regulation of CDR1 (207).

ERG6 encodes C-24 sterol methyltransferase, which catalyzes a reaction not found in the cholesterol biosynthetic pathway (Fig. 7), making it an attractive target for antifungals. Its disruption in *S. cerevisiae* confers pleiotrophic defects that include slow growth, poor mating, poor uptake of tryptophan, increased permeability, and increased cation and antifungal susceptibilities, suggesting increased membrane permeability (34, 138, 249, 572). Mutations in CaERG6 confer resistance to nystatin, and the gene is upregulated by exposure to FLZ and in an azole-resistant clinical isolate, even though it is not upregulated by Upc2p (121, 611). Its disruption in *C. albicans* conferred hypersusceptibility to terbinafine, cycloheximide, fenpropiomorph, and tridemorph (but not to azoles), and resistance to amphotericin B (AMB) (212). The authors surmise that these hypersusceptiblities were due to increased permeability. However, permeability was not directly assayed, and the effects may have also arisen from reduced efflux by the efflux pump Pdr5p (237). Unexpectedly, overexpression of CaERG6 on our multicopy plasmid confers resistance to azoles but not to terbinafine. A presumably inactivating mutation in CgERG6 increases susceptibility to AMB (551). Antifungal inhibitors that target ERG6 should be potent synergens with existing antifungals, unless overexpression of CDR1 or MDR1 can override the potentially permeabilizing effects of inhibiting Erg6p.

Fig. 7 C-24 sterol methyltransferase, encoded by ERG6

ERG24 encodes C14-sterol reductase, which is inhibited by the fungicide fenpropimorph. In *S. cerevisiae*, this inhibition results in the accumulation of ignosterol (ergosta-8,14 dien 3β-ol) (Fig. 8). Therefore, downstream enzymes Δ 8–7 isomerase, Δ 5-desaturase, and the Δ 22-desaturase are inactive on sterols that retain the C14=C15 double bond. This also suggests that ignasterol is not tolerated. It perturbs the membrane and inhibits uptake of glucose and pyrimidines. Mutations in ScERG24 confer resistance, and ScERG24 disruptants are aerobically viable, but only on defined media, a reflection of their dependency on their increased Ca^{++} in the media. Suppressor mutants of ScERG24 resistance mutants, *fen1* and *fen2*, have been reviewed (85, 400). These encode a fatty-acid chain elongase (383), and a plasma membrane H^+-pantothenate symporter (528). Mutations in either gene result in a reduction in membrane very-long-chain fatty acids and sphingolipids. These and other observations (486) suggest that the fungicidal effect of the sterol intermediates that accumulate after inhibiting Erg24p depends on their interactions with these lipids.

CaERG24 was cloned by complementation of an *S. cerevisiae* erg24 mutant. Disrupting the gene *C. albicans* induces slow growth, slight resistance to azoles and nystatin, and hypersusceptibilty to cycloheximide, cerulenin, fluphenazine, and brefeldin A, all consistent with increased permeability. Disruptants were unable to germinate and showed reduced virulence in a mouse-disseminated candidiasis model (217). These results indicate that CaErg24p is similar to ScErg24p and is a potential target for next-generation antifungals.

Nothing is published about mutations in or overexpression of CaERG24. It is coordinately upregulated by Upc2p (121, 611). Its overexpression on our multicopy plasmid confers resistance to azoles; these transformants have not been tested yet for resistance to fenpropomorph (unpublished results).

ERG25 encodes the essential C4-sterol methyloxidase, which acts with Erg26p to sequentially remove the two C4-methyl groups. Its disruption in *S. cerevisiae* results in

Fig. 8 Formation of zymosterol from sequential actions of C14-sterol reductase, C4-sterol methyloxidase, and C4-decarboxylase, encoded by ERG24, 25, 26. Formation of ignasterol by inhibition of C14-sterol reductase with fenpropimorph (left branch). Structure of fenpropimorph is from http://www.hclrss.demon.co.uk/fenpropimorph.html

sterol auxotrophy, indicating that the accumulated 4,4-dimethylzymosterol is not a tolerated membrane sterol. This can be suppressed by a second mutation in ERG11 or by azoles. In this situation, azoles actually promote growth of the *erg25* mutant. The intermediate that accumulates is 24-methylenelanosterol, which apparently cannot be partially acted on by Erg3p to create a toxic sterol (140).This suggests that another route to azole resistance is by inactivating mutations in, or transcriptional repression of, ERG25. An intriguing extrapolation to *C. albicans* is that downregulation of ERG25 may confer phenotypic resistance during azole exposure. However, CaERG25 is upregulated in strains with hyperactive Tac1p (230); it is not upregulated by Upc2p, but its sister gene ERG26 is (121, 611). The cloned CaERG25 gene is able to rescue *erg25* mutants in *S. cerevisiae* (244), but whether disruptants and azole suppression will be the same in *C. albicans* has not yet been determined.

Inhibitors that block C4 demethylation have similar effects in *S. cerevisiae* and *C. albicans*. A natural antifungal agent, PF1163A, derived from Penicillium, reportedly inhibits ScErg25p, since wild-type cells are inhibited but cells overexpressing Erg25p are not (374). The agent also inhibits *C. albicans*, but resistance has not been reported (375). Another agent, 6-amino-2-n-pentylthiobenzothiazole (APB), inhibits *C. albicans* in vitro and is effective in treating systemic candidiasis in mice (55, 56). It blocks C4-demethylation, preferentially the second demethylation, in *C. albicans* and *S. cerevisiae*, as determined by an accumulation of C4-methylated intermediates (262, 263). This could result from inhibition of Erg25p or Erg26p.

ERG27 encodes the 3-keto reductase enzyme required for sterol C-4 demethylation by Erg25p/26p. Its disruption in *C. albicans* results in coordinate loss of Erg7p and shutdown of ergosterol biosynethsis (419). This and its low degree of homology to the human ERG27 make it an attractive target in search of an inhibitor. It is upregulated by Upc2p (121, 611). Its overexpression on our multicopy plasmid makes it hypersusceptible to azoles, in contrast to the ERG24 overexpression, and resistant to echinocandins, especially anidulafungin.

HMG1 encodes the single *C. albicans* homolog encoding β-hydroxymethylglutarate reductase, the rate-limiting and committed step in cholesterol biosynthesis in humans (Fig. 9). Human and fungal enzymes are inhibited by statins such as Lovastatin and Zocor (156). In *S. cerevisiae*, paralogous genes HMG1 and HMG2 each encode this activity, and are differentially regulated; together, they are essential and can be complemented by the human enzyme (40, 63). Overexpression of an active but truncated of HMG1 resulted in accumulation of normal levels of squalene, but near-normal levels of downstream intermediates. This suggests that Hmg1p is rate limiting for the early portion of the pathway, but that other factors are rate limiting after squalene (422).

Few studies have reported mutations in ScHMG1/2 that confer resistance to statins (33); none for CaHMG1 has been reported. However, Lovastatin inhibits growth of *C. albicans* and acts synergistically with FLZ to reduce the minimal inhibitory concentration (MIC) (FIC 0.08). However, this requires high concentrations of the statin, and the synergy is media dependent. What effects inhibitory concentrations of Lovastatin had on sterol composition and whether Lovastatin alone or in combination was fungicidal have not reported. Lovastatin alone did not appear to cause changes in expression levels of HMG1 or selected genes in the ergosterol pathway, and changes in these genes after combined treatment approximated the modest changes after FLZ alone (517). Consistently, in *S. cerevisiae*, Lovastatin is highly synergistic

Fig. 9 HMG-CoA reductase, encoded by HMG1

3-hydroxy-3-methylglutaryl-CoA

$CH_3C(OH)(CH_2COOH)CH_2C(=O)\text{-}SCoA$

↓ HMG1, HMG-CoA reductase

$CH_3C(OH)(CH_2COOH)CH_2CH_2OH$

mevalonate

with azoles and results in inhibition of sterol esterification (307). Speculatively, this synergy could result from a modest induction by Lovastatin of ERG3 (106), which would potentiate azole effects. The lack of upregulation of HMG1 in yeasts by statins or azoles suggests fundamental differences in pathway regulation from humans. Regardless, overexpression of HMG1 (and CDR1) in our high-copy *C. albicans* plasmid confers robust resistance to statins.

Statins may be clinically useful in combination antifungal therapy, but a more potent fungal-specific statin derivative would likely be more useful and potentially fungicidal than Lovastatin. There is hope for this, since even similar commercial variations of statins differ widely in their ability to inhibit various fungal species (unpublished observations), and since some fungal species are much more sensitive to statins than others (66, 432, 453, 580). Inhibition of Hmg1p activity with statins could also reduce flow to isoprene pathways branching from the ergosterol pathway. Prenylation appears to be essential for *C. albicans*, since disruption of RAM2, encoding a subunit of farnesyltransferase, is lethal. However, mammalian inhibitors of this enzyme are not effective on *C. albicans* (518).

2.1 Amphotericin B

This polyene forms a complex that integrates into the plasma membrane and has a higher affinity for membrane ergosterol than for cholesterol, accounting for its specificity. Binding by this complex is fungicidal, resulting in cell permeabilization (54, 280). In addition, AMB binds to vacuoles, despite their relatively low levels of ergosterol, and prevents their fusion, interrupting vacuolar sorting and trafficking (234).

Despite its toxicity, AMB is still the drug of choice for aspergillosis, mucormycosis, fusariosis, and cryptococcosis meningitis. Lipid formulations of AMB have reduced toxicity, but whether these have increased potency is not clear. Mycosamine modifications to the core structure can overcome AMB resistance in *C. albicans* as well as reduce toxicity (396). Tetracycline has been shown to act synergistically with AMB and TER in *C. albicans* and other fungi, perhaps a result of interference with mitochondrial function (384). The garlic deriviative allicin acts synergistically with AMB, apparently by enhancing AMB-induced vacuolar disruption, not plasma membranes (381); other synergens are also being discovered (382). Whether these modifications or synergens will enhance the inhibition or killing of resistant species (*Trichosporon beigelii, Aspergillus terreus, Pseudallescheria boydii, Malassezia furfur,* or *Fusarium* spp.) is not yet known.

AMB-resistant isolates from clinical samples of *C. albicans* are rare, although some surveys show recent increases (596), and it is difficult to obtain resistant mutants in vitro with single-step selection. While that is great news for patients, the lack of a large inventory of resistant strains has hampered understanding of the cellular response to AMB. Resistance occurs in almost all clades of *C. albicans* (~5% among oral isolates from human immunodeficiency virus (HIV) patients), but at a higher incidence (16%) in Clade SA (South Africa) (51); this association has not yet led to clues about the resistance mechanisms. To an unknown extent, the rarity of resistance to AMB may be an artifact of susceptibility assays. Broth-based assays for AMB susceptibility are problematic in that resistance is media and pH dependent and the range in concentration from susceptible to resistant is low. One representative study shows that standard microdilution assays using RPMI or AM3 media failed to detect resistance in *C. glabrata* isolates, which was detected by the agar-based E-test; E-test resistance correlated with resistance in an animal model (261). Since 2004, E-tests on Mueller-Hinton agar (MHA) with 0.5% methylene blue have the NCCLS (National Committee for Clinical Laboratory Standards) recommendation for AMB susceptibility tests of *Candida* spp.

Inhibition of ergosterol biosynthesis with azoles results in subsequent phenotypic resistance to AMB, consistent with the model that ergosterol is its primary binding site (293, 552, 553). Consistently, mutations (ERG3, ERG11, ERG1) that deplete *C. albicans* or *C. glabrata* of ergosterol result in AMB resistance in laboratory (150, 515, 537) or patient isolates (240, 241, 372). As already mentioned, disruption of ERG6 in *C. albicans* or *S. cerevisiae* results in AMB or nystatin resistance and azole hypersusceptibility (139, 212). AMB resistance resulting from ERG6 disruption has also been documented more recently for *C. lusitaniae,* but this does not alter FLZ susceptibility (601). The latter study also showed that most AMB-resistant clinical isolates of *C. lusitaniae* overexpress ERG6, the opposite of what is expected from the ERG6 disruptions; the clinical isolates, of course, may have multiple mutations in addition to those resulting in ERG6 overexpression. AMB resistance in *Leishmania donovani* clinical isolates is associated with defective expression of ERG6 (423). ERG2 is also implicated in AMB resistance, by the finding that AMB-resistant clinical isolates of *C. lusitaniae* accumulate sterols above fecosterol, suggesting downregulation or inactivation of Erg2p (412).

Some studies implicate the fungal cell wall in AMB resistance. *C. albicans* shows increasing transient resistance, "phenotypic resistance", or PR, to AMB as it moves into the stationary phase. This change is not seen if cell walls are removed with zymolyase or other wall-degrading enzymes, or weakened with mercaptoethanol (141–143). Ultrastructural changes in the periplasm and wall correlate with increasing PR (64). We showed that intrinsic AMB resistance in *C. lusitaniae* is regulated by a high-frequency switching mechanism. Cells of most strains switched from resistant to susceptible, concomitantly from round to elongate in cell shape, at a frequency of about 1 per 100 to 1,000 cells (600). These results have been confirmed and extended (129, 338). Susceptible elongate cells were hypersensitive to zymolyase, and zymolyase rendered resistant cells susceptible to AMB (600). Consistently, in *A. flavus*, AMB-resistant mutants were isolated by stepwise selection. Mutant spheroplasts were as susceptible as wild type, but intact cells were resistant, suggesting that cell-wall alterations conferred resistance (485).

These data gave rise to the prediction that mutations that alter cell-wall structure, or perhaps alter key targets within the cell wall, will alter susceptibility to AMB. Only a handful of spontaneous or mutagen-induced mutants have been studied in *C. albicans* or *S. cerevisiae*, but in both organisms it seems that AMB and nystatin invoke different resistance mechanisms, surprising given their structural similarity. In *C. albicans*, a nystatin-resistant mutant and a cytochrome P-450 mutant were cross-resistant to AMB and had sterol alterations, suggesting ERG2 defeciency. In contrast, two AMB-resistant mutants had normal sterols (52). In *S. cerevisiae*, one of two mutants was nystatin resistant and showed alterations in membrane sterols, and was suppressed by ERG3. The second AMB-resistant mutant had none of these features, and was resistant to cell-wall-degrading enzymes. Nystatin, but not AMB, was able to permeabilize wild-type cells to propidium iodide, a measure of extensive membrane damage (174). Absence of sterol changes is also characteristic of AMB-resistant *Ustilago maydis* mutants (221). Extrapolating, alterations in cell walls seem the preferred route to AMB resistance in *S. cerevisiae*, but even here detailed mechanisms are uncharacterized.

A number of links to individual genes involved in AMB susceptibility have been established by gene disruption. Interference with *O*-mannosylation of wall proteins has had a variable, species-specific consequence. Homozygous disruption of PMT1,-4, or -5, and heterozygous PMT2/pmt2 disruptants, did not show altered susceptibility to AMB in *C. albicans* (428), in contrast to the hypersusceptibility of PMT4 in *C. neoformans* (386). KEX2 encodes an endoproteinase needed for processing several wall-associated glycosidases; KEX2 disruptants in *C. glabrata* were hypersusceptible to AMB, azoles, and detergents but had normal expression profiles for efflux genes (22). Constituitive activation of the cAMP pathway, by disruption of PDE2, encoding the high-affinity cAMP phosphodiesterase results in hypersusceptibility to AMB, azoles, and detergents, with a 30% increase in membrane sterols and a 22% reduction in wall glucans and wall thickness, as well as diminished virulence and ability to respond to stress (222, 581). Induction of *C. albicans* CDR1 by adriamycin resulted in tolerance to AMB (379), but clinical strains that specifically overexpress CDR1 or MDR1 are generally not resistant to polyenes; presumably the adriamycin effect on AMB was independent of its upregulation of CDR1.

Microarray analysis of an AMB- and FLZ-resistant derivative of *C. albicans* also implicated cell-wall maintenance genes. The mutant was isolated by stepwise selection on increasing concentrations of AMB (36). The resistance was not stable past 28 generations of growth in the absence of selective pressure, so it may have been "phenotypic". Nonetheless, the derivative strain was depleted of ergosterol and instead had predominantly lanosterol and 24-methylene lanosterol. Microarray analysis of the mutant, in the absence of AMB, showed some increases in ERG6, ERG25, and ERG5, and an increase in cell-wall maintenance gene PHR2. However, these experiments need further validation.

Much of the AMB-resistance pathway is still unknown. Consistently, we recovered five different genes from our *C. albicans* overexpression library, whose overexpression confers resistance to AMB. Only one of these is wall associated, one may be a stress-response sensor, and the rest have no associated functions to date. Lack of progress on this front probably stems from the perceived unimportance of AMB resistance clinically (e.g., 598); however, we suggest that AMB resistance mechanisms in vitro are important tools for identification of new antifungal targets, and for a better understanding of intrinsic resistance in some fungal species.

2.2 Regulation of Ergosterol Biosynthesis Genes

Studies of the regulation of ERG genes in *C. albicans* are just beginning. Regulatory responses of *S. cerevisiae* ERG genes to inhibitors were summarized in our earlier review (3). ScErg9p, encoding squalene synthase, is the first enzyme in the pathway dedicated just to sterols as opposed to earlier points that feed the isoprenoid pathway. Therefore it should be and is a focal point of regulation, since sterols are needed in vast excess of other isoprenoid derivatives (242, 243, 19). However, studies of the regulation of CaERG9 are not yet reported.

Expression studies in *C. albicans* have generally confirmed the negative regulation seen in *S. cerevisiae,* since inhibitors or downstream mutations result in upregulation of several ERG genes. Which genes are upregulated depends on

how the individual study was conducted. *C. albicans* strains with mutations in post-ERG11 genes, notably ERG6 or ERG24, show increases in expression of ERG11, ERG7, and ERG25 (418). In a reverse transcriptase–polymerase chain reaction (RT-PCR) study, azoles and terbinafine induced expression of ERG9, ERG11, ERG25, and ERG3 from 1 to 5 h after exposure and prevented downregulation of ERG1 and ERG7 (189). Some of these changes were shown to require a histone deacetylase activity, since its inhibition by trichosatin A prevented the ERG gene inductions (510). In a promoter-fusion study, ERG11 was induced by azoles and terbinafine after a lag period, suggesting that the induction resulted from the depletion of ergosterol occurring in the first 4–5 h after exposure (516). In an older microarray study, 24-h exposure to itraconazole resulted in upregulation of most of the ERG genes (89). However, this study may be flawed in that it compared expression levels to parallel, untreated, 24-h cultures which were in post-log phase by that time. Baseline levels of the ERG genes would be reduced under those conditions, inflating the levels of the itraconazole-treated culture.

More recent microarray studies have focused on comparing resistant to susceptible cultures, or comparing effects of very-short-term exposures to azole; results are still confusing. In a microarray study, ERG3, ERG6, and ERG25 were upregulated four- to sixfold in strains overexpressing CDR1, but were not upregulated by short-term exposure to fluphenazine (230). In contrast, the ERG genes did not show major changes in expression among lineages that evolved resistance to azoles after in vitro selection, other than a threefold decrease in ERG1 in one of the adapted strains (83). In stepwise-selected azole-resistant strains examined by Roger et al., only ERG2 showed an increase (449). Ketoconazole induced several ERG genes, mostly acting downstream of ERG11 (ERG2, ERG3, ERG10, ERG25) (296). Since these microarray-based analyses of different azole-resistant strains do not show common alterations in specific ERG genes, and also differ from similar studies in *S. cerevisiae*, where, for example, ERG8 and ERG13 are downregulated (38), the implication is that they do not play pivotal roles in the resistant phenotypes. Alternatively, in individual strains, individual changes in ERG gene expressions may be important only in the context of other mutations that are lineage specific. ERG9 did not appear in these global studies to play a dominant role in resistance or altered response to inhibition.

A master regulator of the CaERG genes has been identified. CaUPC2, homologous to sterol uptake genes ScUPC2 and ScECM22, is important for ERG gene inductions and antifungal resistance (313, 501, 576). Disruption of this zinc finger transcription factor encoding gene confers hypersusceptibity to azoles, terbinafine, and lovastatin, as well as cell wall-acting agents, nikkomycin Z, and calcofluor white. Disruptants are unable to upregulate ERG2 or ERG11 in response to fluconazole and show depleted levels of ergosterol as well as reduced ability to import labeled cholesterol. Thus, CaUPC2 may regulate expression of ergosterol biosynthesis and sterol uptake genes. The authors identified a potential consensus-binding sequence upstream of ERG2 and ERG11 which is also present upstream of many ERG genes. These may also be targets of Upc2 regulation. This is part of an azole response element (ARE) upstream of ERG11, shown to be necessary and sufficient for upregulation by Upc2 (385). These studies suggest that global regulation of ERG genes is important in maintaining normal levels of susceptibility to antifungals. Recently, hyperactive alleles of UPC2 have been identified in clinical resistant isolates; this mutant gene is coordinately upregulated with ERG11 in the resistant isolates, and introducing the mutant UPC2 into susceptible strains confers FLZ resistance (121). Global expression studies show that Upc2 upregulates many ERG genes, as well as other targets including CDR1 and MDR1 (121, 611).

It is likely that *C. albicans* also uses transcriptional repressors to control ergosterol biosynthesis. For example, ScMOT3 negatively regulates expression of ERG2, -6, and -9 (197), but no studies are reported for the closest gene in *C. albicans*, orf19.2724. CaEFG1 encodes a helix–loop–helix transcriptional activator which is known as a regulator of morphogenesis and virulence (292, 299, 527). It apparently represses transcription of ERG3, since its disruption results in a 2.5-fold upregulation of ERG3 (300). However, the authors' claim that this overexpression is responsible for FLZ resistance is at odds with results from our overexpression study (Table 2) and with the expectation that overexpression of ERG3 should increase susceptibility, not resistance, to azoles, by increasing production of the toxic sterol intermediate (Fig. 5). It is likely that the resistance of

Table 2 Overexpression of ERG and PDR genes alters susceptibility to antifungals

Gene	FLZ	TER
ERG1	C	R
ERG5	C	C
ERG9	C	C
ERG20	C	C
ERG3	HS	C
PDR16	HS	C
ERG27	S	HS
ERG6–7	R	C
ERG11	R	C
ERG24	R	C

Genes were amplified with Elongase and cloned as *SphI* fragments into pMPA9MAL1
C transformants show the same susceptibility as controls; *HS* hypersusceptible; *S* slightly more susceptible; *R* resistant compared to controls. Clones were verified by sequencing

EFG1 disruptants results from altered transcription of some of the several hundreds of other genes regulated by Efg1p.

Other aspects that control ergosterol metabolism seem likely sources of azole or AMB resistance. There is much new information on the transport of ergosterol to the plasma membrane and uptake of exogenous ergosterol or cholesterol when its biosynthesis is blocked, via sterol-binding proteins (126, 483, 484). Whether mutations in these pathways provide resistance mechanisms is an open question.

2.3 ERG Gene Overexpression Study

Our laboratory has initiated a study on the effects of overexpression of CaERG genes on antifungal resistance. Each gene (Table 2) was amplified from a susceptible *C. albicans* genome by PCR and cloned into our high-copy plasmid (119). Recombinant plasmids were transformed into *C. albicans*, and tested for susceptibility in an agar-based assay. Overexpression of ERG1 resulted in terbinfine resistance without cross-resistance to FLZ, as expected. Likewise, ERG11 or ERG 6 overexpression conferred azole resistance, and ERG3 overexpression conferred hypersusceptibility.

There were some surprises in this preliminary study. We did not expect from existing work that overexpression of ERG24 would confer resistance, since its disruption confers slight azole resistance (217). Overexpression of CaPDR16 conferred hypersusceptibility, but was expected to confer resistance, since disruption of ScPDR16 confers hypersusceptibility (545). ERG27 transformants were hypersusceptible to TER and slightly more susceptible to FLZ, but were more resistant to the combination of FLZ and cyclosporin A, and resistant to the echinocandins. However, these obervations were, atypically, dependent on the orientation of the gene in the vector; these phenotypes may stem from a net antisense expression rather than overexpression.

Overexpression of genes upstream of ERG11 might confer azole hypersusceptibility if they resulted in increased production of the toxic sterol by increasing the overall volume of flow through the pathway. Alternatively, the overexpression might confer resistance if they resulted in increased levels of Erg11p. Neither was observed, since overexpression of Erg1p, Erg9p, and Erg20p had no effect on azole susceptibility. This suggests that intermediates generated by these enzymes are not rate limiting in the production of lanosterol, or that any increase that these do generate is countered by downstream regulation. These results point out another difference in regulation compared to *S. cerevisiae,* since upregulation of ScERG9 confers azole resistance (242, 243).

3 Efflux of Antifungals as a Resistance Mechanism

3.1 ABC Transporters

CDR1 and CDR2 are ABC transporters, transmembrane efflux pumps that use adenosine triphosphate (ATP) to move a variety of small hydrophobic compounds out of the cell (Fig. 10). There are the major efflux mechanisms implicated

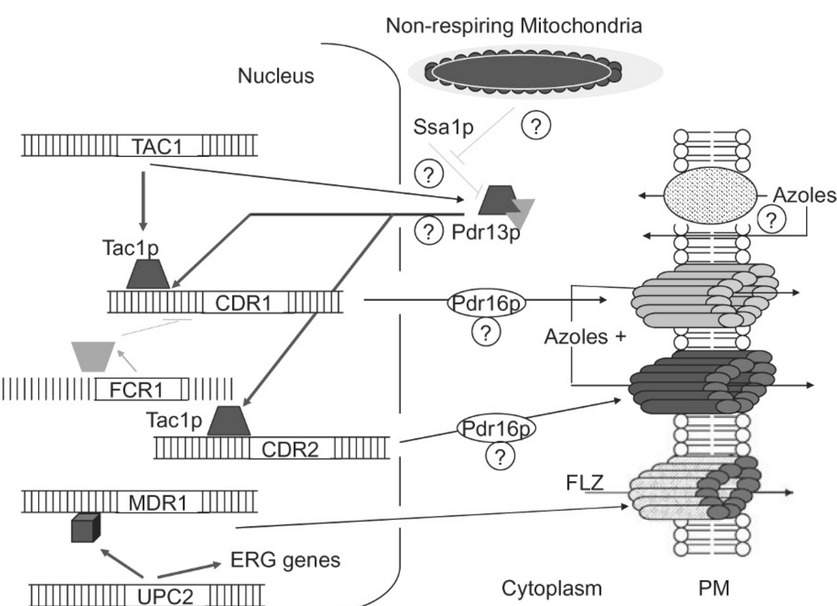


Fig. 10 Proposed regulation of CDR1, CDR2, and MDR1 involved in efflux of antifungal drugs. See text for explanation. Speculative interactions are depiced with a "?". Arrows indicate activation, blocked lines indicate inhibition

in overall resistance mechanisms in azole-resistant *C. albicans* clinical isolates. They are homologous to *S. cerevisiae* efflux pump PDR5 and were identified by their ability to complement *pdr5* mutants (426, 472). They are related to the transmembrane human P-glycoprotein encoded by MDR1. How such a broad group of structurally unrelated compounds can be effluxed by a single pump is now at least partly explained by the floppase activity of this class of pumps. The plasma membrane of *C. albicans* is asymmetric with repect to phospholipids, with phosphatidylethanolamine predominantly (96%) in the cytoplasmic leaflet. Disruption of CDR1 and CDR2 results in a further decrease in the amount of this phospholipid in the external leaflet-(109). Cdr1p and Cdr2p moved fluorescently tagged phospholipids derived from PE, PC, and PS from the inner to the outer leaflet of the plasma membrane. In contrast, Cdr3p moved these from the outer to the inner leaflet. Azoles and other CDR1/2 substrates inhibited the in-to-out transfer of phospholipids, but had no effect on Cdr3p activity. This data are consistent with a model in which efflux results from phospholipid exchange from the cytoplasmic to the external leaflet, carrying along hydrophobic agents embedded in the cytoplasmic leaflet (512). However, there are also direct interactions of substrates with transmembrane domains in the protein. This is similar to the mechanism proposed for mammalian P-glycoprotein (488), and is consistent with the homologies between these proteins and the DRS2 family of phospolipid translocases in *C. albicans*, which include orfs 19: 6778, 23, 24, 6595, 2680, 323, 6224, 783, and 932. For example, CaDrs2p (orf19.6778) is 23% identical and 41% similar to Cdr1p over its entire length.

In *S. cerevisiae*, there are at least 31 genes encoding ABC proteins, 11 in the PDR family (41). In the *C. albicans* genome, there are only six members of this family that have both dual nucleotide binding domains (NBDs), consisting of two conserved Walker domains separated by a signature sequence (Table 3). CDR1 and CDR2 are about ~1,500 amino acids long, 100 kb apart on chromosome 3, and are 83% identical and 91% similar (219). Four other genes in *C. albicans* share extensive homology. CDR99 on Ctg19–10079 is the closest in sequence, 69% identical, 81% similar to CDR1, followed by CDR4 on chromosome 1 at 59% identical, 73% similar, CDR3 on chromosome 4 (53% identical, 68% similar), and SNQ1 on chromosome 6 (39% identical, 57% similar). CaO19.4531 has an additional seventh TM domain in the *N*-terminal half, and is only 24% identical, 42% similar to CDR1. ADP1 has a half-transporter structure with one NBD and a six transmembrane domain, which are 22% identical and 44% similar in this region to CDR1. Each member of this family has nearly identical patterns of TM domains; presumably those with six transmembrane domains act as dimers. Each member also has highly conserved NBDs (Table 4). No other proteins in the *C. albicans* database show the conserved NBDs characteristic of this family.

Overexpression of CDR1 or CDR2 confers resistance to azoles including fluconazole, ketoconazole, miconazole, voriconazole, and itraconazole, as well as to terbinafine and cycloheximide. Overexpressing strains have increased abilty to efflux nystatin (260), but this did not confer resistance to nystatin (364). Some clinical strains that overexpress CDR1 or CDR2 are susceptible to posaconazole, implying that this azole is not an effective substrate (71). However, overexpression of either gene in a hypersusceptible *S. cerevisiae* host clearly increases resistance to all these azoles, but not to nystatin or to the echinocandins (360, 363, 472, 480, 564). Deletion of CDR1 confers hypersusceptibility to azoles in *C. albicans* (473). Overexpression of CDR1 and CDR2 is common among resistant clinical isolates (306, 315, 573, 574) and in laboratory isolates selected for azole resistance (11, 83, 84), although some susceptible clinical isolates also overexpress CDR1 (574). Deletion of CDR2 in *C. albicans* confers hypersusceptibility, but only in strains already deleted for CDR1 (472). Expression of CDR2 is elevated in revertants of hypersusceptible mutants in which CDR1 had been disrupted (471). In addition to the standard antifungals, many other agents are apparently effluxed by CDR1 or CDR2, and it is clear that the two pumps have only partly overlapping specificities (reviewed in (427)).

In contrast, CDR3 and CDR4 have no apparent role in antifungal resistance. Overexpression in *S. cerevisiae* of CaCDR3 did not confer a resistant phenotype. In *C. albicans*, neither CDR3 or CDR4 is induced by FLZ, and their disruption does not confer hypersusceptibility (28, 134). However, these disruptions are inconclusive since they were done in a CDR1 host, and it is formally possible that heterologous overexpression fail because of a negative interaction of these proteins with a *S. cerevisiae* protein. The implication that neither gene functions in multidrug resistance is therefore likely but premature. Further support for this implication is that Cdr3p moves phospholipids from the external to the cytoplasmic leaflet, opposite of the direction induced by Cdr1p and Cdr2p, discussed later in this review.

Structurally, CDR1 is a typical ABC transporter in many respects, composed of a pair of tandemly duplicated six-pass transmembrane domains, downstream of conserved, nonidentical NBDs with ATPase activity. These have a highly conserved ABC signature motif with flanking Walker A and B motifs (134). These genes encode the fungus-specific cysteine in the conserved Walker sequence of NBD1, instead of the invariant lysine found in nonfungal ABC transporters (Table 4). Replacement of this cysteine with a lysine in CaCDR1, placed in an *S. cerevisiae* hyperexpression system, diminishes ATPase activity and confers hypersusceptibility to antifungals, without altering protein localization or stability. Replacement of the conserved lysine in the Walker box of NBD2 has similar but not identical effects (232). Site-directed mutagenesis of C193 or

Table 3 ABC transporter genes in *C. albicans* and *S. cerevisiae*

	CA gene	CA gene ID	SC homolog	% ID (similar)	Function of SC gene	Function of CA gene
PDR family						
1	CDR1 (12)	CaO19.6000	PDR5	54(71)	PDR	PDR
2	CDR2 (12)	CaO19.5958	PDR5	53(69)	PDR	PDR
3	CDR3 (12)	CaO19.1312	PDR5	48(65)	PDR	Unknown, no resistance[a], opaque-specific
4	CDR4 (12)	CaO19.5079	PDR5	51(68)	PDR	Unknown, no resistance[a]
			PDR11	33(49)	Sterol uptake	
			PDR12	44(62)	Weak acid efflux	
			YNR070W	55(71)	Unknown	
5	CDR99 (12)	19.8533,4	PDR5	56(73)	PDR	Unknown
6	SNQ2 (12)	CaO19.5759	SNQ2	54(73)	Drug resistance, not azoles, partially overlaps PDR5	Unknown
			AUS1	34(52)	Sterol uptake	
7	ADP1 (6)	CaO19.8090	ADP1	50(65)	Unknown	Unknown
8	UNNAMED (6)	CaO19.4531	YOL075c	34(53)	Unknown	Unknown
MRP family						
9	YCF1	CaO19.13832	YCF1	55(71)	Vacuolar glutathione-conjugate-bilirubin, cadmium transporter activity	Unknown
			VMR1	35(51)	Vacuolar metal resistance	
			NFT1	25(46)	Unknown	
10	MLT1	CaO19.5100	BPT1	38(57)	Like YCF1	Unknown
11	unnamed	CaO19.6382		43(62)		Unknown
12	YOR1	CaO19.1783	YOR1	47(64)	Efflux organic anions, oligomycin resistance	Unknown
			YBT1(BAT1)	32(52)	Bile acid transporter	
MDR family						
13	ATM1	CaO19.1077	ATM1	64(75)	Mito Fe/S transporter	Unknown
14	HST6		STE6	30(51)	Secretes α-factor	Probably secretes mating peptide
15	MDL1	CaO19.10146	MDL1	46(64)	Mito peptide transporter	Unknown
16	MDL2	CaO19.5600,5599	MDL2	38(54)	Mito peptide transporter	Unknown
ALD family						
17	PXA1	CaO19.7500	PXA1	42(57)	Peroxisomal transport LCFA	Unknown
18	PXA2	CaO19.12720	PXA2	40(67)	Heterodimer with PXA1	Unknown
Unclassified						
19	UNNAMED	CaO19.10632	NONE		Unknown half transporter, conserved in many fungi	Unknown

Modified from (3). Information regarding *S. cerevisiae* genes and family groupings is based on (41). *C. albicans* genes were listed based on blast searches with each *S. cerevisiae* gene. Genes in the PDR family were also searched by blasting the conserved NBD domains in the CDR family (135) against the *C. albicans* genome database (219). Values within parentheses denote number of transmembrane helices. % identities and similarities of the homologs from *C. albicans* versus *S. cerevisiae* were from blast alignment results. Functions associated with the *C. albicans* genes are discussed in the text; functions associated with *S. cerevisiae* genes are readily accessed at http://www.yeastgenome.org/. *PDR* polymorhphic drug resistance

[a]Incomplete analysis does not implicate resistance

K901 in Walker box 1 and 2 (outside the most conserved sequences shown in Table 4) diminishes ATPase activity, whereas changes at other conserved positions in the boxes do not (427). Another structure–function study shows that a mutation in transmembrane domain 11, converting threonine at position 1351 to phenylalanine, blocks resistance to antifungals and FLZ efflux, without altering ATPase activity, nucleotide/substrate binding, or protein localization and stability (496).

In vitro mutagenesis of CDR1 has identified some functional domains but has not produced a hyperactive allele. TM12 seems important for substrate specificity. ΔTM12 strains (expressed from a multicopy plasmid in *S. cerevisiae pdr5* mutant) resulted in a drug-specific loss of resistance: resistance to azoles, oligomycin, chloramphenicol, and benomyl was retained, but resistance to cycloheximide, anisomycin, and nystatin was lost. The ΔTM12 strain retained the CDR1-dependent ability to efflux estradiol and

Table 4 Conserved nucleotide binding domains in the *C. albicans* CDR family

	NBD1			NBD2		
	Walker A	**ABC Signature**	**Walker B**	**Walker A**	**ABC Signature**	**Walker B**
	200	320	340	910 920	1020 1030	1040 1050
CDR1	LGRPGAGCSTLL	SGGERKRVSIAE	QCWDNATRGLD	MGASGAGKTTLL	NVEQRKRLTIGV	LLFLDEPASGLD
CDR2	LGRPGAGCSTLL	SGGERKRVSIAE	QCWDNATRGLD	MGASGAGKTTLL	NVEQRKRLTIGV	LLFLDEPTSGLD
CDR3	LGRPGAGCSTFL	SGGERKRLSIAE	QCWDNSTRGLD	MGASGAGKTTLL	NVEQRKRLTIAV	LVFLDEPTSGLD
CDR4	LGRPGAGCSTFL	SGGERKRVSIAE	QCWDNSTRGLD	MGASGAGKTTLL	NVEQRKRLSIGV	LVFLDEPTSGLD
CDR99	LGRPGAGCSTLL	SGGERKRVSIAE	QCWDNSTRGLD	MGATGAGKTTLL	NVEQRKRLTIGV	LLFLDEPTSGLD
SNQ2	LGRPGAGCTTFL	SGGERKRVSIAE	YCWDNATRGLD	MGESGAGKTTLL	NVEQRKKLSIGV	LLFLDEPTSGLD
4531	MGGSGSGKTTLL	SGGEQRRVSLAI	LFLDEPTTGLD	MGPSGSGKTTLL	SGGEKRRVSIAI	VLFLDEPTSGLD
ADP1				MGGSGAGKTTLL	SGGEKRRVSIAC	ILFLDEPTSGLD
10632				MGPSGCGKSTLL	SGGQKRRVSIAS	ILFLDEPTSGLD

Most sequences in this domain are identical. Conserved changes are *highlighted in gray*, unconserved changes relative to CDR1 are *white letters on black background* (3)

to hydrolyze ATP (260). Point mutations in TM10 of ScPDR5 alter substrate (azole) and inhibitor (FK506) specificity (123); effects of analogous mutations in CDR1/2 have not yet been reported. However, site-directed mutagenesis of CaCDR1 overexpressed in *S. cerevisiae* showed that some mutations in TMS11 or in the sequence between Walker box A and signature C in NBD1 conferred hypersusceptiblity to anisomycin, cycloheximide, fluconazole, miconazole, and nystatin. Mutations in TMS6 or in the sequence between Walker A box and signature C sequences in NBD2 conferred hypersusceptiblity to a subgroup of these substrates. One mutation in TMS6 caused mislocalization of the protein unless the cell was grown in cycloheximide (427). Clearly, many more site-directed mutations are needed to tell this story.

Mutations in CDR1 among clinical isolates have also been documented, but none is uniquely associated with resistance (Fig. 11). Haque et al. (175) analyzed genes from 18 azole-resistant isolates to find 53 SNPs; 47 were synonymous, and among the six nonsynonymous mutations, only two were seen only in resistant isolates, T842S and F1399Y. A mutant protein generated by site-directed mutagenesis of F1399Y in a wild-type CDR1 showed no difference in susceptibilities when overexpressed in *S. cerevisiae*, compared to overexpressed wild-type CDR1. Holmes et al. (195, 196) looked at fewer isolates, but also characterized CDR2. They also found many nonsynonymous SNPs in CDR1 and CDR2, including between alleles in the same wild-type strain, but most did not alter antifungal susceptibility. Most of these occurred in unconserved regions of the proteins; their incidence was much higher in CDR2. One exception, a double mutation G1473A/I1474V, was found as an allelic variant present in most isolates in CDR2; the AV allele was associated with twofold higher FLZ resistance than the GI allele when introduced into *S. cerevisiae*. The authors speculate that most mutations in either gene are deleterious, and predict that point mutations that increase azole resistance via creating a hyperactive protein will not be found or will be rare. The chances for this are higher in CDR2, which is apparently a more recently diverged paralog and is still evolving. The fact that hypersusceptible SNPs were not found among clinical isolates but were found by mutagenesis of lab strains supports the argument that the CDR genes have functions other than antifungal resistance and that loss of this function reduces fitness in vivo. There is evidence in *C. glabrata* that Cdr1p and the related Pdh1p are activated by protein kinase A (PKA) phosphorylation (560, 561). If this is also true in *C. albicans*, potential hyperactivating mutations could act by mimicking the phosphorylated conformation. Despite the lack of hyperactive alleles, cis-acting mutations in CDR1 of two resistant clinical isolates have been identified, which increase its transcription sevenfold over wild type and stabilize its mRNA threefold, resulting in overexpression (319).

Cdr1p interacts directly with its substrates. Heterologously overexpressed CaCDR1-GFP pump binds to photoaffinity reagents that bind to human P-glycoprotein. Binding of these reagents is competed out by putative Cdr1p substrates nystatin and miconazole, in a manner suggested at least by two substrate binding sites (498). Similarly, Cdr1p and Cdr2p cross-link to a photoaffinity reagent derived from rhodamine 6G, showing their direct involvement in efflux and enabling the localization of binding sites. They cloned the genes into a high-copy vector behind the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, and expressed the proteins in *S. cerevisiae* transformants; CDR2 had been modified to alter CTG codons to TCT to maintain serines in those positions. The transformants were resistant to fluconazole, ketoconazole, and itraconazole, and showed increased efflux of rhodamine 6G (149), as predicted in earlier work (472, 480). They went on to show that the photoaffinity reagent IAARh123, known to cross-link to human MDR1, specifically cross-linked to both CDR1 and CDR1. They then cloned and expressed *N*- and *C*-terminal "halves" of CDR2 in their *S. cerevisiae* system, and showed that each

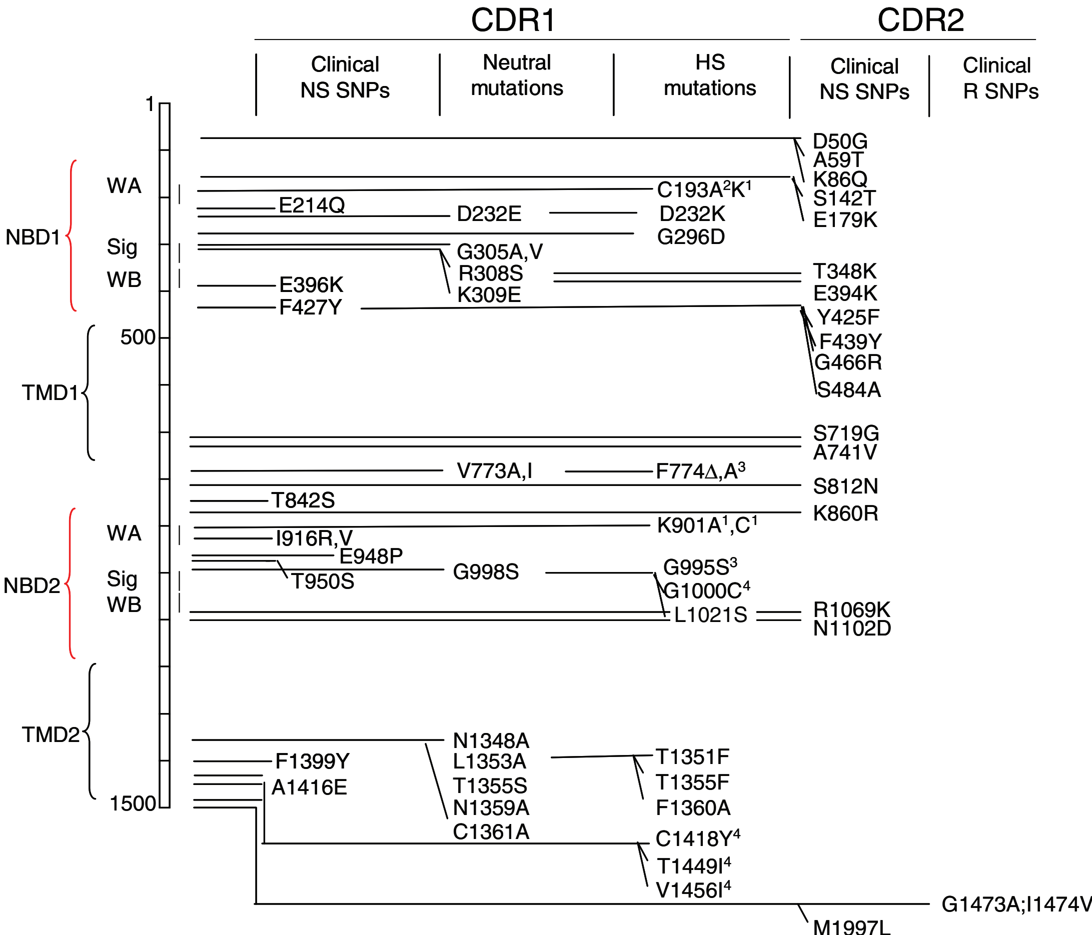


Fig. 11 Diagram of sequence changes in CDR1 and CDR2. Nucleotide binding domains (NBDs) and their conserved WalterA/B and signature sequences (Sig) are indicated at their positions along the 1501/1499 amino acid proteins, along with the transmembrane domains (TMD). Clinical nonsynonymous (NS) SNPs are those changes found in clinical isolates, not associated with azole resistance either because they are also found in susceptible isolates or because site-specific mutagenesis discounts their involvement. Neutral mutations, not altering susceptibilities, and hypersusceptibile mutations (HS) were introduced by site-specific mutagenesis. Note that only a single paired mutation at 1473–4 in CDR2 is responsible for slightly increase azole resistance. 1 Intermediate susceptibility, partial ATPase activity; 2 inactivate ATPase 3HS to CYH, FLZ; 4 HS to MIC; 5 Elevated FLZ MIC in a *S. cerevisiae* overexpression strain. Data are pooled from (175, 195, 196)

half could bind rhodamine 6G independently, although both halves were required for resistance. Cells expressing CDR1 were hypersusceptible to FK520, an immunosuppressant shown to compete for binding sites on human MDR1, whereas cells overexpressing CDR2 were resistant to FK520. Furthermore, cells expressing CDR1, but not CDR2, showed strong synergy between fluconazole and FK520 at subinhibitory doses (149). Similar results were reported by Schuetzer-Muehlbauer et al. (481). Cells expressing CDR2, but not CDR1, were hypersusceptible to hydrogen peroxide and resistant to diamide (149). Thus, there are clear differences between these two highly homologous pumps.

Recently, CDR1 has been highly purified as a his-tagged protein from an overexpressing *S. cerevisiae* strain, and reconstituted with lipids into an active conformation (497). Cdr1p has natural fluorescence due to high Trp content, and conformational changes that further expose these residues increases fluorescence. The authors were able to show that antifungal substrates interact specifically with the purified, reconstituted pump. Specificity was demonstrated by showing that nonsubstrates do not alter fluorescence, and that mutant proteins defective in ATP hydrolysis do not undergo ATP-dependent shifts. However, these mutant proteins still bind antifungal substrates and undergo the same conformational changes as the wild-type protein. They also showed that substrate binding did not stimulate ATP hydrolysis, in contrast to the interaction in mammalian MDR transporters. The purified Cdr1p bound and hydrolyzed all four NTPs equally.

Specific overexpression of CDR1 in *C. albicans* has confirmed its importance in conferring multidrug resistance (364). Its overexpression was achieved by creating

a fusion of the *N*-acetylglucosamine-inducible, glucose repressible HEX1 promoter to the coding region of CDR1. This construct was cloned into a shuttle plasmid and transformed into *C. albicans* strain CAI4. Transformants showed *N*-acetylglucosamine-dependent increases in CDR1 mRNA and CDR1p antigen, and rhodamine 6G efflux, during 3 h of induction, with no corresponding increases in glucose-grown cells. Cells grown in *N*-acetylglucosamine showed increased resistance to typical CDR1 "substrates", including azoles and terbinafine, confirming that the elevated level of CaCdr1p had the same effect in *C. albicans* as it did it *S. cerevisiae*.

3.1.1 ABC Pump Inhibitors

Studies cited above suggest that resistance in isolates from clinical samples to azoles, largely mediated by overexpression of CDR1/2, might be overcome by simultaneous treatment of "ABC inhibitors" such as FK506, FK520, or a propafenone GP382 (149, 481, 496). From a clinical perspective, this approach may be limited, since the inhibitors appear to target only Cdr1p, not Cdr2p, and since resistance mutations arise at high frequency. Inhibitors of mammalian MDR1 are either not effective on the fungal target or are cytotoxic. Screening of a peptide library led to identification of D–NH_2–RRRFWWFRRR–$CONH_2$, which inhibits *S. cerevisiae* plasma membrane ATPase Pma1p and sensitizes *Cryptococcus* and *Candida* species to fluconazole, including azole-resistant strains overexpressing efflux pumps (342).

Disulfiram is another candidate for a CDR antagonist, since it appears to inhibit human MDR1 (303, 477). This oxidant, known for its use as a deterrent-based treatment for alcoholism, inhibits human acetaldehyde dehydrogenase, resulting in the accumulation of acetaldehyde and associated nausea following ethanol consumption. Shukla et al. (499) showed that disulfiram treatment of plasma membranes isolated from *S. cerevisiae* that overexpress CaCDR1 results in inhibition of its ATPase- and nucleotide-binding activities. They further show that disulfiram acts synergistically with antifungals that are substrates of CDR1 to inhibit the CDR1 overexpressing strain. The authors, however, imply that disulfiram reverses CDR1-mediated resistance by specific reactions with CDR1. The agent likely inhibits activities of many enzymes, not just CDR1, the only activity looked at in the study; hence it is fungicidal alone at slightly higher concentrations than when used in the synergy study. This potential lack of specificity may be a deterrent to its clinical use, as is its lack of fungicidal or fungistatic effects on *C. albicans* in ex vivo blood cultures, even at much higher doses (unpublished observations).

Systematic searches for direct CDR efflux inhibitors, looking for inhibitors that restore fluconazole susceptibility to *C. albicans* strains overproducing Cdr1p and Cdr2p, have resulted in isolation of two promising classes of specific inhibitors. One class is composed of natural products related to milbemycin, a widely used macrolide, antiprotozoal agent for heartworm, modified to compound MC 510027 (Essential Therapeutics, Inc.). These were promising synergens in many species of *Candida* and in most, not all, isolates of *A. fumigatus* (361). Specific derivatives block resistance conferred by CDR1 or CDR2, but not MDR1 (Table 6). The second class is a set of synthetic derivatives of quinazolinone. Initial derivatives were limited by solubility problems and because they were too specific, e.g., inhibiting one but not other species of *Candida* (289). These problems are being minimized by derivatives with greatly improved synergy and with good serum binding and clearance profiles (569, 570). However, results on a broader spectrum of species are not yet reported it detail, although they are referred to as effective against all clinically relevant species of *Candida*, and even the most potent deriviatives for Cdr1p are not effective against Cdr2p. In vivo studies are pending.

3.2 Regulation of CDR1/CDR2

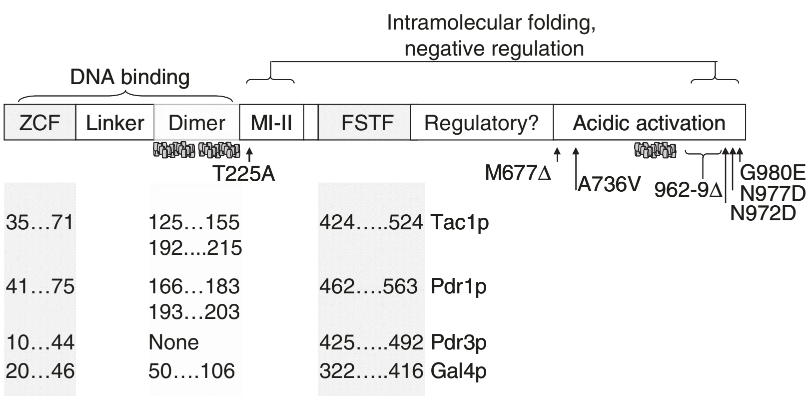
3.2.1 Regulatory Sequences

Understanding how CDR1 and CDR2 are regulated is clinically important, since in resistant isolates these genes and others are often upregulated together. This is now known to be most often due to mutations in a regulatory gene that activate expression of both efflux genes. Cis-acting regulatory sequences in CDR1 and CDR1 have been identified and discussed in our previous review (3). In addition, sequences within the ORF of CDR1 from resistant clinical isolates increase transcription initiation and extend mRNA stability (319).

3.2.2 Regulatory Proteins: TAC1

Two regulators of CDR1 have been described. The first is TAC1, identified in a screen of Zn(2)-Cys(6)-encoding genes that were linked to the mating MTL locus on chromosome 5. TAC1 is required for both basal and upregulated expression of CDR1 and 2. Its disruption results in hypersusceptibility to azoles and inability to express CDR1 or CDR2, both at basal consituitive levels or when exposed to the inducer fluphenazine. Tac1p binds to DRE elements upstream of CDR1 and CDR2 (81); extrapolating from other genes in this superfamily, binding occurs via the conserved zinc finger domain, and specificity is imposed by additional contacts throughout the DNA binding domain (Fig. 12). Only the zinc-binding

Fig. 12 Hyperactive mutations in the TAC1 regulatory gene that upregulate expression of F target gene CDR1. Mutations are indicated by upward-pointing arrows, Various domains of the 2,946 bp ORF are indicated, including the zinc finger DNA binding domain (ZCF), the conserved fungal specific transcription factor domain, and the variable but acidic carboxy terminal activation domain, in which many of the hyperactive mutations are located (79–81, 478, 610)



domain is conserved; about half of the genes also have a moderately conserved "fungal-specific transcription factor domain" (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), which corresponds to a middle homology region, MHR (478). We are not aware of any functions associated with this region. The remaining domains (Fig. 12) are conserved in function but not in primary amino acid sequence. The dimerization domain has stronger coiled-coil potential than Pdr1p.

Selection for resistance to genes that are effluxed by genes regulated by TAC1 results in hyperactive mutations in TAC1. TAC1 sequence is highly variable among strains and alleles with in isolates, having 26 polymorphisms not associated with changes in function (positions 47, 104, 131, 170, 189, 199, 206, 207, 232, 317, 377, 396, 558, 572, 683, 764, 772, 776, 829, 869, 895, 904, 935, 937, 941, 944) and six possible gain-of-function hyperactive mutations (Fig. 12). Two of these, N972D and N977D, are sufficient for hyperactivity and resistance, as demonstrated by site-specific mutagenesis in *C. albicans* (80, 610). Hyperactive mutations result in constituitively high levels of CDR1 and 2, so that cells are prepared in advance of exposure. Most hyperactive mutations identified thus far are downstream of the fungal-specific transcription factor conserved domain, and are located in the carboxy-terminal activation domain, as are similar mutations in ScPDR1 and 3 (504). The single TAC1 hyperactive mutation that is not in the activation domain, T225A, is located in a region corresponding to transcription inhibitory motifs in Pdr1p and Pdr3p, and other proteins in the superfamily (376), suggesting that a similar motif is present in Tac1p, but with an unconserved sequence (79). This is consistent with a still unproved model in which the amino acids in the 225 region interact with those in the *C*-terminal activation domain, masking the latter until inducer binding interferes with the interaction, exposing the activation domain. Some of these mutations may act by interfering with binding of a repressor, Ssa1p, based solely at this point on speculations in *S. cerevisiae* (487).

More mutations in TAC1 positions are likely to be found, since only 23 alleles have been characterized to date. To date, selections have been for resistance and hence for hyperactive alleles; selection for loss of function is more tedious, since the phenotype would be hypersusceptible to azoles. However, this was done in *S. cerevisiae* by selecting for resistance to a lethal gene whose expression was driven by a PDR5 promoter, in turn controlled by a GAL promoter-driven PDR3 gene. One interesting loss of function mutation, D853Y, suppresses hyperactive alleles when overexpressed (500).

Hyperactive mutations are codominant; strains or constructs with both wild-type and hyperactive alleles are susceptible or show intermediate levels of resistance to FLZ and TER and expression of CDR1 and CDR2, particularly at unexposed, constituitive levels, compared to wild-type or strains carrying only hyperactive alleles. Therefore, growth under selective pressure drives homozygosity at TAC1 and at the linked MTL1 (79, 80), consistent with and accounting for the observation that homozygosity at the linked mating-type locus is strongly correlated with azole resistance (456). Progression to azole resistance was seen to occur via a complex scenario, first involving point mutations in TAC1 and the linked ERG11, then loss of homozygosity to allow expression of resistance. This loss occurred either by mitotic recombination, gene conversion, or chromosome duplication, followed either by loss of the chromosome carrying the susceptible allele or the creation of an isochromosome with duplicated left arms carrying the ERG11 and TAC1 resistant alleles (79, 80). Presumably, these hyperactive alleles are not dominant.

In *S. cerevisiae*, both PDR1 and PDR3 regulate PDR5, whereas in *C. albicans*, TAC1 seems to be the sole zinc finger regulator of CDR1. Pdr1p is present at ten times the level of Pdr3p and responds to different signals. The two genes, 36% identical, are regulated by different signals; for example, PDR3 is activated by mitochondrial signaling, and they regulate many partially overlapping sets of target genes (162). Both must be disrupted to confer hypersusceptibility, whereas only TAC1 disruption is required in *C. albicans*. Pdr1p and Pdr3p may form heterodimers with each other and with other ZCF proteins, including Stb5p, in order to target different sets of genes. Data show that TAC1 is necessary for CDR1 and 2 expression; nevertheless, it may not be sufficient,

at least under some circumstances. Other genes may be needed, in response to different signals, to activate CDR expression.

3.2.3 Tac1p Regulon

Tac1p regulates many genes in *C. albicans* in addition to the CDR efflux proteins. This regulon has been analyzed (297) using complementary tools of global expression analysis and direct promoter binding, using global chromatin immunoprecipitation and subsequent intergenic microarray analysis. Expression analyses used Affymetrix-based microarrays, and looked at expression level changes that were common when comparing four clinical-azole resistant isolates to matched putative parent strains that were susceptible. Three of these four were known to have hyperactive TAC1 alleles. Expression analysis used RNA from mid-log phase cultures that were not challenged by azoles, a strategy partly justified by the known constituitive activation of Tac1p. Thirty-one genes were upregulated by at least 1.5-fold in all four comparisons, and most of these upregulations depended on Tac1p, evidenced by comparison to a TAC1 deletion strain. These included TAC1 itself, suggesting autoregulation as seen for PDR3, the known targets CDR1, CDR2, IFU5, HSP12, and RTA3, GPX1 (putative glutathione peroxidase), CHK1 (histidine kinase), LCB4 (sphingoid long-chain base kinase), NDH2 (NADH dehydrogenase), SOU1 (sorbose dehydrogenase), orf19.3047 (transcription cofactor), and orf19.4531 (ABC transporter). Twelve genes were downregulated in all four comparisons, including several membrane transporters and cell-wall proteins. Four of the upregulated genes were confirmed by qPCR, which showed about tenfold greater changes than the microarray data, suggesting that more genes are in the regulon, but are hidden by the less sensitive microarray data.

In a complementary approach, this same international collaboration (297) performed ChIP on Chip analysis to determine which promoters directly bind Tac1p in vivo. In this case, however, the test strain produced a normal Tac1p (although modified by the HA tag), not the hyperactive protein. Only six genes showed enhanced binding greater than twofold higher-than-background levels, and only one of these was also upregulated in the collection of Tac1p-hyperactive strains. Autoregulation of TAC1 was missed in the microarray, possibly because the binding domain of Tac1p to itself is >1 kb upstream of the ORF; qPCR of the bound versus unbound fragments showed very strong Tac1p binding to this domain.

Correlations between genes upregulated by Tac1p and genes whose promoters are bound by Tac1p were poor. Only 26% (8 of 31) of upregulated genes, and none of the 12 downregulated genes, showed Tac1p binding in ChIP-Chip assays. Conversely, only 19% of the genes identified as Tac1p binders showed altered expression levels. First, normal Tac1p may bind only a subset of possible genes recognized by activated Tac1p. Second, some genes showing altered expression may do so as an indirect consequence of Tac1p hyperactivation. Third, some of the Tac1p binding may be artifactual, not biologically relevant. In this context, only 46% (17 of 37) of genes bound by Tac1p had Tac1p consensus binding sequences (CGGN$_4$CGG or CGGN$_3$CGG motifs). Most conservatively, only six genes met all three criteria (binding, consensus sequence, and altered expression), and another three showed both binding and altered expression (Table 5).

Table 5 Genes in the TAC1 regulon identified which show Tac1p-dependent upregulation, binding, and consensus sequences

ORF	Gene	Function	Fold ↑ MA	Fold↑ PCR	Fold ↑ WT	BR MA	BR qPCR	Consensus
Genes showing expression changes, binding, and consensus sequences								
orf19.3188	TAC1	ZCF regulatory	3.1	3		nd	200	CGGAAACGG
orf19.6000	CDR1	Efflux	2.9	9	4.4	10.5	30	CGGATATCGG
orf19.5958	CDR2	Efflux	33.1	400	50.5	1.7		CGGAAATCGG
orf19.23	RTA3	Phospholipid flipping ATPase; induced by fluphenazine and other drugs	29.8		41.0	1.8		CGGAACTCGG
orf19.2568	IFU5	Unknown., membrane protein, induced by fluphenazine	3.9		5.4	3		CGGAAATCGG
orf19.4898		Unknown	2.3		3.4	1.8		CGGGTGACGG
Genes showing expression changes and binding								
orf19.1887	YEH1	sterol esterase	2.6		4.5	1.7		nd
orf19.5257	LCB4	D-Erythro-sphingosine kinase	2.5		4.0	1.6		nd
orf19.86	GPX1	Glutathione peroxidase	2.9		4.0	2.8		nd

Data derived from (297). Fold ↑ MA is the fold increase in expression levels in microarray experiments, averaging increases from four pairs of strains. Fold ↑ PCR is the fold increase in expression determined by qPCR. Fold ↑ WT is the change from microarray experiments compairing strains expressing wild-type Tac1p. BR is the binding ratio, amount of wild-type Tac1p bound to each promoter-gene relative to non-specific binding. *nd* not detected; *blank entries* indicate no data available

S. cerevisiae uses YRR1, another Z2C6 transcription factor, to regulate azole resistance. This gene is also regulated by PCR1/3, and acts on a partially overlapping set of target genes (284). While there is no paralog to ScYRR1 in *C. albicans*, it remains possible that it has similar second-tier regulators waiting to be identified.

In *S. cerevisiae*, the chaperone Hsp70 protein Ssa1p negatively regulates Pdr3 activity. This protein probably maintains Pdr3p in a relatively inactive state most of the time, and its release signals activation of PDR5 transcription. Signaling from defective mitochondria depends on Ssa1p, perhaps by creating multiple binding sites for this protein (487).

3.2.4 NDT80

Potential activators of CDR1 were also screened by integrating a hybrid CDR1 promoter/lacZ fusion into *S. cerevisiae*, and then transforming with a *C. albicans* library on a multicopy plasmid. This identified CaNDT80, homologous in its DNA-binding domain to the novel DNA-binding domain of a meiosis-specific transcription factor in *S. cerevisiae*. Homologs have also been identified in *Neurospora crassa* and in *Aspergillus nidulans* (100, 235); the common function associated with this family is regulation of stress imposed by nutrient limitation. Disruption of CaNDT80 confers hypersusceptibility to azoles and decreases the azole-induced expression of CDR1 (72), although the degree of hypersusceptibility is not dramatic. Data from the TAC1 studies indicate that Ndt80p cannot induce transcription of CDR1 in the absence of Tac1p. CaNDT80 is upregulated by exposure to antifungals. The ScNDT80 DNA binding domain can substitute for the *C. albicans* domain in inducing CDR1 promoter in *S. cerevisiae*, but their dissimilar activation domains are not interchangable. A mutation in the *C. albicans* DNA-binding domain, R432A, which corresponds to an inactivating mutation in ScNDT80, blocks its ability to induce the CDR1 promoter in *S. cerevisiae* and to complement a ΔΔNDT80 strain of *C. albicans* (567).

It is possible, since Tac1p binds to DRE elements, that Ndt80p binds to the basal regulatory element (BRE) in the CDR1 promoter, identified by Sanglard's group (93). However, the putative binding site for Ndt80p, based on its *S. cerevisiae* homolog (GNCRCAAA(A/T), does not correspond to the region defined as the BRE by Sanglard's or Prasad's group. Therefore, the data from the three laboratories suggests that three activators may be required for CDR1 expression, with Tac1p being the required factor. Verification of this will require identification and mutation of the binding site(s) for Ndt80p and disruption of the gene encoding the DNA-binding protein that binds to the BRE detected by Gaur et al. (148).

Serum slightly increases azole susceptibility and proportionally downregulates CDR1 by twofold. The reduced level corresponds to the level seen by disrupting CaNDT80; serum has no further effect in the disruptant (597). These observations suggest that Ndt80 activation is negated by serum. However, Ndt80p could respond to signals, not yet identified but potentially different from those acting on Tac1p, to more fully activate expression. An argument against this, however, is that resistance in clinical isolates by mutation in NDT80 is not yet reported, although it has not been investigated systematically. The subtle nature of effects of disruption on azole resistance and CDR1 expression, as well as the role its homologs play in other species, is consistent with the hypothesis that its role in this process is tangential, possibly a cross-talking between resistance and starvation stress pathways.

Older approaches to analyzing the candidate regulators of resistance genes in *C. albicans* have been to identify and clone homologs of regulatory genes in *S. cerevisiae*, and then transform *S. cerevisiae* strains, deleted for those genes, with the cloned *C. albicans* homolog. This is a powerful method, but has its limitations, as the following examples show. In *S. cerevisiae*, yAP1 regulates expression of a large number of genes in response to oxidative stresses and to azoles. Notably, it upregulates ScFLR1, an MFS protein, to confer resistance to FLZ. The *C. albicans* homolog CAP1, complements yAP1 function in deletion strains (6). However, overexpression of CAP1 in *C. albicans* does not confer FLZ resistance; in contrast, it downregulates MDR1 (7). More recently, a sceening of a *C. albicans* library in *S. cerevisiae* detected three zinc finger protein genes (CTA4, ASG1, and CTF1) that substituted for the deleted PDR1/3 genes to activate expression from the PDR5 promoter and increased antifungal resistance independent of PDR5. However, deletion of these genes in *C. albicans* did not alter antifungal susceptibility or change expression of CDR1, CDR2, or MDR1 (82). These examples underscore that functions of these regulatory proteins evolve rapidly among fungal species.

ScPDR1 and ScPDR3 are Zn_2C_6 type zinc finger-encoding transcription factors that regulate expression of many resistance genes including ABC transporter PDR5 (30, 99, 236, 335), the homolog of CaCDR1, which confers FLZ resistance when overexpressed (31). Three *C. albicans* genes have been identified that complement PDR1/3 deletion strains, restoring PDR5-dependent FLZ tolerance. One of these, FCR1, was another Zn_2C_6-type zinc finger regulatory protein. However, its deletion in *C. albicans* confers in vitro and in vivo hyperresistance to FLZ, suggesting that it represses CDR1, the opposite of its effect in *S. cerevisiae*. Its expression is downregulated by exposure to FLZ, which apparently allows an adaptive response to FLZ. Its overexpression confers hypersusceptibility and blocks efflux and upregulation of CDR1 by FLZ (490, 532). There is no clear homolog to CaFCR1 in *S. cerevisiae*. A second complementing

gene, FCR3, encodes a basic leucine zipper (bZip) domain, and directly upregulates ScPDR5; its homolog is ScYAP3. The role that CaFLZ3 plays in *C. albicans* has not yet been reported (594).

Regulation of expression by PDR1 in *S. cerevisiae* requires other proteins or signals that regulate the activity of the transcription factors. ScPDR13 in *S. cerevisiae* was discovered by screening a multicopy plasmid library for transformants that were cross-resistant to oligomycin and cycloheximide, normally mediated by two different ABC transporters YOR1 and PDR5, respectively. Both transporters were upregulated and required in transformants that overexpressed PDR13, now SSZ1. Resistance also required a functional PDR1. A point mutation in a PDR13 gene was identified that had the same effect as overexpression of the wild-type gene. Overexpression of PDR13 did not elevate the level of Pdr1p, nor did it require the native PDR1 promoter for its effect, suggesting that its interaction with Pdr1p was post-translational. PDR13 encodes a heat shock protein in the Hsp70 family, so an initial model was that this protein is needed to refold Pdr1p, and likely other targets, to an active conformation. Consistently, PDR13 disruptants are cold sensitive and slow growing (169), and fail to induce transcription of other genes not regulated by PDR1, for example, CUP1 and CRS5, normally induced by copper stress (247). Subsequent studies showed that select hyperactive mutants of PDR1 are resistant without the need for Pdr13p (171). Pdr13p/Ssz1p is interacts with a DnaJ-related Hsp40 chaperone subunit encoded by ZUO1, complexed with Ssb1p on the ribosome (336). Pdr13p/Ssz1p still confers resistance if they are expressed in a manner that allows them to be free of association with the ribosome, or if its peptide-binding domain is deleted (124). These changes also still allow Pdr13p/Ssz1p to function as part of the ribosome chaperone complex (78). One model is that the Pdr13p/Ssz1p complex acts as a stress sensor, to fold Pdr1p into a more active conformation, which is mimicked by point mutations in hyperactive Pdr1p. There are no reports yet on the *C. albicans* homolog CaPDR13/SSZ1, other than that it is slightly downregulated in some FLZ-resistant clinical isolates (591).

3.3 Drug Efflux and Membrane Composition

There is supportive evidence for floppase activity of CDR1 in *C. albicans*. Strains selected for resistance to gradually increasing concentrations of FLZ overexpress CDR1, CDR2, and ERG11, and their membranes were increasingly fluid membranes with less ergosterol and externalized phosphatidylethanolamine (253). Similarly, eight azole-resistant clinical isolates had enhanced membrane fluidity, decreased ergosterol content, and elevated levels of PE in the outer leaflet of the plasma membrane (340). These changes may both be a consequence of increased efflux and also contribute to resistance by facilitating the floppase activity. Our earlier review summarized data from many papers showing that mutations, mostly in ERG genes, alter membrane fluidity and also alter efflux and antifungal susceptibility (3).

Closer scrutiny, however, suggests that these effects are mediated by their alterations of lipid rafts, detergent-resistant islands rich in ergosterol and sphingolipids. There is evidence that activity of HuMDR1 requires its localization to rafts (9, 154, 308, 536). Tagged Cdr1p expressed in *S. cerevisiae* has been shown to localize to rafts, and disruptions in ergosterol or sphingolipid biosynthesis resulted in poor surface localization of Cdr1p, but not Mdr1p (353, 354, 402). These data suggest that it is not the altered fluidity or permeability of the plasma membrane per se that increases susceptibility, but rather the disruptions to ergosterol-sphingolipid rich raft domains, which appear to be essential for CDR1 localization function.

3.4 Efflux by Major Facilitators

CaMDR1, alias BEN1, is an MF protein that is specific for FLZ among the azoles, not to be confused as a homolog of human MDR1, encoding P-glycoprotein, an ABC transporter. Major facilitators are proton antiporters whose energy derives from proton gradients established by independent proton-translocating ATPases. There are at least seven proteins in this family in *C. albicans*; only MDR1 has been shown to play a role in antifungal resistance. It effluxes a variety of compounds, but the only clinically used antifungal that it effluxes is FLZ, as reviewed (3).

New information about MDR1 mostly concerns its regulation. Using a β-galactosidase reporter system in *C. albicans*, the MDR1 promoter has been analyzed. There is a benomyl-response element (BRE) at −296 to −260, required for induction by benomyl and for high-level constituitive upregulation, which also specifically binds transcription factors in vitro. On the basis of its sequence, its activator may be the MCM1-encoded MADS box transcriptional activator. A second regulatory sequence at −561 to −520 is the hydrogen peroxide response element (HRE), not required for constituitive expression (450). Genomewide expression analysis has recently shown that a zinc cluster transcription factor encoded by MRR1 (formerly ZCF36) was upregulated coordinately with MDR1 in resistant clinical isolates. Mutations identified in the resistant isolates' MRR1 genes identified P683S and G997V mutations. Introduction of these hyperactive mutant genes into susceptible strains caused MDR1 overexpression and multidrug resistance. Thus, MRR1 is a major regulator of this resistance pathway, which includes

many other genes, notably oxidoreductases that may further protect against collateral damage induced by FLZ (347).

3.4.1 New Tools for Analysis of Drug Efflux

There is an important tool that allows rapid accessment of substrates and inhibitors of candidate efflux genes expressed heterologously in *S. cerevisiae* (279). The host strain in this assay system has been deleted of multiple efflux genes (PDR5, PDR10, PDR11, PDR15, SNQ2, YOR1, and YCF1) and transcriptional activator PDR3. It is transformed with an integration vector that targets the PDR5 locus, and-the cloned candidate gene is driven by the PDR5 promoter whose expression is activated by host PDR1–3 hyperactive transcriptional activator. The vector also includes GFP, FLAG, and HIS tag options. This system allows very high levels and membrane targeting of single efflux candidate genes. Among many uses, this system facilitates structural studies of the proteins, and screens specific inhibitors of pumps. For example, overexpression of six individual membrane proteins conferred resistance to one or many antifungal agents (Table 6), mostly consistent with profiles established previously. Notably, elevated CaERG11 conferred resistance to most azoles except miconazole, and elevated CaMDR1 conferred only weak resistance to ketoconazole, none to the other azoles, under these conditions. Elevated CaCDR1-mediated resistance was the most susceptible to reversal by chemosensitizers. No single agent sensitized the host to elevated levels of all three *C. albicans* pumps; however, several agents in combination with FK506 were fully chemosensitizing. These strains will be valuable in screening new antifungal agents. However, it should be noted that chemosensitizing in this assay does not imply direct inhibition of the pumps. For example, FK506 chemosensitizes by inhibition of the calcineurin stress response pathway. Presumably, when this pathway is blocked, there is enough intracellular FLZ to inhibit growth when CDR1 is overexpressed, but not when CDR2 is. This may be due to higher expression levels of Cdr2p in this system.

4 Evidence that Resistance in Clinical Isolates of *C. Albicans* Is Complex

It should be emphasized that there are numerous reports of clinical isolates whose resistance seems to result from efflux mechanisms that are not attributable to CDR1, CDR2, or MDR1. We reviewed several publications that document that evolution of FLZ resistance in vitro or in longitudinal isolates recovered from the same patients is a complex and sequential selection for multiple mutations in many genes, only some of which are currently known (3). This is consistent

Table 6 Resistance and chemosensitizers of individual overexpressed *C. albicans* membrane resistance genes in *S. cerevisiae*

OVX gene:	Cne MDR1	Ca ERG11	Sc PDR5	Ca CDR1	Ca CDR2	Ca MDR1
Antifungal						
FLZ	R	R	R	R	R	S
ITZ	R	S	R	R	R	S
MCZ	S	S	R	R	R	S
KTZ	R	R	R	R	R	r
NYS	S	S	S	S	S	S
R6G	R	S	R	R	R	S
CHX	S	S	R	R	R	R
CER	S	S	R	R	R	R
TX100			R	R	R	S
Chemosensitizer of sub-MIC FLZ		MIL α11	No	Yes	No	No
		MIL α20	No	Yes	Yes	No
		MIL α25	No	Yes	Yes	No
		MIL β9	No	Yes	No	No
		MIL β11	No	Yes	Yes	No
		ENN	Yes	Yes	No	No
		FK506	Yes	Yes	No	Yes
		OLI	No	No	No	No

Summary of agar disk diffusion assays using *S. cerevisiae* host AD1-8u-, deleted for 8 efflux or resistance genes, transformed with overexpressing (OVX) clones of the indicated genes. *S* susceptible, no change relative to control; *R* resistant relative to control; *r* slightly more resistant than control; *Cne C. neoformans*; *FLZ* fluconazole; *ITZ* itraconazole; *MCZ* miconazole; *KTZ* ketoconazole; *NYS* nystatin; *R6G* rhodamine 6G; *CHX* cyclohexamide; *CER* cerulenin; *MIL* milbemycin; *ENN* enniatin; FK506; *OLI* oligomycin (279)

with complex alterations seen in matched resistant clinical isolates by microarray (230). We also reviewed observations that collectively suggest that nonmutational, physiological, adaptive responses to antifungals (phenotypic resistance, trailing, or tolerance) may represent pathways that are more important clinically than stable resistance mutations, since most patients who fail to respond to treatment are infected with susceptible isolates.

4.1 Tolerance Pathways

These proposed inducible pathways for tolerance are not hypothetical. Two pathways to date are implicated in tolerance: cAMP–PKA, and Ca–calmodulin–calcineurin pathways. A third, the protein kinase C–cell integrity pathway (184), is likely involved in the response to echinocandins and in "paradoxical" resistance to these drugs, as discussed later.

The calcineurin pathway in fungi is a stress-response, signal-transduction pathway essential for tolerance of *C. albicans* to FLZ (Fig. 13). Calcineurin is a heterodimeric phosphatase; calcium-dependent calmodulin binds to calcineurin to activate its phosphatase. In *S. cerevisiae*, Ca^{2+}-bound calmodulin binds to calineurin, which can then remove an inhibiting phosphate on ScCzr1p, allowing it to enter the nucleus and activate transcription of stress response genes. The pathway is conserved in many fungi, although the phenotypic consequences of its inactivation vary with the species (455). In *C. albicans*, calcineurin subunits are encoded by CMP1 (CNA1) and CNB1. While disruption of both alleles of CNA1 is not lethal, disruptants are killed when exposed to FLZ and other azoles, terbinafine, amorolfine, calcofluor white, Congo red, caffeine, SDS, brefeldin A, and mycophenolic acid. Disruptants were more sensitive to Na^+, Li^+, and Ca^{2+}, could not survive in serum, and were avirulent in a mouse infection model (23, 49, 470), athough they were still virulent in vaginal or pulmonary models (24). Similar effects result from inhibition of calcineurin by cyclosporin A, which binds to cyclophilin and the complex binds and inhibits calcineurin, or by FK506, which binds to FKBP12, preventing it from tethering calcineurin to its target proteins (87, 316, 388, 529, 543). The fungicidal synergy of cyclosporin A and FLZ is not dependent on transporters CDR1, CDR2, MDR1, or FLU1, since the synergy is still evident in disruptants (320). These are the only likely targets for these two agents, since the deletion of cyclophilin and FKBP12 results in lack of synergy of either inhibitor with FLZ. These observations indicate that the pathway is essential for responding to a variety of stress signals.

Consistent with findings in *S. cerevisiae*, disruption of CaCNA1 altered colony morphology. CaFKS1, a β-glucan synthase subunit, and CaPMC1, a calcium efflux protein, were upregulated by calcium or FLZ in a calcineurin-dependent manner, whereas CaCDR1, CaFKS3, CaPMR1, and CaPMR2 were not. Deletion of CaPMC1 conferred sensitivity to Ca^{2+} but not to Li^+ or FLZ (470) (23). Which gene, regulated by CNA1/CNB1, is required for tolerance to FLZ is therefore not known. Similar phenotypes were conferred by disruption of CNB1 (57). These authors point out that inhibitors affecting wall structure, in contrast to those

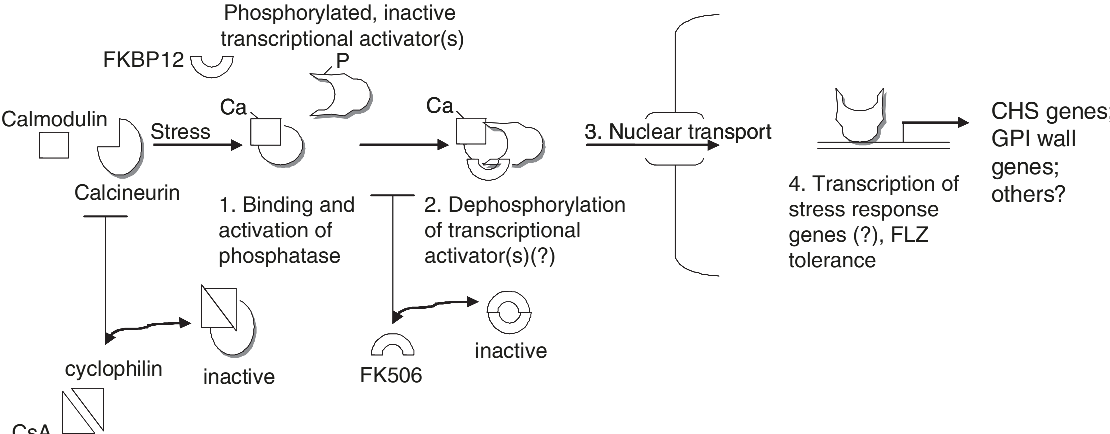


Fig. 13 Calcineurin pathway for inducing tolerance of FLZ by activation of stress response genes. FLZ exposure imposes a stress which mobilizes Ca^{2+}, activating calmodulin, which binds and activates the phosphatase calcineurin (1). With the help of FKBP12, activated calcineurin binds and dephosphorylates transcriptional activators, not yet defined in *C. albicans* (2). This allows nuclear import of the activator (3), which upregulates expression of stress response genes (4). Which of these is critical for survival in FLZ is not yet known. Cyclosporin (CsA) blocks the pathway by binding to endogenous cyclophilin to inhibit calcineurin, and FK506 blocks by complexing with and inhibiting FKBP12. Modified from (3)

affecting membranes, are not synergistic with CsA. They show that a FLZ-resistant mutant with a mutation in ERG11 is susceptible to the FLZ-FK506 synergy, whereas FLZ-resistant mutants showing overexpression of CDR1 are not.

CaCZR1, on the basis of its homology and ability to substitute for ScCZR1 in *S. cerevisiae*, was predicted to be the likely downstream target of calcineurin in *C. albicans*. Although CaCRZ1 is a transcriptional activator of about 60 genes and depends on calcium and calcineurin, it does not seem to mediate azole tolerance or virulence since its deletion confers only modest changes (231, 389). These observations show either that CaCRZ1 is not the primary target of calcineurin, or that there is a redundant gene. Overexpression of CRZ1 on our multicopy plasmid confers hypersusceptibility to azoles and TER.

Some targets of regulation by the calcineurin pathway have been identified in *C. albicans*. For example, the "paradox" effect, in which high concentrations of echinocandins are less inhibitory than lower concentrations, is blocked by calcineurin inhibitors and by deletion of MKC1, the mitogen-activated protein kinase of the cell-wall integrity pathway (566, 578). It regulates expression of the chitin synthase genes, whose upregulation is the likely cause of the paradox effect (355). It also regulates expression of cell-wall GPI proteins UTR2 and CRH11 required for normal wall integrity and susceptibilities (397).

4.2 cAMP–Protein Kinase A Pathway

This pathway in *S. cerevisiae* is required for growth, carbohydrate synthesis, and recovery after stress, and is an antagonist of the calcineurin stress response pathway (534). This antagonism is mediated by phosphorylation of the nuclear localization signal of ScCrz1p, preventing its activation of calcineurin response genes (225). The cAMP–PKA pathway in *C. albicans* (506) may facilitate the recovery process following inhibition by FLZ. Disruption of either CDC35, encoding the adenyl cyclase enzyme, or CAP, the cAMP-associated protein, results in hypersusceptibilty to azoles and terbinafine, as does incubation of wild-type strains with adenyl cyclase inhibitor MDL-12330A. These hypersusceptibilities were overcome with exogenous cAMP. The FLZ-induced expression of CDR1 was blocked by the deletions or inhibitor (208). The cAMP response protein (CREB) has been recently identified in *C. albicans*, which should enable further genetic analysis (505).

On this basis, we speculate that phenotypic resistance to FLZ may be a two-step process: a tolerance response mediated by a calcineurin-induced mechanism, to allow survival, then resumption of growth from the inhibited state, mediated by cAMP–PKA activation of targets that remain to be identified. In this model, the apparent antagonism between the two pathways is actually a timing mechanism. Activated PKA phosphorylates-the *C. albicans* equivalent of ScCrz1p to shut down the calcineurin pathway once the stress response has achieved its goals.

4.3 Histone Deacetylases as Targets of Azole Adaptive Response

Histones are deacetylated by histone deacetylases (HDAC) as an important mechanism of gene activation from yeast to man, and inhibitors of these enzymes are actively being explored as chemotherapeutic agents (343, 344). These histone modifications are clearly global and important in *S. cerevisiae* (270–272, 445). In *C. albicans*, Trichostatin A (TSA) and other HDAC inhibitors alter gene expression, as evidenced by their ability to increase the frequency of white-to-opaque switching, adhesion, and yeast-to-hyphal transition (248, 503). They also cause increased susceptibility to azoles and block the trailing response (317, 511). These effects were limited to inhibitors of ergosterol biosynthesis and were not seen with caspofungin, fluorocytosine (FC), or AMB, and they were not seen in some yeasts. TSA reduced azole-mediated induction of ERG1, ERG11, CDR1, and CDR2, indicating that trailing is mediated by upregulation of these and possibly other genes (511). This also indicates that upregulation by this mechanism of other target genes is not an option for decreasing susceptibility for the non-ergosterol inhibitors. Disruption of HDA1, encoding the deacetylase, had the same affects as the inhibitors, showing that this was their primary target, and inhibitor effects were consistent with phase-specific effects of disruption of individual HDAC genes (521).

One of the lessons in progress from studies of HDAC inhibitors on cancer is that specific HDAC inhibitors often have very specific effects; e.g., they are effective only on select cancer types. This likely stems from the large numbers of these enzymes, which probably act on specific sets of genes, and which are probably differentially affected by each inhibitor. So, the challenge for HDAC as antifungals or synergens to antifungals is whether derivative forms can be found or synthesized that are both specific to fungal enzymes and yet target conserved motifs that suffice to confer broad-spectrum activity. Their potential is to confer susceptibility to strains and species that are resistant to azoles via CDR1 overexpression. A posted but unpublished candidate is MGCD290, which shows impressive synergies with azoles against many species of *Candida* and *Aspergillus* (http://www.methylgene.com/images/gestion/posters/poster078.pdf); since it is slated for phase I clinical trials, it presumably is selective for fungal HDACs.

4.4 Novel Mechanisms for Azole Resistance?

CaPDR16 is part of the Tac1p regulon, by several criteria: upregulation in clinical isolates with hyperactive Tac1p (90, 297), downregulation in Tac1p deletion strains, direct binding by Tac1p to its promoter (297, 610); and coregulation with CDR1 and CDR2 by fluphenazine or estradiol (74, 459). Importantly, PDR16 disruption increases, and overexpression decreases, fluconazole susceptibility in *C. albicans*. However, these changes only alter the MIC by twofold either way (459). These results are consistent with those seen in *S. cerevisiae*; additionally, PDR17 disruption acts synergistically with disruption of PDR16. Their combined disruptions alter phospholipid and sterol composition (107, 545). In contrast, *C. albicans* transformants overexpressing PDR16 on our high-copy plasmid are hypersusceptible to FLZ and resistant to AMB (unpublished observation). The basis for this difference may be in the different levels of overexpression, or in the more fundamental functional differences between the *C. albicans* versus *S. cerevisiae* genes. PDR16 encodes a putative phosphatidylinositol transfer protein (PITP) in the Sec14p family. These proteins are still enigmatic and intriguing; Pdr16p could, by extrapolation (160, 201, 349, 460, 507), facilitate vesicular trafficking or correct insertion of plasma membrane proteins such as the efflux pumps or even membrane-bound ERG proteins, and thus have a mechanistic role in azole resistance.

ALK8 is a *C. albicans* homolog to alkane-inducible cytochrome P450 genes, and data show that its overexpression in ABC pump-disrupted strains of *C. albicans* or *S. cerevisiae* conferred multidrug resistance, including azoles. Alk8p was shown to hydroxylate lauric acid in vitro, a reaction that was competed out with fluconazole. Like FLU1, no correlation of overexpression of ALK8 with resistance among clinical isolates was established, but information on how this correlation was sought is lacking (394). Nevertheless, these data suggest that another mechanism for resistance in *C. albicans* may be drug detoxification initiated by P450-mediated hydroxylations. However, no modifications to antifungals have yet been directly documented.

Circumstantial evidence has been presented for FLZ resistance by inhibiting uptake, but so far only in *C. lusitaniae* (371). However, one study does show that uptake of FLZ in *C. albicans* is by facilitated diffusion (energy independent and saturable) (8), and therefore that an as-yet-unidentified carrier protein is involved and is another potential target for resistance by mutation or tolerance by regulation. This mechanism may not apply to the more hydrophobic azoles, which may enter more readily by passive diffusion.

Conversely, one study provides strong evidence that sequestration of FLZ into vesicular vacuoles is at least part of a resistance mechanism (314). Resistant isolates recovered from a cancer patient after prolonged FLZ treatment showed increased levels of FLZ accumulation, mostly into a high-speed pellet subfraction, correlated with dramatically increased density of vesicular vacuoles seen by electron microscopy. Sequestering of toxic agents into vacuoles is not a new mechanism of resistance. For example, the yeast ScYCF1 ABC transporter is a vacuolar pump that moves arsenite from the cytoplasm into vacuoles, acting in synergy with a plasma membrane transporter to reduce susceptibility (155).

Is FLZ mutagenic? In one study, *C. albicans* exposed in vitro to FLZ-generated FLZ-resistant derivatives showing loss of one copy of chromosome 3 or 4 via non-disjunctions. The implication is that these chromosomes carry recessive resistant alleles. Since these mutants have normal expression levels of CDR1, CDR2, MDR1, and ERG11, alternative mechanisms of resistance are probably involved (404). Another study reported a strong correlation among clinical strains between FLZ resistance and loss of chromosome 5, generating homozygosity at the mating type locus (456). This loss would also generate homozygosity at ERG11 and TAC1. However, when loss of chromosome 5 is selected for by sorbose selection (211), or screening for mating type homozygotes, instead of FLZ selection, there is no correlation (287, 429). Together, these results suggest that chromosome loss is a common result of growth inhibition, but that this may facilitate the selection for recessive mutations that confer FLZ resistance.

Is there any evidence that FLZ is directly mutagenic at the DNA sequence level, by induction of some form of adaptive mutagenesis? Adaptive mutagenesis has been redefined from its original heretical forms into one in which cells increase mutation rates in response to growth-inhibiting stress, so that mutant genes can allow growth to resume. Mutations are not targeted to those genes in any fundamental way (191). Adaptive mutagenesis in *S. cerevisiae* has been shown to be dependent on mutagenic nonhomolgous end joining of dsDNA breaks and on error-prone translesion DNA synthesis by polymerase ζ (180–182). In *C. albicans*, there are no studies. ERG11 genes from FLZ-resistant strains are a richer source of DNA polymorphisms than those from susceptible strains in some (321), though not all, studies (302). This suggests adaptive mutagenesis, and data being collected in large-scale multilocus sequence analysis projects may provide new data and insight.

4.4.1 Mitochondrial Respiration and Antifungal Susceptibility

Susceptibility to FLZ in *C. glabrata* and in *S. cerevisiae* is dependent on mitochondrial function. Petite mutants arise at very high frequencies, and are induced by ethidium bromide (133, 378), in which some or all mitochondrial DNA is

deleted. These respiratory defective strains are FLZ resistant (47, 53, 98, 172, 256, 467, 468). At least part of the basis for this seems to be a retrograde downregulation of expression of PDR5, the *S. cerevisiae* homolog of CDR1, by a functional F_0 component of the mitochondrial ATPase, in respiring cells. This downregulation is lost in petites or in strains with point mutations in the ATPase, resulting in upregulation of PDR5 (605). This defective-mitochondrial signal acts by activating Pdr3p, which autoregulates its own upregulation. This upregulation depends on Lge1p, which is a component of a histone ubiquitination complex that targets lysine of H2A; this modification permits efficient methylation of H3-lysine 4. Loss of LGE1, but not the other genes involved in the complex, blocks upregulation of efflux genes (350, 605) and confers resistance to rapamycin (586). This suggests that Lge1p has a second role, independent of its function in the ubiquitination complex, perhaps interacting directly with and activating Pdr3p. There are no *C. albicans* homologs to ScLGE1, nor any genes associated with histone ubiquitination, so a corresponding gene will have to be found via functional analysis.

The same link between mitochondrial function and efflux gene expression exists in *C. glabrata* (53, 466). Furthermore, FLZ-resistant *C. glabrata* petites need not have irreversible mtDNA deletions. Petites that arose from insertion mutations in several mitochondrial biogenesis genes were reversibly FLZ resistant. Their respiratory deficiency was not due to alterations in the mitochondrial genome. The deficiency and the FLZ resistance reverted at a very high rate, suggesting that an epigenetic mechanism was determining respiratory competence (238).

It is possible that there is more to mitochondrial-based resistance than induction of CDR1. It has been suggested that sterol-depleted mitochondria spew out reactive oxygen species (ROS) that inhibit or kill the cell, and therefore that dysfunctional mitochondria are benign. However, there is some evidence to the contrary (255). Another perplexing link between mitochondrial function and sterol metabolism involves ERG3 mutants in *S. cerevisiae*. Respiratory competent cells with ERG3 deletion are resistant to fluconazole, whereas petites with ERG3 deletions are not; petites are resistant only with a functional ERG3 gene (256). Extrapolating from *Paracoccidioides brasiliensis*, there may also be a mitochondrially localized ABC transporter whose function contributes to azole susceptibility (159).

The above studies argue that there is a complex link between mitochondrial function and antifungal susceptibilities. The demonstration that a reversible mitochondrial dysfunction in *C. glabrata* is responsible for FLZ resistance raises the question of whether a similar effect may occur in *C. albicans*. However, the relationship in *C. albicans* is difficult to study, since forming petites in this species is difficult. There is a report of induction of mitochondrial dysfunction by ethidium bromide in *C. albicans*, accompanied by a slight increase in tolerance for AMB (151), but there was no demonstration of an altered mitochondrial genome and no testing for azole resistance. Inhibition of mitochondrial protein synthesis and cytochrome function with erythromycin also resulted in increased tolerance for AMB (152). Similarly, respiratory-defective *C. albicans* were isolated after exposure to acriflavin, and were resistant to histatin (166), but again, defects in the mitochondrial DNA were not demonstrated. In our hands, it is possible to isolate ethidium bromide–resistant mutants, but these are not respiration defective (unpublished data). Other reports show reduced adhesion or virulence of putative *C. albicans* petites, but do not characterize changes in mitochondrial DNA nor alterations in antifungal susceptibility (14–17). Growth of *C. albicans* in defined anaerobic media does not require ergosterol and results in almost complete conversion of yeast to hyphal forms which are highly resistant to azoles, AmB, terbinafine, and zaragozic acid (120). This observation, and the efflux pump–independent resistance of *C. albicans* within anaerobic biofilms (433), strongly indicate that there is a link between respiring mitochondria and antifungal susceptibility in *C. albicans* that warrants further study.

5 Hitting Targets outside the Ergosterol Pathway

Because of the prospect of increasing azole resistance, it is important to better understand mechanisms of action of other antifungals, either so that they may be used instead of, or to synergize with, azoles, or so that new fungal-specific genes involved in those mechanisms may be targeted by next-generation antifungals. Some of the antifungal agents discussed in this review may never be used clinically, but identifying genes and pathways that respond to these may uncover new targets or help understand actions of and fungal responses to clinical antifungals. Others, e.g., FC, could be used more effectively if we could block potential resistance mechanisms or at least prescreen effectively for resistant isolates before treatment.

5.1 Echinocandins

5.1.1 The Drugs

Echinocandins are natural cyclic lipopeptides (Fig. 14) that now include synthetic derivatives, notably caspofungin, micafungin, and anidulafungin (103). Each inhibits synthesis of the major wall polysaccharide, β-1,3-D-glucan, by

caspofungin

micafungin

anidulafungin

Fig. 14 Structures of echinocandins. From http://www.doctorfungus.org/thedrugs/Glucan_synth_inhibitors.htm

inhibiting β-glucan synthetase (275, 387), a plasma transmembrane protein encoded by FKS1. They have broad-spectrum fungicidal activities against yeasts and fungistatic activities against aspergilli in vitro and in vivo (12, 115, 192, 281, 393, 406, 413–416, 579). Notably less susceptible species include *Cryptococcus neoformans* and *Fusarium*, and most Zygomycetes (103). Clinical resistance to echinocandins is still rare in this early stage of its clinical use, and is most often due to mutations in the FKS1 gene (406). Echinocandins are also very promising in that resistant mutants are not cross-resistant to other classes of antifungals, and conversely, clinical isolates that are resistant to other antifungals, notably FLZ, are not typically cross-resistant (61, 417, 441). However, some species of *Candida, C. parapsilosis* and *C. guiellermondii*, have higher in vitro MIC values, puzzling given that they are typically treated successfully in vivo. In general, the relationship between in vitro susceptibility and in vivo outcome has been uncertain, but there are reports of apparent selection for resistance during therapy, including one patient in whom different mutations in FKS1 arose independently during treatment (168, 233, 261, 282, 283, 337, 346, 416).

5.1.2 The Target: FKS1 Encoded β-Glucan Synthetase

The lack of cross-resistance of echinocandins results from their inhibition of synthesis of a unique target, the essential

β-1,3-D-glucans in the fungal cell wall. The primary targets in *S. cerevisiae* are β-glucan synthase subunits encoded by FKS1 or a paralogous gene encoded by FKS2. They are presumed to be alternate catalytic subunits; FKS1 encodes the major activity in *S. cerevisiae* and is cell-cycle regulated; FKS2 is normally not expressed unless the population enters stationary phase or is exposed to stress, and is regulated by the cell integrity pathway (PKC1) and mediated by calcineurin (290). The enzyme is a plasma membrane protein with 16 putative transmembrane domains. Point mutations in either ScFKS gene confer resistance (116, 117, 334). However, only small numbers of mutants have been analyzed in this way; systematic study is lacking. The activity of the synthase is regulated by ScRho1p GTPase (333, 431), a master integrator of multiple pathways dealing with stresses that affect wall function, and an activator of Fks1 (290).

In *C. albicans*, the same target enzyme (55% identical) is encoded by a single essential ortholog with many names, CaFKS1/GSC1/GSL21/GAC1 (orf19.2929); we will refer to it hereafter as CaFKS1. It encodes a plasma membrane protein with 16 transmembrane domains. There are two related *C. albicans* genes; nonessential CaGSL2 (orf19.3269) is ~29% identical to CaFKS1, ScFKS1 or ScFKS2; it encodes a full-length protein, but its expression level is nearly undetectable under normal conditions. The second related nonessential gene is CaGSL1/GSL22 (orf19.2495) (339). CaGSL1 is most similar to ScFKS3, which has no known role in glucan synthesis or resistance, and appears to be truncated, containing only 10 transmembrane domains, and is presumably a pseudogene. Despite this, CaGSL1 is regulated by caspofungin (296), and it should be pointed out that its similarity to ScFKS3 is only marginally higher than to the other FKS genes. More work is needed to understand what roles these nonessential genes may play, for example, under stress conditions.

Most work in *C. albicans* operates on the assumption that only the essential CaFKS1 encodes active enzyme, since mutations to resistance occur in this gene. Four of four independent spontaneous mutants selected in vitro for resistance to semisynthetic echinocandin L-733,560 showed cross-resistance only to other echinocandins and had in vitro resistant β-glucan synthase activity (274). Disruption of the resistant CaFKS1 allele in each mutant, using an integrative plasmid containing a fragment of CaFKS1, negated this resistance. This shows that mutation in either CaFKS1 allele is sufficient for resistance (114). These caspofungin-resistant mutants were fully virulent in a mouse-disseminated candidiasis model. Surprisingly, in this model, even resistant mutants were effectively treated with caspofungin (274). However, transformants in which the susceptible allele had been disrupted, leaving only one resistant allele, were highly resistant in vivo (114).

More recent studies show that resistance mutations occur in *C. albicans* at eight positions in two hot spots, HS1 and HS2 (Fig. 15). Four of four spontaneous laboratory-resistant isolates, and four of four clinical-resistant isolates had at least one of these mutations. Furthermore, the S645P mutation was introduced by site-specific mutagenesis into the homologous position in a susceptible ScFKS1 gene; introduction of this into *S. cerevisiae* disrupted for FKS1 resulted in resistance (398). These mutations were detected in another 85 of 85 spontaneous resistant isolates from two laboratory strains using a molecular beacon assay that focused only on the hot spot, the 600–700 region (29). These mutations putatively conferred 30- to 1,000-fold increases in MIC to caspofungin and micafungin, but only 16- to 125-fold increases to anidulafungin. Mutant enzymes were ~1,000-fold less sensitive to caspofungin by in vitro glucan synthase assays, and resistant strains require 100- to 1,000-fold higher doses to reduce kidney fungal burden in a murine candidiasis model (398, 406). Strains that were heterozygous for these FKS1 mutations were significantly less resistant in murine candidiasis assays than homozygous mutants, indicating that genetic screening of isolates is important to anticipate a much more likely second mutation to full-blown in vivo resistance (398, 406).

It is still possible, in these mutational studies of *C. albicans*, that mutations in genes other than FKS1 are required for high-level resistance. Laboratory mutants were isolated in a manner that would have permitted a two-step selection, and clinical resistance strains have uncertain progressions to resistance. Contributing to this uncertainty is the fact that the site-specific mutagenesis studies were done in *S. cerevisiae,* not *C. albicans*. Also, the hot spots in FKS1 are defined by full-length gene sequencing of only eight *C. albicans* isolates (398) in a one *C. krusei* isolate (226), supported by hot spot-only analysis in larger numbers of *C. albicans* mutants (27, 29, 282). Therefore, other mutations within FKS1, not yet detected, could modify the degree or specificity of resistance.

Resistance-associated mutations in hot-spot positions of FKS1 in other yeasts and molds have also been reported

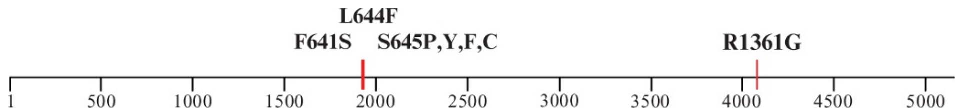


Fig. 15 Positions of hot-spot mutations in *C. albicans* FKS1 that confer echinocandin resistance. Data is compiled from (6, 39)

(226, 261, 406, 446). Polymorphisms at 649 and 642 likely account for reduced susceptibilities of *C. parapsilosis* and *C. guilliermondii*, respectively (29, 398, 406). Preliminary analysis of in vitro selected resistant *C. parapsilosis* isolates in our laboratory indicates that mutations that confer even higher levels of echinocandin resistance are not located in hot-spot regions, and at least some may not reside in CpFKS1 (unpublished observations). Engineering of the S645Y mutation into the *A. fumigatus* FKS1 gene conferred resistance (147). In contrast, in resistant species *Cryptococcus, Fusarium*, and in zygomycetes, the mechanism is not clear (167, 535).

It has been suggested that clinical resistance to echinocandins requires more than just elevated in vitro MIC values, to also include detection of specific hot-spot mutations in FKS1, and a demonstration of reduced sensitivity of glucan synthase in vitro (406). Presumably, the incidence of resistance to echinocandins will rise as its use becomes more widespread; in this case, rapid detection of the mutations by PCR offers the best short-term solution.

5.1.3 Differential Resistance to the Three Echinocandins

Even though very little has been done on this, it is clear that all echinocandins are not alike in their response to resistant mutations arising in clinical isolates. In vitro resistance putatively due to FKS1 hot-spot mutations is less pronounced with anidulafungin (16- to 125-fold increase in MIC) than for caspofungin or micafungin (30- to 1,000-fold increase) (406). This difference may be more pronounced in other species. For example, a multidrug-resistant *C. parapsilosis* isolate was recovered from a patient with endocarditis. While highly resistant to micafungin and caspofungin, the isolate was susceptible to anidulafungin (348). This indicates that unknown mutations interact differently with each echinocandin, and that anidulafungin may have the advantage. These observations also suggest that further modifications to the existing echinocandins could sidestep FKS-1 mutation-based resistance, should this become prevalent in the future. Resistance due to mutations in other genes may also prove to be drug specific.

5.1.4 Resistance outside FKS1

Despite the apparent predominance of FKS1-mutations in echinocandin resistance, there are many other potential mechanisms of resistance to echinocandins based on the complexity of regulation of the fungal cell wall (Fig. 16). However, unlike with azole resistance, efflux seems to play an unimportant role. Overexpression of CDR1, CDR2, or MDR1, or disruption of the entire family of ABC transporter genes, had only subtle effects on caspofungin or micafungin susceptibilities, mostly only in agar-based assays (363, 480).

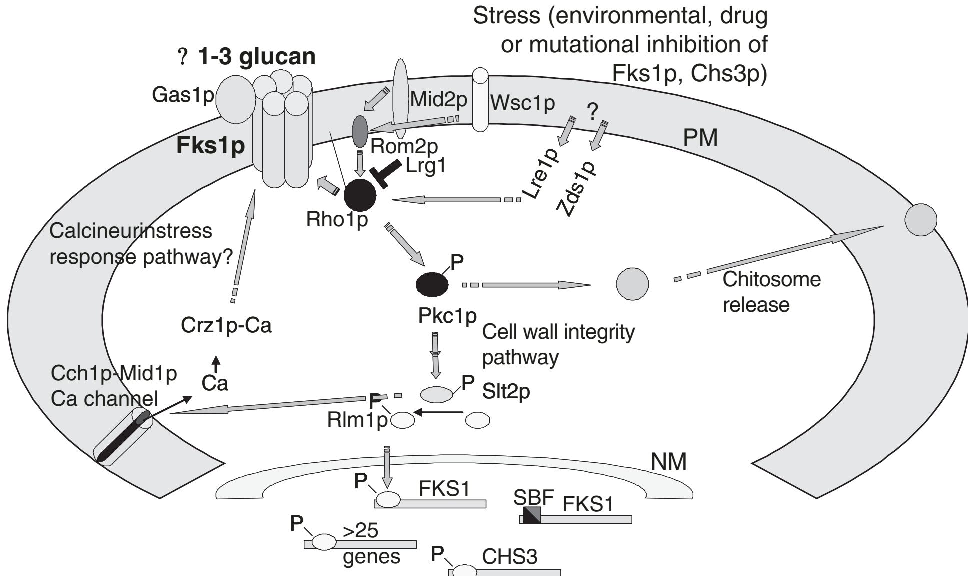


Fig. 16 Model for cell wall regulation in *C. albicans*, extrapolated from *S. cerevisiae*. The catalytic β 1–3 glucan synthase, Fks1p, is activated by G protein Rho1p, in turn activated by Rom2p and inactivated by Lrg1p. Rom2p is regulated by sensors of wall stress, Wsc1p, and Mid2p, and perhaps others. Activated Rho1p also activates Pkc1p to initiate the cell-wall integrity pathway (CWI). This includes release of chitin from chitosomes and Ca^{2+}, the latter activating the calcineurin stress response pathway, which may contribute to FKS1 expression. CWI activates transcription factor Rlm1p, to upregulate many genes, including FKS1 and CHS3, the major chitin synthase. Modified from (104, 290)

Consistently, azole-resistant mutants that overexpressed these genes were susceptible to echinocandins (20). At least at low concentrations, both uptake and efflux of caspofungin by *C. albicans* appear to be mediated by a high-affinity, energy-independent facilitated transporter (392). Although mutants in this putative function have not yet been reported, it is another potential resistance mechanism.

5.1.5 Global Approaches to Resistance Analysis

Using an overexpression approach, one group overexpressed *S. cerevisiae* cDNA clones in *S. cerevisiae* transformants and selected for caspofungin resistance. They isolated only a single gene, SBE2, a golgi protein required for cell-wall assembly, that conferred specific resistance when overexpressed, and hypersensitivity when deleted (391). It remains to be seen whether this protein may be involved in processing one or more of the transmembrane proteins involved in cell-wall regulation, including Fks1p (Fig. 16). We have also used overexpression in *C. albicans* to identify genes that alter echinocandin susceptibility; many genes increased or decreased susceptibility, notably ERG27 (Table 7).

In *S. cerevisiae*, the commercially available set of knockout strains encompassing all nonessential genes (>4,000) were tested for alterations in caspofungin susceptibility (324). Nine disruptants were resistant, and five of these encoded cell-wall or signal-transduction genes. Twenty disruptants were hypersusceptible; 11 were involved in the PKC cell integrity pathway, and chitin, mannan, and ergosterol biosynthesis, including the target gene FKS1. Consistently, PKC inhibitor staurosporine was synergistic with caspofungin against *A. fumigatus*, *A. nidulans*, and *A. flavus* isolates that were resistant to caspofungin alone.

Using the same approach, a different group identified an overlapping set of *S. cerevisiae* genes whose disruption altered susceptibility to caspofungin (291). Disruption of 53 genes resulted in hypersusceptibility, another 39 in resistance, using a less stringent definition of altered susceptibility than in the competing study. Notably, deletion of FKS2, but not FKS1, conferred resistance, as did CZR1, the calcineurin-dependent upstream activator of FKS2 (291). One expects that FKS1 disruptants would be susceptible, since FKS2 is normally not expressed unless FKS1 is deleted, and since Fks2p is more sensitive to caspofungin than Fks1p. It is not clear why FKS2 disruptants would be resistant to caspofungin. In the absence of additional information, it would seem that these strains would have the same susceptibility as wild type, since both express FKS1. From this open question, it seems that there is still much to be learned about the regulation of cell-wall biosynthesis and its regulation.

From these two disruption studies in *S. cerevisiae*, it appears that agents that interfere with the PKC cell integrity pathway, and those conditions or mutations that inhibit compensatory changes in cell-wall biosynthesis, may act synergistically with caspofungin and allow effective treatment of strains and species that are relatively insensitive to caspofungin alone.

Global effects of caspofungin on gene expression in *C. albicans* microarrays have also been documented (296). This study found no changes in FKS1 expression following a 3-h exposure to a subinhibitory concentration of caspofungin, and an overall downregulation of stress response genes. In followup validation by RT-qPCR, the authors noted that some genes were upregulated by more than 14-fold, compared to less than twofold by microarray. This calls into question the sensitivity of the assays used here, and raises the possibility that many important genes may have been missed. The study also suffers from looking at only the single 3-h exposure and from lack of comparison of wild type to resistant mutant strains to help distinguish changes directly related to resistance.

Table 7 summarizes global studies in *S. cerevisiae* and *C. albicans* using disruption and overexpression libraries, microarrays, and synthetic lethals.

These early studies suggest that there are potentially many novel mechanisms for resistance to echinocandins. Some of these genes may become important in resistant clinical isolates as echinocandins become more widely used, and they could be present and required along with FKS1 mutations. In addition, they provide new potential targets for future antifungals and broaden our understanding of the morphogenesis and regulation of cell-wall functions. Whether genes that confer resistance to one echinocandin always also confer resistance to the others is an important but largely untested question because of the current paucity of clinical resistant strains. Preliminary data from our *C. albicans* library indicate that overexpression of zinc finger regulatory genes and genes with no known function or relationship to cell-wall biogenesis confers resistance to echinocandins.

5.1.6 Paradoxical Resistance

Another resistance mechanism may underlie the "paradoxical" resistance to caspofungin (524, 577). In this, 16% of clinical isolates of *C. albicans* are susceptible to caspofungin at low concentrations, but resistant at higher concentrations. Over half of tested clinical isolates are killed at low concentrations but are tolerant at higher concentrations. These effects reverse to wild type upon subculture. This effect was not seen for other echinocandins, or in other species (524, 526). The data suggest that a compensatory pathway is induced, but only at higher concentrations of caspofungin. A strong candidate for this is chitin biosynthesis, since mutations in that pathway can confer echinocandin resistance

Table 7 (A) Cell-wall genes implicated in echinocandin susceptibility in *S. cerevisiae* or *C. albicans*. (B) Transcription factor genes implicated in echinocandin susceptibility in *S. cerevisiae* or *C. albicans*

Sc gene	Phenotype	Ca gene	Ca ORF19	Ca MA	Ca OVX	Putative or Sc function
(A)						
FKS1	HS	FKS1	2929			β-1,3-Glucan synthase subunit
SLG1	R, SL	WSC1[a]	5897		C	Sensor for the PKC1-SLT2 cell integrity pathway
ECM33		ECM33	3010	3.3d		GPI-anchored surface glycoprotein
CRH11		CRH11	2706	2.7		Glycosyl hydrolase
FKS2	R	GSL2(?)	3269	2.6		β-1,3-Glucan synthase subunit
GAS1	SL	PHR1	3829	2.5		GPI-anchored surface glycoprotein, lengthens glucan
CNE1[a]		IPF8537	5300	2.3		ER sorting protein? Calnexin-like
KRE1	SL	KRE1	4377	1.7		Cell wall protein involved in β-1,6-glucan synthesis
CHS3	HS, SL	CHS3	4937			Chitin synthase III
CHS4	HS, SL	CHS4	7349			Activator of Chs3p
CHS5	HS, SL	CHS5	807			Involved in Chs3p transport from the late Golgi to chitosome
CHS6	HS, SL	CHS6	5155		R	Involved in Chs3p transport from the late Golgi to chitosome
CHS7	HS, SL	CHS7	2444			Facilitates exit of Chs3p from the ER
MNN10	HS, SL	MNN10	5658			Subunit of the Mannan polymerase II complex
SIT4	HS	SIT4	5200			Ser/Thr Protein phosphatase that negatively regulates Slt2p
TUS1	R	ORF	6842			GDP-GTP exchange factor for Rho1p
SMI1	HS, SL	SMI1	5058			Regulator of β-1,3-glucan synthesis
BCK1	SL	BCK1	5162			MAPKK-kinase of the cell integrity pathway
BEM2	SL	BEM2	6573			GTPase-activating protein for Rho1p
MID2	SL	?				Sensor for the PKC1-SLT2 cell wall integrity pathway
ROM2	SL	ROM2	906			GDP/GTP exchange factor for Rho1p
ERG3		ERG3	767	3.3d	C	C-5 sterol desaturase
ERG7		ERG7	1570		R	Lanosterol synthase
ERG27		ERG27	3240		R	C3-sterol ketoreductase
(B)						
CRZ1	R	CRZ1	7359		HS	Calcineurin responsive zinc-finger TF
TYE7		TYE7	4941	4d		bHLH TF, glycolysis in Sc, antifungal response in Ca
NHP6		NHP6	4623	3.3d		Chromatin protein
RRN3		RRN3	1923	2.3		RNA polymerase I core TF
ASG1[a]		IPF19920.3/ZCF24	4524	2.1	C	TF involved in cell wall
HAP1[a]		IPF13021/ZCF14	2647	1.8	C	TF involved in heme and oxygen response
SNF2	HS	SNF2	1526			TF acting in the SWI/SNF chromatin remodeling complex
SWI6	HS		1475			Component of SBF and MBF TFs, G1/S transition
SLT2/MRK1	R	MKC1	7523			CWI map kinase
RIM20	SL	RIM20	4800			TF involved in stress resistance
RLM1	SL	RLM1	4662		HS	TF mediating cell integrity pathway response
SWI4	SL	SWI4	4545			TF mediating the cell integrity pathway response
HAP5	SL	HAP5	1973			TF required for activity of the CCAAT-binding complex
QRI5	SL	?				Transcription profile suggests involvement in stress responses

Sc S. cerevisiae; *Ca C. albicans*. Phenotype: Resistant (R) or Hypersusceptible (HS) in gene deletion strain. SL = synthetic lethal with FKS1, FKS2, GAS1, and/or SMI1 (291). *CA MA C. albicans* microarray data, showing fold increase or decrease following 3 h caspofungin; genes with no entry value showed no (<2-fold) significant changes (296). Ca OVX = *C. albicans* overexpression (this application); *C* control level of susceptibility. *TF* transcription factor. RER genes (regulators of echinocandin resistance) are all ZCF genes that cannot be assigned as individual Sc orthologs and are not associated with any function or expression level. Note that 5 Ca echinocandin resistant genes recovered from our transformant library do not correspond to any of the genes in this table or in (296) or (292) and are not yet associated with any function. LDG ORFs 9–11 collectively have activity that cannot be separated by subcloning

[a]tenuous, divergent ortholog

(290), disruptions in ScFKS1 induce chitin biosynthesis (145), and ScFKS1 disruption results in hypersusceptibility (291, 324). Consistently, it was recently shown in *C. albicans* that high concentrations of caspofungin increased chitin production by ninefold, as glucan production fell fourfold (525). This effect has been seen in a mouse model, but not reproducibly, so it has been inferred that it is not a factor in clinical failure (76). This perspective needs more study, but even taken at face value, the lack of relevance may be due to blocking of induction mechanism by the in vivo environment, or to lack of induction because of lower effective concentrations of the drug, noting that serum increases the MIC of echinocandins, or to avirulence of the induced subpopulation. Only the latter of the three mechanisms would render mutations that hyperactive the induction mechanism irrelevant as potential clinical threats. The key mechanism questions include what is sensed by the excess of caspofungin? To trigger what response pathways? The calcineurin stress response and cell-wall integrity pathways are involved (577); are there others, and what are the targets of these regulatory events? Does the excess drug further repress Fks1p to trigger the response, or does it allow binding to a secondary, lower-affinity target? The key question to keep in mind on the clinical relevance issue is whether the paradoxical response represents a mechanism that can be induced by means other than drug concentration in vivo, and therefore constitute an adaptive mechanism, and, by mutation, another potential resistance mechanism. Experiments have not yet addressed this question.

5.1.7 5-Fluorocytosine and Fluoroorotic Acid

These pyrimidine analogs are suicide inhibitors that must be modified by susceptible cells by enyzmes in the pyrimidine salvage pathway to be toxic. The pathway for FC, outlined in Fig. 17, includes a cytosine-purine permease for uptake, a deaminase that is not present in humans, thereby explaining the basis for fungal specificity, and a uracil phosphoribosyltransferase, to generate the toxic intermediate F-UMP. This is incorporated into RNA via F-UTP, presumably inactivating its template function and also inhibiting RNA synthesis. It is also converted by ribonucleotide reductase to F-dUMP, which inhibits thymidylate synthase and DNA replication (273, 421, 565). Fluoroorotic acid (FOA) has long been used to inhibit orotidlyate decarboxylase or orotate phosphoribosyltransferase, encoded by URA3 and URA5, respectively.

Clade-associated resistance to FC was reviewed (3). Molecular studies have confirmed the role of UMP pyrophosphorylase, now called uracil phosphoribosyltransferase. Resistance in most, but not all, clinical strains of *C. albicans* is most likely due to a mutation in the FUR1 gene, formerly FCY1, encoding this enzyme. The mutation at C301T alters a conserved amino acid, is homozygous in FC-resistant strains, is heterozygous in strains with intermediate levels of resistance, and is confined to a single lineage, Clade I (108, 199, 430). The model is that a defective or deficient Fur1p cannot effectively convert FC to the toxic F-UMP, thereby providing resistance. We have confirmed this model by introducing wild-type FUR1 into FC-resistant strains containing homozygous C310T mutations, and showing that the transformants were susceptible to FC (unpublished observations). Consistently, disruption of FUR1 conferred resistance to both FC and FU (587).

Evidence that mechanisms other than FUR1 inactivation are operative in *Candida* is suggested by early biochemical and genetic studies of *C. albicans* and *C. glabrata*. These studies point to deficiencies in cytosine permease and cytosine deaminase, or alterations in thymidylate synthase activities (128, 345, 565). One of 25 clinical isolates showing FC resistance had a homozygous mutation in cytosine deaminase FCY1, although no evidence was presented that this was responsible for its resistance (199). Disruption of FCY2, the permease, conferred resistance to FC, but not to FU, which can enter the cell by other means (587). Our observation is that strains that are homozygous at FUR1 C310 still spontaneously mutate to FC resistance at a high frequency, suggesting that some other gene whose loss of activity confers resistance is non-allelic or heterozygous. We have identified a putative nucleotidase gene, termed here NUC1, by selecting for FC resistance among a library of *C. albicans* transformants overexpressing wild-type *C. albicans* genes, whose overexpression confers resistance to both FC and FOA. These transformants are not auxotrophic (unpublished observations). Presumably, this resistance results from depletion of the pool of toxic F-UMP (Fig. 17).

Studies of FC resistance in *C. lusitaniae*, a species in which genetic analysis is possible, surprisingly offer clues about FLZ uptake. Clinical isolates are resistant owing to mutations the the FCY2 permease. These mutants are susceptible to FLZ alone, but are resistant to FLZ at high

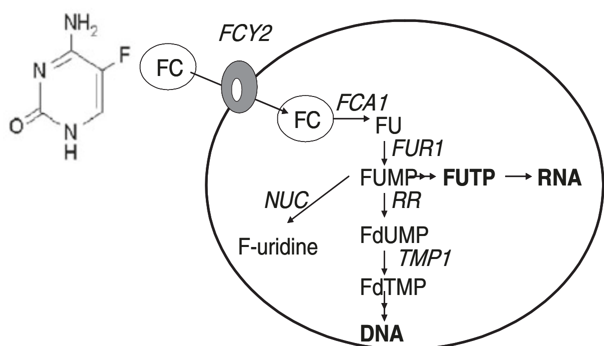


Fig. 17 Pyrimidine salvage pathway in *C. albicans* responsible for susceptibility to flurocytosine (FC)

concentrations of FC (371). This has been confirmed using targeted disruption of FCY2 in a wild-type strain, eliminating the argument that the clinical strains had other mutations responsible for the phenotype (70). Noel et al. proposed that the basis for this effect is a competition for a common uptake facilitator. This facilitator normally is the means of entry for FLZ, but in the absence of a functional Fcy2p, FC competes with FLZ for binding to the putative facilitator, even though FC is not imported. Independently, Papon et al. showed that disruption of either FCY1(deaminase) or FCY2, but not FUR1, conferred the cross-resistance pattern (395). While this detracts from the uptake model, it is still a possibility. Lack of deaminase may increase cytosolic pools of FC and effectively block further uptake by the permease, generating the same circumstance as permease disruption. However, this proposed mechanism is not the only one that could account for the observations, and data directly showing FC-mediated inhibition of FLZ uptake in the permease mutants has not been shown. Neither has it been asked whether the combined drugs induce a known FLZ-resistance mechanism only in the permease mutants. Whether the FC-resistant *C. albicans* FCY2 disruptant (587) shows this type of cross-resistance has not been reported.

Rapamycin (= sirolimus) is a macrolide antibiotic produced by *Streptomyces hygroscopicus* which inhibits a serine-threonine kinase encoded by TOR1 (target of rapamycin). This intriguing pathway plays a pivitol role in nutrient sensing and complex responses to restore and maintain cell growth in *S. cerevisiae*. Tor1 is part of a multisubunit complex TORC1 which acts at at least three levels. First, it blocks nutrient scavenging by inhibiting autophagy (ATG pathway) and, via Tap43-mediated inhibition of a PPN2 phosphatase complex, inhibiting Ntr1 and Gln3 alternative nitrogen source uptake and catabolism. Second, it blocks stress responses by inhibiting transcription factors Msn2/4 and Rtg1/2. Third, it activates protein synthesis by blocking inhibitors of translational initiation factors, by upregulating rRNA transcription and processing and expression of ribosomal proteins, and by promoting mRNA stability (Fig. 18). TOR interacts with the PKA pathway in complex ways; either can activate biosynthetic genes in response to nutrient signals, so hyperactivation of RAS can compensate for inhibition of TOR; both functions are needed for glucose fermentation, and TOR activates respiration in the absence of PKA inhibition (26, 73). In many ways, there is mutual antagonism with the calcineurin stress response pathway, so that only one is active at a given time, and the TOR pathway is needed to recover from inhibitions established during stress responses. Thus, it is pivitol to cancer biology and has been studied extensively in yeast and man in the past few years (1, 18, 88, 96, 97, 122, 203, 318, 325, 332, 424, 451, 479, 583). There are still a number of unknowns in the pathway established in *S. cerevisiae* TOR pathway, and very little of it has been verified in *C. albicans*, so there is potential for differences that may be important in virulence or in vivo persistence.

In most or all eukaryotes, including *C. albicans*, rapamycin binds to and promotes binding of FKBP12 to TOR kinase, inhibiting its function. The TOR pathway can be thought of as a stress recovery pathway, and/or a growth maintenance pathway; its inhibition results in cell arrest in a G_0 state. Rapamycin (sirolimus) is used clinically as an immunosuppressant, not as an antifungal. Non-immunosuppressive derivatives have been made and in some cases are more effective antifungals than the parent agent (86, 105). Rapamycin has antifungal activity in a mouse-disseminated *Aspergillus*

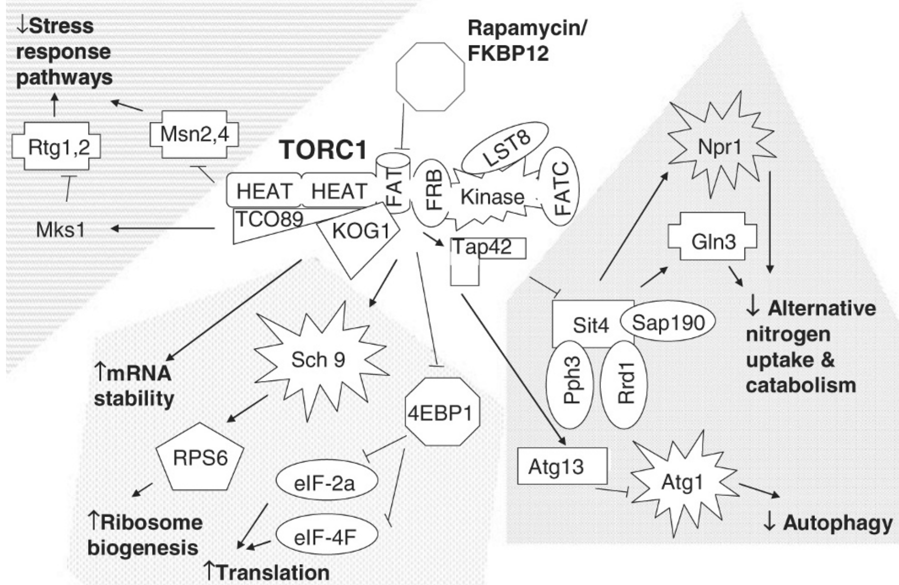


Fig. 18 Rapamycin inhibitng the TOR stress recovery pathway. Rapamycin inhibits the pivotal TORC1 kinase. Active TORC1 kinase regulates a variety of pathways that are needed to resume proliferation after stress. It blocks nutrient scavenging by autophagy (ATG pathway) or inductions of alternative nitrogen uptake and catabolism (GLN3) and promotes general uptake of abundant, preferred nutrients via Npr1 promoted permeases. It increases translation by inducing ribosome biogenesis and increasing mRNA stability. It represses antagonistic stress response pathways

model (193). It is contraindicated for use with voriconazole because of increased bioavailability of rapamycin, but can be used together at a reduced dose of rapamycin (331).

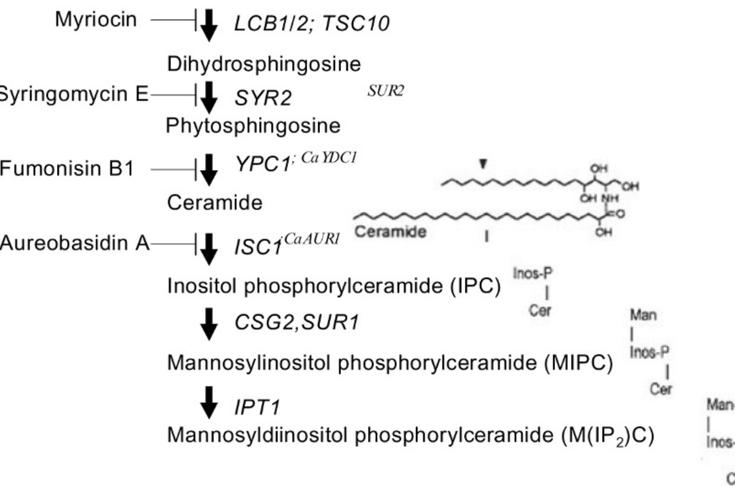
The potential for synergy with rapamycin and any fungistatic agent is that the agent will inhibit growth and rapamycin will prevent an adaptive outgrowth. Unfortunately, there are only a few studies of even in vitro synergy of antifungals with rapamycin. One (127) reports a modest synergy with elaiophylin, a cation channel former (161), and with the potassium ionophore nigericin (425), also produced by *Streptomyces hygroscopicus*. Rapamycin was not synergistic in vitro with caspofungin, voriconazole, or AMB against *A. fumigatus* or *C. albicans* (522, 523). Interactions in recalcitrant molds have not been reported. Furthermore, synergy with rapamycin may involve more than just reduced MIC, including converting fungistatic to fungicidal, or prolonging postantifungal effects, and they may require sequential, rather than simultaneous exposure.

In spite of the multifaceted mechanisms of action of TOR, resistance to Rapamycin can result from mutations in only one of the many divergent pathways affected by TOR (Fig. 18), including inactivation of NPR1, GLN3, MKS1, SAP190, or RRD1. Resistance can also occur by mutation within the TORC1 complex; for example, disruption of TOR89 or heterozygous disruption of the essential KOG1 confers hypersusceptibility to Rapamycin. TOR also activates Apg13 to indirectly inactivate protein kinase Apg1, a key kinase responsible for induction of autophagy, explaining the rapamycin induction of autophagy (228, 595). Overexpression of APG13 may confer tolerance to rapamycin. In addition, many components in the complex and the affected pathways are essential for viability; some of these gene products could be targets of next-generation antifungals.

Preliminary data from our *C. albicans* overexpressant library indicate that overexpression of several genes with unknown functions are resistant to rapamycin, suggesting that there is much to be discovered in this pathway.

Aureobasidin A is a cyclic depsipeptide, produced by *Aureobasidium pullulans* R106 that inhibits inositol phosphorylceramide (IPC) synthase, which catalyzes a late step in the synthesis of sphingolipids (Fig. 19). It is active in vitro against a wide spectrum of fungi, including *Candida, Crytococcus, Histoplasma,* and *Blastomyces*, but notably not *Aspergillus* or Zygomycetes, and was tolerated and effective in mouse-disseminated candidiasis (531). However, its development was halted in clinical Phase I trials owing to limited activity against *Candida species*. Sphingolipids IPC, MIPC, or M(IP_2)C are essential for yeast viability, and the downstream forms are required for normal tolerance to calcium. Intermediates, especially ceramide, are potent inhibitors at low concentration. Sphingolipids and select precursors are implicated in heat stress responses, endocytosis, cell integrity pathway, and cell signaling (380). Polarization of the plasma membrane into ergosterol- and sphingolipid-rich "raft" domains is thought to be a basis for collecting proteins, especially GPI-anchored proteins, ABC transporters, and MFS transporters, which in turn are needed for morphogenesis, hyphal formation, and antifungal resistance in *C. albicans* (326). Synergy between azoles and aureobasidin A is therefore expected, but is not observed.

The enzyme targeted by aureobasidin A is encoded by the essential gene AUR1 (ISC1); select point mutations in, or overexpression of, this gene results in resistance (179, 183). This enzyme complexes the ceramide chains to inositol phosphate, rather than to choline phosphate as in mammals, accounting for its specificity (380). Aureobasidin A is active against many species of *Candida*. In *S. cerevisiae*, it results in cell death by loss of membrane integrity (125). It also effectively inhibits IPC synthase in *A. fumigatus*, but the organism is resistant, apparently because of ABC



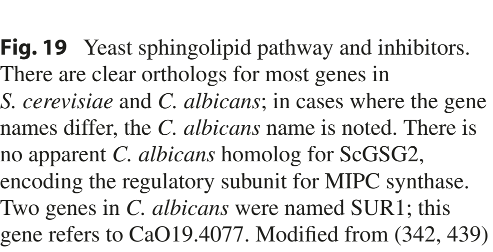


Fig. 19 Yeast sphingolipid pathway and inhibitors. There are clear orthologs for most genes in *S. cerevisiae* and *C. albicans*; in cases where the gene names differ, the *C. albicans* name is noted. There is no apparent *C. albicans* homolog for ScGSG2, encoding the regulatory subunit for MIPC synthase. Two genes in *C. albicans* were named SUR1; this gene refers to CaO19.4077. Modified from (342, 439)

transporter–mediated efflux (606). Zygomycetes are resistant because they do not form sphingolipids, but rely on their alternative neutral glycosphingolipids (13).

Screening of our library of *C. albicans* overexpressants identified CaIPT1, not CaISC1, as a gene capable of conferring resistance to aureobasidin A (unpublished observation). The two genes encode similar phospholipase C-like enzymes, and both transfer inositol phosphate, albeit onto different substrates, and Ipt1 is inhibited in vitro by aureobasidin A, but at 1,000-fold higher concentrations. Perhaps the elevated level of Ipt1p catalyzes sufficient ceremide→IPC synthesis under conditions in which aureobasidin A is inhibiting Isc1p. It has been shown that disruption of CaIPT1 results in abnormal localization of membrane efflux pump CDR1 and hypersensitivity to azoles and terbinafine, which are are normally effluxed by Cdr1p (403); our overexpressing CaIPT1 transformants were not resistant to azoles. In *S. cerevisiae*, CDR1 homolog ScPDR5 and ScIPT1 are coregulated by PDR-1 and -3, as are several other genes (LCB2, SUR2, LAC1) in the sphingolipid pathway (350), making a regulatory tie between genes encoding the pump and components of the rafts in which they reside.

Another connection between sphingolipid metabolism and the PDR resistance pathway is in the regulation of toxic ceremide-derived metabolites that accumulate under stress. Inhibition of ScPDR5 results in upregulation of RSB1 via PDR1; Rsb1p effluxes excess toxic long-chain bases from the perturbed sphingolipid metabolism. (170). The *C. albicans* homolog RTA2 has not been analyzed functionally, but is upregulated by ketoconazole and caspofungin, and in an AMB/FLZ-resistant mutant (37, 269, 296). This suggests that it does play a role in stress responses, but perhaps that it is regulated differently than in *S. cerevisiae*.

Nonsystematic approaches in *S. cerevisiae* show that suppressor mechanisms can override loss of sphingolipid biosynthesis. For example, mutations in ScSLC1 allow synthesis of an alternative analog of the phosphoceramide backbone which can substitute for mannosylphosphorlyceramides; these mutants are hypersensitive to azoles and amphotericin B [10]. Also, overexpression of ScHOR7 confers tolerance to Ca^{2+} in cells that are deficient in synthesizing the last two MIPCs [11]. Identifying these and other pathways in *C. albicans* should lead to identification of new antifungal targets, and inhibition of some of these should be synergistic with auriobasidin A, possibly synergy with defensins (2)

5.2 Peptides

5.2.1 Histatin

Histatins, notably Hst3 and Hst5, are histidine-rich cationic peptides (Table 8) present in human saliva. They have antimicrobial, anti-candidal activity in vitro, and their in vivo levels are inversely correlated with oral yeast carriage (209, 390). They are active against azole-resistant *C. albicans* (539) and most other species of *Candida*, as well as A. *fumigatus* and C. neoformans (186, 538); broader ranges of susceptibility have not been reported.

The mechanism of action of Hst5 is intriguing (Fig. 20), and is not, as with other cationic peptides, due to formation of membrane pores (454). Instead, Hst 5 binds to the membrane-associated heat shock protein ScSsp1p (295). Only a 12-amino-acid fragment of His5, P-113, is required for this effect (452). This interaction is necessary for most of the killing effect of Hst5 (294). The mechanism of killing once this occurs is not yet clear.

Death mediated by Hst5 depends on the release of K^+ and ATP from the cell (258, 259), primarily mediated by Trk1p, the plasma membrane K^+ transporter (25). However, this release is selective and does not involve cell lysis (259). The

Table 8 Natural anticandidal peptides and derivatives

Aa		
Histatins		
His3	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN	32
His5	DSHAKRHHGYKRKFHEKHHSHRGY	24
P-113	AKRHHGYKRKFH	12
Dh-5	KRKFHEKHHSHRGY	14
Dhvar5	LLLFLLKKRKKRKY	14
Lactoferrins		
hLF1–11	GRRRRSVQWCA	11
β-Defensins		
hBD-2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	41
hBD-3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	45
Cathelicidins		
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37
RK-31	RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	31
KS-30	KSKEKIGKEFKRIVQRIKDFLRNLVPRTES	30

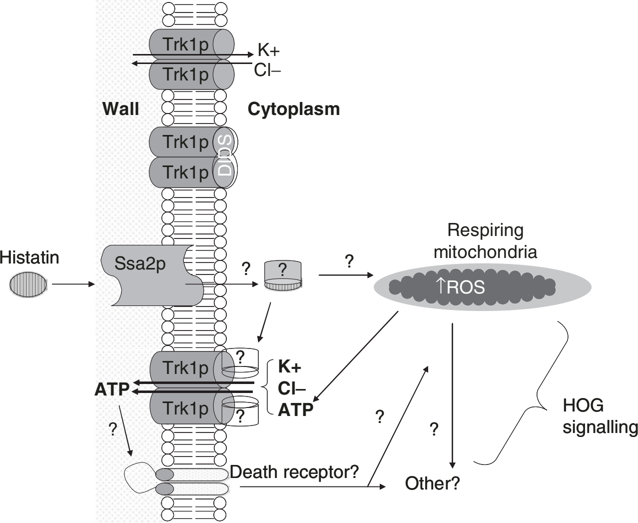


Fig. 20 Cell-mediated death by histatins. See text for explanations

resulting depletion of intracellular ATP is not responsible for cell death, since anaerobically grown cells show similar ATP depletion but are Hst5 resistant (259). Instead, it is speculated that the extracellular ATP binds to and activates a protein that cross-reacts with human P2X(7) receptor (258). In some human tissues, this protein may act as a cell death receptor which triggers massive Ca^{2+} influx, lipolytic signaling, and caspase-mediated cell death (144, 210, 444). This hypothesis is consistent with several observations of Hst5-mediated death in *C. albicans*, including that it is partially mimicked by exogenous ATP and its analogs, that mere loss of ATP is not sufficient to explain death, and that a cross-reacting protein is detected (258). However, the P2X7 model analogy fails on several levels. Histatin-mediated killing is not dependent on extracellular Ca^{2+} (258, 259), nor on classic fungal apoptotic mechanisms (584). Binding and activity of Hst5 is inhibited by low concentrations of Ca^{2+} present in human saliva, masking its antifungal activity (113). Despite the detection of a *C. albicans* protein that cross-reacts with P2X7 antibody, no homolog or domain in the current *C. albicans* database is evident, and no follow-up reports confirm this model.

In vitro stepwise selection on Hst3 identified resistant mutants that still bound and internalized Hst3, and still effluxed ATP (132). The need for stepwise selection suggests that more than single mutations are needed. These mutants were lost; however, subsequent analysis of dervatives of these stepwise selections showed that stably resistant mutants did show reduced ATP efflux and oxygen consumption (131). It will be important to reisolate resistant mutants that maintain ATP efflux to determine whether any of these are defective in a hypothetical extracellular ATP receptor. Some mutants showed reduced levels of Trk1p, consistent with the model in Fig. 20. One mutant had normal Trk1p levels, nevertheless did not efflux ATP upon exposure to histatin, implicating intermediates connecting imported histatin to Trk1p. Proteome analysis of these mutants shows that many, complex changes have occurred, reflecting their stepwise selection; overall, these changes are consistent with partial mitochondrial dysfunction.

Mitochondria play a complex role in response to histatin (185, 454). In *C. albicans*, respiring mitochondria are essential for the fungicidal effect of Hst5, since respiration-defective mutants are resistant (165) and since inhibition or uncoupling of oxidative phosphorylation blocks killing by Hst5 (95, 165, 185, 259). Unaccountably, the latter is not true in *S. cerevisiae*, even though respiring cells are more susceptible than fermentative cells (95). A similar pathway is implicated for Hst3, from which Hst5 is derived (132, 588). Hst5-affected mitochondrial membranes are eventually depolarized (185) and release ROS, but this is not the mediator of cell killing (554, 584). Mitochondrial swelling is an early morphological response to histatin treatment, although accompanied and followed by many other changes (204).

Histatin resistance in *C. glabrata* is unusual among *Candida* species (188, 366). This is not due to differences in regulation or activation of CgCDR1 or CgCDR2, since their disruptions do not alter histatin susceptibility (188). The lower levels of susceptibility to his5 and derivatives in *C. glabrata* parallel those of *S. cerevisiae*; perhaps this is due to their shared abilities to repress mitochondrial functions (95, 508).

In response to exposure to histatins, *C. albicans* activates its HOG (high osmolarity glycerol) stress response, consistent with the proposed mechanism of action. Inactivation of HOG confers hypersusceptibility; other stress response pathways are less important. Preexposure to osmotic stress adapts cells to tolerate subsequent histatin treatment (557), suggesting that resistance to histatins could occur by mutations that upregulate this pathway.

Clearly, more work is needed to describe the mechanisms and regulation of histatin-mediated cell death, and this will lead to an understanding of fundamental and novel processes, and subsequent development of new antifungal targets. Clinically, systemic uses for histatins may depend on its targeting fungal cells in a protective matrix, or by modifications such as multisite amino acid substitutions (187), or branching (607), by even by gene therapy strategies (377). Their potential as synergens for other antifungals has been reported only once, where it was synergistic with and extended the spectrum of AMB, but had no synergy with azoles or FC against *Candida* and *Aspergillus* species (544). This study should be extended to other antifungals, notably the echinocandins.

5.2.2 Lactoferrin

Lactoferrin (LF) is another human glycoprotein with broad-spectrum antimicriobial activities. It is present in milk, saliva and various exocrine secretions and in neutrophils, which

can release LF at wound sites. Its anti-*Candida* effects have been documented in many laboratories, in which it acts synergistically with FLZ or AmB, and is notably more effective on *C. krusei*, which is typically more resistant to antifungals (42, 266, 267, 358, 369, 462, 463, 519, 542, 562, 563, 590). At high doses, it is effective in a mouse-disseminated candidiasis model (309).

LF's mechanism of antifungal action remains enigmatic. LF scavenges iron even at low pH, creating a challenging environment for microbes at wound sites (568). However, its antifungal mechanism is not likely dependent on this ability, since iron-free LF, and an amino terminal proteolytic derivative, lactoferricin, and hLF1–11, a loop of 18 amino acid residues formed by a disulfide bond between cysteine residues 20 and 37 of human lactoferrin, retain antifungal activity without binding iron (267, 311). "Activated" lactoferrin, formed by immobilization onto pectins, is a more effective antifungal than LF in solution (359). Although ambiguous, inhibitor studies suggest that LF causes a large-scale influx of intracellular Ca^{2+} into mitochondria, resulting in loss of mitochondrial membrane potential and influx of Ca^{2+} from organelle stores and from the environment (310). Despite sequence differences, hLF1–11 has many features in common with histatins, including low-level K^+ release without cell lysis, inhibition by mitochondrial inhibitors and by Ca^{2+}, release of ATP, and inhibition of killing by ATP antagonists (311, 555, 556). In its "activated", immobilized form, LF inhibits adhesion of *C. albicans* to epithelial cells (358, 359). LF may interact with cell-wall mannoprotein(s), since their inhibition by pre-exposure to tunicamycin blocks inhibition (367, 368). Resistance and resistance mechanisms to LF and derivatives remain to be explored.

Defensins are arginine-rich peptides (36–47 amino acids) with six cysteines that form three intramolecular disulfide bridges (Table 8). They are made in various epithelial tissues, where they concentrate in phagolysosomes or are secreted into mucus membranes as an innate defense against microbes; β-defensins hBD-2 and -3 are fungicidal to many species of *Candida* (94). They are upregulated in response to oral colonization by *C. albicans* (246). These two peptides have many effects on *C. albicans* in common with histatins, including binding to Ssa proteins, ATP efflux without overt lysis of the membrane, and dependence on respiration (558, 559). However, defensins do not require Trk1p (558).

Resistance to all candidicidal peptides is understudied. *S. aureus* has acquired an innate resistance to defensins via Mpr-F, an enzyme that adds a lysine to membrane phosphatidylglycerol. Presumably, the reduced membrane charge prevents its interaction with the cationic defensin (409). Other bacteria have acquired charge-related modifications to their membrane, or efflux mechanisms, which confer resistance to these peptides (408). Potentially, similar mechanisms might confer resistance in *Candida*.

Cathelicidins are a heterogeneous group of peptides synthesized in epithelial cells in a number of tissues, notably in sweat and saliva, where they provide initial lines of defense. They are grouped and named because of a common proprotein region cathelin, a cathepsin L inhibitor, and a conserved signal sequence (94). The most characterized of these, LL-37 (Table 8), has modest antifungal activity (288, 439, 602), which is enhanced by proteolysis in sweat (164, 252, 304, 356). Upon contact with the plasma membrane, they adopt an α-helical conformation that penetrates into the bilayer, and most have a charged head that remains on the face, resulting in extensive membrane permeablization (101). Kinetics of permeablization matched that of killing. While KS-31 and RK-31 differ from parent LL-37 in which they localize (LL-37 remains at the cell surface, but RK-31 enters the cytoplasm), they each cause permeabilization and ATP release (102).

Plant-derived oils from a variety of sources, notably thymus, melaleuca (tea tree), and cinnamon, have rapid cidal effects on human fungal pathogens. Where investigated, these seem to act as membrane-disrupting agents and are likely limited to topical use (65, 173, 420, 461, 493, 494, 502). Whether these oils have mechanisms related to lytic peptides remains to be seen. Resistance to these agents is not documented.

Synthetic peptide libraries have been constructed and screened for fungicidal activity. These have been cationic peptides, to mimic natural antifungal peptides and derivatives. For example, Monk's group made a 1.8 million member D-octapeptide library that contained cationic peptides $D–NH_2–A–B–X3–X2–X1–RRR–CONH_2$. A peptide that seems to act by inhibiting Pma1p, the major plasma membrane ATPase, was fungicidal and, at lower doses, sensitized *C. albicans* to FLZ (342). Other synthetic antifungal peptides have also been identified (251, 252, 365, 399). Though promising, the studies in general are limited to in vitro susceptibilities, so their in vivo efficacy is not known. Resistance mechanisms to these peptides have not generally been investigated, yet.

5.2.3 Aminoacyl tRNA Synthetase Inhibitors

Icofungipen (formerly PLD-118, BAY10–8888) represents another class of antifungal compounds, cyclic β-amino acids (Fig. 21), which apparently target aminoacyl synthetases and inhibit growth in vivo. Preliminary studies suggest that yeasts are susceptible to icofungipen, because of a combination of its ability to accumulate in the cytoplasm and its ability to inhibit isoleucyl-tRNA synthetase. In vitro studies show that isoleucyl-tRNA synthetase activity is inhibited by more than 90% at 10 mM icofungipen, with a corresponding decrease in protein synthesis. Furthermore, adding isoleucine to media precludes inhibition by icofungipen, suggesting, in this

Fig. 21 Structures of cyclic amino acid inhibitors

PLD-118

H_2N COOH

cispentacin

H_2N COOH

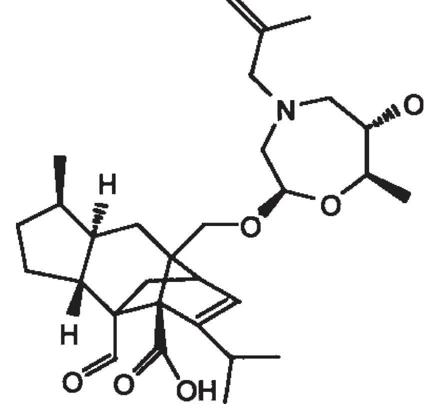


Fig. 22 Structure of sordarin derivative R-135853 (417)

model, that isoleucine concentrations compete for both uptake and synthetase (608). Consistently, but not conclusively, increased expression levels of the synthetase among *Candida* species and mutants of *C. albicans* are correlated with resistance (609). More convincingly, the levels of accumulation of icofungipen are much lower in the resistant *C. albicans* mutants, suggesting that uptake is blocked by a defective permease, or that efflux is increased in the mutants. Consistent with this model, in vitro inhibition is blocked by isoleucine or leucine in the media.

In vitro susceptibility tests with icofungipen are generally "fussy" and show a wide MIC range from 1 to 64 μg/mL among 69 *C. albicans* isolates, despite using very low innocula; seven of these were resistant. In contrast, in vivo susceptibility in a disseminated-mouse model was very robust, at least using a strain showing low in vitro MIC values, showing 100% survival with 10 mg/kg over 10 days, not affected by isoleucine (178). Icofungipen was shown to be effective in eradicating FLZ-resistant *C. albicans* in a rabbit OPEC model (411). The drug was safe and well tolerated in the animal model (401, 410). Phase II clinical trial of OPC showed good control of *C. albicans* infections, but not eradication (130). No current clinical trials are posted on the GlaxoSmithKline Clinical Trials Register site http://ctr.gsk.co.uk/medicinelist.asp.

Because icofungipen has a different target than clinical antifungals, one would expect an additive or synergistic interaction with azoles, echinocandins, etc., but no data is available. Combination therapy, therefore, is worth considering and testing, to exploit possible synergies and to preclude breakthrough of resistant isolates.

Another cyclic β-amino acid analog, cispentacin, or (1*R*,1*S*)-2-aminocyclopentane-1-carboxylic acid (Fig. 21), is taken up by *C. albicans* via the inducible proline permeases, and probably by other permeases, and accumulates to millimolar concentrations (60, 214). Uptake is competitively inhibited by proline, and it inhibits proline tRNA synthetase and protein synthesis. This static agent was effective in a mouse-systemic candidiasis model (254).

5.2.4 Sordarins

Sordarins are natural products from the sordariomycete *Graphium putredinis*. They inhibit microbial but not human translation elongation factor 2 (eEF2) (59, 110–112). Derivative forms are effective in vivo against yeasts other than *C. glabrata*, *C. parapsilosis*, *C. lusitaniae*, and *C. krusei*. In disseminated infections in mice, these derivatives were effective against *C. albicans* but less so against *Aspergillus* (327–329). A derivative (Fig. 22) with a1,4-oxazepane ring moiety was effective against FLZ-susceptible or -resistant *C. albicans*, *C. glabrata*, *C. guilliermondii*, and *C. neoformans*, but not against *C. parapsilosis*, *C. krusei*, and *Aspergillus* spp. (229). Its in vivo efficacy against both OPC and systemic *C. albicans* was impressive. The limited spectrum has been the biggest current problem for sordarins, not specificity or toxicity. However, that is changing. A new, novel natural sordarin moriniafungin has a broader spectrum, failing to inhibit only *C. lusitaniae* in this admittedly limited collection, and synthetic derivatives of hypoxysordarin act on all but *A. fumigatus*. Azasordarins are effective against all species of *Candida* except *C. krusei* (39). Because of this idiosyncratic spectrum, perhaps combinations of sordarins with complementing profiles could be exploited.

Sordarin binds to *C. albicans* and *S. cerevisiae* eEF2 in vitro and its binding is enhanced by the presence of ribosomes, which suggest a complex interaction between eEF2 and ribosomes (112, 489). Consistently, resistance to sordarin in *S. cerevisiae* is conferred by mutations in eEF2 which result in loss of drug binding in extracts (59, 223, 489). Resistance is also conferred by alterations (chimeras, site-specific mutagenesis) in ribosomal proteins which interact with eEF2 (157, 224, 476). These alterations are outside the

points of contact between eEF2 and the proteins (277), indicating that the interaction is complex and allosteric. Studies of resistance to sordarins are therefore of fundamental interest to the molecular mechanics of translation (513).

5.2.5 CAN-296

A complex polysaccharide isolated from *Mucor rouxii*, CAN-296, has rapid lytic, fungicidal effects on many pathogenic yeasts, regardless of susceptibilities to other antifungals, although it is not effective on *Aspergillus* (44). On the basis of the premise that its high molecular weight implies a wall or membrane target, a group at Wayne State University showed that CAN-296 inhibits proton pumping (media acidification) in susceptible but not resistant *Candida* isolates. This implicated the membrane H^+-ATPase; however, the H^+-ATPase activity of membrane fractions was not affected by CAN-296 (45). CAN-296 binds to cell-wall fractions of *C. albicans*, and the binding is reversibly inhibited by Ca^{2+} (46). Together, the data suggest that inhibition of proton pumping by CAN-296 is an indirect effect, and therefore that the true target is not yet known. Its in vivo binding may limit its clinical use to topical antifungals, where it is effective (43). It is unfortunate that no further studies on this dramatically fungilytic agent have been reported; identification of its target and mechanism are goals still worth pursuing.

5.2.6 Steroidal Saponins

Steroidal saponins are a diverse group of glycosidic steroidal agents derived from plants, which likely act by targeting and disrupting membranes. A subset of these are specific to fungi, presumably related to ergosterol content, and are surprisingly nontoxic to mammalian cells. A leading candidate, CAY-1, was fungicidal against multiple *Candida* and *Aspergillus* species, *C. neoformans*, and *P. carinii* without being antibacterial or cytotoxic to multiple mammalian cell lines (92, 440). It showed modest synergy to indifference with AMB or ITZ against *Aspergillus* species and *C. albicans* (91). Similar results were seen with other steroidal saponins (593, 604). One unusual route to fungal resistance to saponins is by enzymatic inactivation. Some species, e.g., *Fusarium*, secrete the enzyme tomatinase which removes the sugar from the saponin α-tomatin; heterologous expression of this enzyme in *S. cerevisiae* confers resistance (75). It is not clear whether activities like this may be widespread or broad spectrum; if so, this would restrict its clinical value. Other resistance mechanisms may arise; it seems likely that conditions that result in downregulation of ergosterol content in fungi would reduce their susceptibilities to saponins. Whether these agents are potent in vivo has not been reported, other than as vaginal topicals (604).

5.2.7 Acetominophen

S. cerevisiae is inhibited by very high concentrations of acetaminophen, and susceptibility is dramatically lowered by deletions in ERG genes. Deletions above ERG3 were more susceptible than those at or below ERG3. Resistance was dependent on PDR1/3 regulation, which could be mediated through YAP1 or through another unknown target of PDR1/3. Erg11p and Erg5p cytochrome P450 enzymes did not play any role in activation or detoxification (520). There are no published susceptibility assays on *Candida* species, although one report shows that acetaminophen-enhanced phagocytic killing of *C. albicans* by human polymorphonuclear leukocytes (163).

6 Resistance in Biofilms

Candida species are capable of forming biofilms, in which "normal" planktonic cells differentiate by global changes in gene expression into sessile cells that elaborate an extracellular polysaccharide matrix whose composition varies with the species. In this matrix, cells grow at a slower rate in a microanerobic to anaerobic environment, and they show high levels of resistance to select antifungals. Excellent recent reviews cover the status of molecular genetics of biofilm development (surface adhesion, cell–cell adhesion, hyphal transition, maturation) (50, 370), but the resistance aspect of biofilms is still enigmatic. This may be due to novel and multiple resistance mechanisms, the complications of possible subpopulations within a biofilm with different resistance levels, and the phenomenology aspect of working with biofilms, which are established using different methods in various laboratories.

In vitro systems for establishing and researching biofilms vary. They have been established by simple adherence to and maturation on polystyrene, e.g., in bottoms of standard 96-well plates or petri dishes. In some cases, these surfaces are coated first, typically with serum. In other systems, dental acrylic or catheter material provides the substrate. In more elaborate systems, adherent cells are constantly exposed to media flow. These systems are designed to more closely mimic in vivo environments, and typically result in thicker and more differentiated biofilms in which cells in the periphery are mostly hyphae or pseudohyphae (69, 198, 352, 435, 436, 438). In animal models, central venous catheters are colonized by systemically infected *Candida* (495). Biofilms are typically assayed by fluorescent laser scanning confocal or scanning

electron microscopy, and viability of embedded cells is monitored in situ by reduction of the redox indicator XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide), which measures activity of mitochondria via mitochondrial enzyme (435). Alternative fluorescent dyes seem superior for this application (137, 198, 218). What is it measuring?

Under most of these conditions, cells in biofilms show resistance to amphotericin B, triazoles, and flucytosine (4, 50, 67–69, 118, 265, 305, 352, 436) and to many metals (copper, cadmium, silver, cobalt, chromium, and selenium) (177). In contrast, caspofungin was found to be effective in vitro against *C. albicans* cells in biofilms formed in vitro, and prevented the formation of biofilm, if used at concentrations above the MIC but within therapeutic range (21, 77, 264). Lipid formulations of AMB are effective against biofilms (264). An in vivo study in rabbits found that AMB reduced and CAS eliminated colonization of central venous catheters by *C. albicans* (495); many antifungals that are ineffective against mature biofilms can prevent their in vitro formation at subinhibitory concentrations (264).

It has been proposed that azole resistance of *C. albicans* in biofilms formed on dental acrylic results from a three-step process (67). In the early attachment phase, CDR1 and CDR2 are transiently induced. As the biofilm advances through aggregation/proliferation and maturation phases, during which they elaborate and become encased in a polysaccharide extracellular matrix, this expression dissipates. Resistance to azoles in later phases is maintained by alterations in the sterol membrane; during the early phase, the sterol biosynthesis pathway bottlenecks at zymosterol, followed by a 30% reduction in ergosterol. These changes are putatively also the basis for resistance to AMB (351). Early phase dependence on efflux as a resistance mechanism is largely based on the observation that mutant cells disrupted at CDR1, CDR2, and/or MDR1 were more susceptible to azoles than were wild-type cells in early phase development (330, 351), but were equally resistant in later phases (351, 434). Northern analyses and promoter reporter assays were consistent with this, showing that CDR genes and MDR1 were induced slightly in the early biofilm phase (330, 351) and CDR genes maintained high-level expression (351, 434). Consistently, rhodamine123 accumulation was reduced only during early-phase biofilm development (351). However, relevance of these expression levels of the pumps is in question, because they do not correlate with the efflux assays.

Several observations, using a flow method to establish very thick biofilms, support the view that the matrix protects embedded cells against antifungals. *C. albicans* cells in biofilms double their wall-associated and secreted β-1,3 glucans by twofold relative to planktonic cells, and their walls are twice as thick. Secreted β-1,3 glucan binds FLZ, potentially reducing the concentration of drug available to inhibit cells. Treatment of biofilms with glucanase sensitizes cells to FLZ, and addition of glucans to the medium protects them from FLZ (362). *C. albicans* biofilms are rich in glucose and generally are less of a barrier than the hexosamine-rich biofilm generated by *C. tropicalis* (4).

However, whether the biofilm matrix or structure per se is responsible for antifungal resistance is controversial. Diffusion of antifungals, at least through thinner biofilms formed in static cultures, was somewhat delayed, but antifungals still achieved penetration and accumulation to high concentrations (5). Resistance is not correlated with thickness of the biofilm. Most importantly, biofilm-associated cells are resistant to fluconazole, even when disrupted and assayed outside of their matrix (407, 434). However, this resistance depended on assaying the cells at high density (407). Furthermore, this resistance to azoles was reproduced in planktonic cells, by merely adjusting their cell density to the high levels of maturing biofilms. It was not dependent on efflux (since it was seen in strains disrupted at CDR1, CDR2, and MDR1), on quorum sensing (since it was seen in strains disrupted at CHK1), or on the calcineurin stress response pathway (since it was not affected by FK506). The effect was not due to conditioning of the medium or inactivation of the drugs, since media from high-density cultures still inhibited low-density cultures. These results should be qualified by the observation that resistance levels of high-density planktonic versus biofilm-dissociated cells were never assayed at extremely high concentrations of any drug (data was reported as MIC > values); therefore, it is possible that the biofilm-derived cells had even higher levels of resistance. Nevertheless, these data indicate that antifungal protection requires only changes in gene expression/physiology of cells induced by growing a high density, not the biofilm matrix or architecture. Collectively, these data do not exclude that biofilm matrix/architecture is a contributing factor to resistance, but they do indicate that it is not essential.

However, interpretations based on common resistance of stationary phase versus biofilm cells may be misleading, because each may involve a unique resistance mechanism. For example, gene expression patterns among ergosterol biosynthesis β-1–6 glucan-related genes differed dramatically between the two cell types (245).

It has been suggested that the differentiation of cells in biofilms or in high-density cultures that confers resistance is a result of adaptation to microaerobic or anaerobic environment occurring especially in the internal microenvironment. Using a nitrogen gas/cysteine/resazurin system to generate, maintain, and verify anaerobiosis in a defined media, Dumitru et al. (120) demonstrated that *C. albicans* could grow and was resistant to many antifungals, including Amphotericin B, Cerulenin, Terbinafine, Zaragozic acid B, and azoles, at concentrations 4- to 40-fold higher than aerobic MIC values. Unfortunately, the study did not test higher concentrations to

allow a fair comparison to resistance levels reported for biofilms, and did not ascertain whether biofilms were a component of this resistance. Anaerobiosis is understudied in *Candida*, and despite decades of analysis in *S. cerevisiae*, many aspects of anaerobic responses are still enigmatic. Nevertheless, it is clear that sweeping adaptations in cell membrane and cell-wall composition occur, involving differential expression of 500 genes and requiring 23 genes specifically needed for anaerobic growth. Sterol biosynthesis requires oxygen, so anaerobic growth in vitro depletes membranes of ergosterol, conferring AMB resistance, and forcing adaptive responses that free cells from their dependence on ERG11 and therefore increasing azole resistance (202).

However, the connection between anaerobiosis and biofilms is suspect, because biofilms form in some well-aerated systems. Biofilms were generated by all tested *Candida* species in YNB-glucose media on polysterene, and in static and shaking anerobic environments created using OXOID Gas Generating Kits Anaerobic systems BR0038B (Oxoid Ltd., Basingstoke, Hants, UK). Several non-*albicans* species formed more extensive biofilms in anaerobic than aerobic environments: *C. krusei, C. tropicalis, C. parapsilosis, C. kefyr*, and *C. guilliermondii*; whereas *C. albicans* biofilms were denser in aerobic and shaking conditions (533). In contrast, Biswas et al. were unable to form biofilm in the same media with *C. albicans* with static or shaking incubations. This group used a different system to generate anaerobic environments: anaerobic grade gas and nitrogen (carbon dioxide 5%, hydrogen 10%, and nitrogen balance), with a catalyst/desiccant water system that binds and removes trace oxygen (48). Thus, it seems that while anaerobiosis and biofilms both result in antifungal resistance, anaerobiosis is not an essential feature of biofilms and therefore induction pathways are likely to be different. However, the two pathways to resistance may share common or partially overlapping mechanisms for resistance, and anaerobic-induced resistance may be equally important as an in vivo adaptive response.

Global expression analysis is also being used to understand biofilm development, and is just beginning to distill into a conceptual scenario of the biofilm process, but not antifungal resistance. Using an Affymetrix microarray-based expression analysis approach, 500 genes were identified to have altered expression withing 30 min of adhesion to polystyrene (357). About half were related to ribosome biogenesis and protein synthesis and about a third have functions not yet identified. Sulfur-metabolizing genes were notably induced, possibly implicating a cysteine-based (GSH) resistance mechanism. Notably missing were efflux genes CDR1/2 and MDR1. Similar results were seen in by Garcia et al. using a microarray that covered only 2,002 genes (146). This group also showed that the altered transcriptosome was remarkably similar among three different biofilm systems (aerated, continuous flow microfermentor; static microtiter plates; static catheter disks). This indicates that the core pathways involved in biofilm formation and resistance are distinct from planktonic growth. Cao et al. (58) used a 3132-gene cDNA microarray to compare 24-h expression of a partially inhibited biofilm to a normal biofilm; partial inhibition was via a low concentration of quorum-sensing farnesol (437). This group noted that partial inhibition resulted in upregulation of resistance transcription factor repressor FCR1 (490), and downregulation of PDR16 (459). The implication is that these mechanisms are downregulated and upregulated, respectively, in the normal biofilm. However, they did not report any assays of resistance in the partially inhibited biofilms, nor whether these two genes were expressed at different levels in the normal biofilm compared to susceptible planktonic cells. Furthermore, downregulating FCR1 should confer resistance by allowing higher levels of CDR1 expression (490), but this is not seen by Cao et al. – or others – in 24-h biofilms. Additionally, the gene they report at downregulated PDR16 is cited as an “ABC family of transporters”, whereas PDR16 is actually a phosphatidylinositol transfer protein in the Sec14p family; therefore it is not clear whether the correct gene is being reported. These issues must be resolved in order to accept that these mechanisms are operative in the normal biofilm, especially since the other microarray groups have not reported differential expression of these two genes. Sohn et al. (514) used microarrays to look and expression differences spanning the short interval required for adhesion and found a that many cell surface genes were differentially regulated, and differently depending on whether adhesion was to plastic or epithelial cells. No connections to or tests of altered resistance were noted. Yeater et al. (599) also used microarrays to compare three different types of biofilms at three time points, comparing each to planktonic cultures. They also did not observe regulation of CDR1 or CDR2, or MDR1, that could account for early phase azole resistance. Furthermore, the only consistent change in ergosterol biosythesis gene expression was an upregulation of ERG10, expected as a consequence of reduced feedback inhibition as sterol content decreases with biofilm maturation. A number of other transporters are regulated, as are putative genes with no annotated function, which could account for resistance and which must be pursued individually by gene disruption and/or overexpression. Therefore, microarray-based expression analysis is not yet offering any simple known pathways to a resistance mechanism. As has been pointed out, a potential limitation to this global approach is that, by extracting RNA from the entire biofilm, it may average expression levels of subpopulations within the biofilm which may be expressing different gene sets, rendering changes in some invisible (10).

However, these global expression analyses do show a shift in metabolism from oxidative to fermentative metabolism,

which is similar to the proteomic analysis of FLZ-resistant mutants (592). It seems that this shift in some biofilms does not require anaerobiosis. Mutations or environmental conditions that induce this shift before exposure to azoles confer resistance, perhaps by reducing flow through the sterol biosynthesis pathway and thereby preventing accumulation of toxic sterol intermediates.

At least two types of cell subpopulations in biofilms which involve differences in resistance have been described. First, cells in the surface monolayer, which are more adherent and more yeast-like than the less adherent population, are reported more resistant to AMB (245). While the differences in resistance (adherent versus less adherent) presented in this chapter are not convincing, their differences in expression levels of specific genes are, and this suffices to demonstrate the reality of these two distinct populations. ERG1 is downregulated fivefold and SKN1 is upregulated 15-fold in the adherent versus less adherent cells: again, not seen in AMB-resistant stationary phase cells. A second type of heterogeneity is the persister cell subpopulation, which confers very high-level multidrug resistance of cells in biofilms, above the level of the bulk of the dominant biofilm population. This subpopulation is rare, only 1 per 50–2,000 adherent cells, randomly distributed throughout the surface. At this low level, they would not cause misinterpretation of resistance in the bulk population using XTT assays. Persisters do not grow, but tolerate high concentrations of AMB, chlorhexidine (276), or EDTA (176). These cells are not stably resistant mutants; instead, they can be isolated and propagated to give rise to new persistent subpopulations at the same rate as parental cells. They form even in a wide range of mutants that do not form mature biofilms, but which do adhere, so it is suggested that they arise as a consequence of adherence, since they are not seen in planktonic growing or stationary phase cultures (276). That persisters are unique to adherent cells bears confirming, given the observations that difference in susceptibility of biofilm cells versus stationary phase cells is essentially an artifact of the cell density at which they are assayed (407). Nevertheless, data establishing the reality of *Candida* persister cells are convincing and are reminiscent of bacterial persisters that achieve resistance by a dormancy process. The possibility should be kept in mind that persister cells may also form in vivo independently of biofilm or adherence, and that there may be strain or species differences in the frequency or means of induction of these intriguing cell types. It should also be remembered that global expression analyses of biofilms are too insensitive to detect changes in this small subpopulation; these must be isolated from the dominant population for analysis or identified by a mutational approach.

Several gene inactivation approaches have been reported, but these have not yet distinguished between genes needed for biofilm formation/maturation and those needed for resistance. Richard et al. (443) screened 197 *C. albicans* strains with single-gene homozygous disruptions for defects in biofilm formation on catheter silicone which did not affect overall growth rates. They found four genes whose disruption blocked early phase biofilm development: NUP85 (nuclear pore, mRNA export), MDS3 (alkaline response regulator), SUV3 (mitochondrial RNA helicase, respiratory competence), and one gene blocking intermediate phase biofilm maturation, KEM1(exoribonuclease, pleiotrophic effects). Extrapolating to the whole genome, they estimate that 114 genes are essential to biofilm development. Although the functions of these first four genes are unrelated, they are all required for hyphal development as well as biofilm development. This same group also systematically disrupted 21 of 25 genes encoding cell-wall-associated proteins (373). Six homozygous disruptants were hypersusceptible to echinocandins, and two of these, SUN41 and ORF19.5412, were defective in biofilm formation. Mutations in SUN41, a putative glucohydrolase, also reduced virulence and hyphal formation. Another study found that disruption of any of a class of cell-wall proteins with cysteine-rich CFEM domains resulted in wall defects and reduced ability to form biofilms (405), but did not analyze antifungal resistance.

That cells recovered from biofilms are still resistant to antifungals suggests that intrinsic changes in gene expression are involved, and that mutational analysis should be able to separate the processes of biofilm formation from resistance. Disruption of the cell-wall stress sensor kinase encoded by MKC1 results in reduced invasive filamentation, but the mutants attach normally and form biofilms, although they are not normal. Importantly, cells in these mutant biofilms are not resistant to azoles (268). Therefore, the mutation partially separates biofilm from resistance and makes the identification of targets of Mkc1p action a high priority (50).

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Section E
Viral Drug Resistance – Mechanisms

Chapter 30
Mechanisms of Resistance of Antiviral Drugs Active Against the Human Herpes Viruses

Clyde S. Crumpacker

1 Introduction

The antiviral drugs against the human herpes viruses provided pioneering insights, which have led to the development of the field of antiviral therapy. The first successful use of antiviral drugs to treat any life-threatening viral infection was Vidarabine (adenosine arabnoside) in 1977 (1). This was followed by the development of Acyclovir as the first specific antiviral drug which required a viral enzyme (thymidine kinase, TK) for activation to a nucleoside triphosphate, which inhibited the viral DNA polymerase and was a chain-terminator of viral DNA elongation (2, 3). When tested against clinical viral isolates, acyclovir was most effective against those herpes viruses which established latency in neuronal tissue (HSV-1, HSV-2, VZV) (4), with some activity against EBV, and very little against clinical isolates of CMV in a plaque reduction assay (4, 5). With the possible exception of influenza A virus and amantadine, this marked the beginnings of antiviral therapy.

From the earliest times, studies of resistance to acyclovir, and other antiviral drugs played an essential role in defining the mechanisms of action of antiviral drugs and elucidated key features of the targets of antiviral therapy. This was especially true of the viral DNA polymerase enzyme, since all of the clinically approved drugs against the herpes virus act on the viral DNA polymerase as the final target. In this chapter, we will review the mechanisms of resistance of the current antiviral drugs against the human herpes viruses. This will also include experimental drugs, which are currently in development, but not yet approved for clinical use.

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2 Thymidine Kinase Herpes Simplex Virus (HSV) Type 1 and Type 2

Two viral-encoded proteins, the viral thymidine kinase (TK) and DNA polymerase (pol) are the only targets for the acyclic nucleoside analog of guanosine, acyclovir, and resistance mutations in the genes for these two proteins account for all of the resistance to acyclovir observed in vitro or in a clinical use of acyclovir (6). Acyclovir is an acyclic nucleoside of guanosine that is preferentially phosphorylated by the herpes simplex virus TK to form acyclovir monophosphate. Human cellular TK enzymes have very little ability to add the initial phosphate group to acyclovir. The human thymidylate kinase enzyme, however, readily adds the second and third phosphate to acyclovir monophosphate to form acyclovir triphosphate. Resistance to acyclovir, which is mediated by the viral TK, occurs by three mechanisms: (1) selection of a TK-deficient mutant; (2) selection of a TK-low producer mutant of herpes simplex; (3) selection of a mutant that produces an altered TK which is capable of phosphorylation of thymidine but no longer phosphorylates acyclovir (7, 8).

In clinical use, selection of TK-deficient mutants is the most common mechanism for development of acyclovir-resistant HSV. This was the mechanism described in the first example of resistance to acyclovir in a human patient in 1982 (9). Mutations that result in thymidine kinase deficiency or low-producing TK mutants can occur in almost any part of the viral TK enzyme. The herpes TK gene contains a run of cytosines (c-cord) and guanosine (g-string), which are essential for function, and mutations in this region of the gene occur commonly in clinical isolates of herpes simplex. These homopolymers result in mutational hot spots that mediate TK deficiency and resistance to acyclovir (10). This results in a truncated TK protein with little ability to phosphorylate thymidine. An analysis of the electrophoretic mobility of the herpes simplex TK enzyme obtained from 13 acyclovir-resistant HSV isolates from patients with AIDS revealed that only one of the TK

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proteins was of full length and others were truncated and severely shortened (11).

The mutant TK that was of full length contained a single mutation in a region of the herpes TK known to contain an α-helix structure, and the proline point mutation is likely to break the α-helix (11). None of the TK proteins produced any significant TK activity. Since a mutation in any part of the HSV TK gene may result in an enzyme, which is able to confer resistance to acyclovir, nucleotide sequencing of the entire viral TK gene is required to detect resistance. The report on an altered substrate specificity as a mechanism for acyclovir resistance showed that prolonged acyclovir treatment of mice selected for a mutant that was not able to phosphorylate radiolabeled acyclovir but was clearly able to phosphorylate thymidine to form thymidine monophosphate (8). The initial demonstration of resistance to acyclovir mediated by the viral TK and DNA polymerase genes was shown in vitro (12, 13) and in mice treated with acyclovir (14).

3 HSV DNA Polymerase

The HSV DNA polymerase gene encodes a 1,235-amino acid peptide that is able to carry out synthesis of the herpes virus DNA from an origin of replication located in the long, unique region of the herpes virus genome. The herpes genome contains two origins of replication, one in the long, unique nucleotide sequence region (ORIL), and one in the short unique sequence region (ORIS), but the origin of replication at ORIL is considered to be the main origin that functions on reactivation from latency (15). The HSV DNA pol was first cloned and expressed in vitro in a rabbit reticulocyte system, and the single pol peptide was functionally able to carry out synthesis of HSV viral DNA by itself (16, 17). A second protein, the product of gene UL42, is a polymerase accessory protein that greatly enhances the DNA synthesizing activity of the HSV pol (18). The UL42 pol accessory protein binds directly to the HSV DNA polymerase and acts to increase the processivity of the HSV pol (19). In addition to the HSV DNA polymerase (UL54) and the polymerase accessing protein (UL42) forming the functional pol complex, five other virally encoded proteins are necessary for replication at the fork of HSV DNA (20). These include the origin binding protein (OBP, UL9), which binds to the origin of replication ORIL and initiates viral DNA synthesis; and the UL30 protein, a single-strand binding protein which keeps the DNA in a single-strand form enabling the pol complex to make a complementary strand of HSV DNA. A helicase primase complex consists of three viral proteins, UL5, UL8, and UL52, carrying out the unwinding at the fork of the newly replicating viral DNA (21). Although these seven viral proteins appear to be crucial in HSV DNA synthesis, when an antiviral drug such as acyclovir is used to treat herpes infection in either tissue culture or in patients, resistance has only been documented in the viral DNA polymerase gene UL54. The first direct evidence that a drug resistance mutation conferring acyclovir and phosphonoacetic acid (PAA) resistance in HSV was due to an altered HSV DNA polymerase function was obtained with HSV-1/HSV-2 intertypic recombinant viruses (22). This study showed that the purified viral DNA polymerase from a drug-resistant virus had greatly altered kinetics for incorporation of nucleotide triphosphate compared to the drug-sensitive HSV polymerase. The cells infected with both sensitive and resistant recombinant viruses produced similar amounts of acyclovir triphosphate, which excluded the viral TK as a source of resistance, and indicated that the altered viral DNA polymerase was the cause of the acyclovir and PAA resistance. The complete nucleotide sequence of the HSV DNA polymerase gene was independently reported by two groups (23, 24). The nucleotide sequence analysis of the HSV DNA polymerase has also revealed the location of amino acids that are involved in substrate and drug recognition (25).

The herpes simplex polymerase peptide and the polymerase of all the herpes viruses contain an exonuclease domain in the polymerase peptide. This is an important editing function, which enhances the fidelity of viral DNA replication and is able to remove falsely incorporated nucleotides. This editing function plays a major role in the decreased mutation rate of the human herpes viruses compared to the mutation rate observed in RNA viruses such as HIV-1 and influenza A. This exonuclease also contributes to the highly conserved genomes of the human herpes viruses compared to RNA viruses. In a project that involved the nucleotide sequence analysis of the CMV DNA polymerase gene from 40 clinical isolates of HCMV from four different locations in the United States, only a 4% incidence of polymorphisms in the CMV DNA polymerase was observed (26). The viral DNA polymerase therefore is the preferred target of all clinically approved antiviral therapies for the human herpes viruses.

The herpes simplex DNA polymerase gene was also cloned and expressed in yeast (27). The polymerase expressed in yeast had functional activity, and could be inhibited by the antiviral drug acyclovir. The herpes virus DNA polymerase is a member of the class of α-DNA polymerases, which includes the human DNA polymerase α and the bacteriophage S6 polymerase. All the human herpes virus DNA polymerases are closely related, and the enzymes possess clusters of highly conserved amino acids (28). The conserved residues are not randomly distributed but are clustered at specific regions. These domains also appear to have strong sequence homology with domains in the DNA polymerases of vaccinia virus and adenovirus type 2 and bacteriophages Ø29 as well (23, 24, 28–30). These conserved regions provide a compelling case for their functional importance, and they are considered major

sites for nucleotide binding and pyrophosphate exchange. The three most highly conserved regions I–III are located in the same linear arrangement on each polypeptide and the distances between the consensus sequences are remarkably similar at around 100 amino acid residues in each case (31).

These regions are designated by roman numerals I through VII. The most highly conserved region I consists of six invariant amino acid residues YGDTDS (884–889), including the aspartate residues DTD, which are essential for nucleotide binding in all RNA and DNA polymerases. To avoid being lethal for viral replication, resistance mutations usually occur at sites that are not directly involved in catalysis such as region I. A study employing site-specific mutagenesis of an in vitro cloned and expressed active HSV DNA polymerase surprisingly showed that the amino acid G adjacent to the DTD complex could be changed to serine G885S and still result in an active enzyme (32). Any change in the DTD amino acids of region I resulted in an inactive enzyme. Another mutation S889A resulted in an acyclovir-resistant polymerase (33).

The drugs PAA and phosphonoformic acid (PFA) are pyrophosphate analogs and very similar in structure (Fig. 3). They work by a similar mechanism as competitive inhibitors of pyrophosphate exchange and bind directly to the viral DNA polymerase (34). They are not incorporated into elongating DNA and do not require activation by any viral enzyme. When drug resistance mutations conferring resistance and hypersensitivity to PAA were mapped by marker rescue, five of six mutations mapped in regions II and III of the herpes simplex DNA polymerase (31). These were Ala 719 Val and Ser 724 Asn in region II, and Asn 815 Ser and Gly 841 Ser in region III. Resistance to acyclovir was conferred by mutations in regions II and III, and cross-resistance to both PAA and acyclovir was conferred by mutations in region II (Ser 724 Asn). The regions II and III have important functional significance because each of these regions contains the sites of mutations that confer resistance to acyclovir. All acyclovir-resistant mutations are found in conserved regions of the DNA polymerase, designated I, II, III, V, VII, and A.

The three-dimensional crystal structure of the herpes simplex I DNA polymerase at 2.7 Å resolution has recently been described for the first time (35). The HSV-1 DNA polymerase has a structural similarity to other α polymerases and has permitted construction of high-confidence models of a replication complex of the polymerase and the DNA chain termination of acyclovir. The analysis of the HSV pol structure provides valuable insight into domain functions, the conformational changes required for catalysis, and an enhanced understanding of herpes virus DNA replication. The structure also permits increased understanding of the relationship of the highly conserved regions of the amino acids to each other. The structure reveals that HSV pol is composed of six structural domains. These six structural domains are a pre-NH_2 domain, the NH_2 domain, the $3^1 5^1$ exonuclease domain, and the polymerase palm, finger, and thumb domains. The polymerase exonuclease domain is essential as an editing function for herpes DNA replication to remove falsely incorporated nucleotides and contains conserved regions exo-I, exo-II (region IV), and exo-III (C region). The highly conserved region III and IV belong to the finger subdomain; regions I, II, and VII are located in the palm subdomain, and the thumb subdomain contains the conserved region V. These domains are assembled to form a disk-like shape around the central hole with the NH_2 and *C*-termini at opposite sides of the protein. In the crystal structure of the herpes DNA polymerase, the two main regions conferring resistance to acyclovir are regions region III, the finger subdomain, and region II located in the palm subdomain (31, 35). The most highly conserved catalytic region I, residues 884–889, is also in the palm subdomain (Fig. 1 DNA Pol).

To avoid being lethal, mutations that confer resistance to acyclovir and other nucleosides usually occur at sites that are not directly involved in catalysis, such as the invariant YGDTD8 (884–889) of region I. Since acyclovir monophosphate incorporation into the DNA duplex alone does not inhibit HSV pol strongly, it has been postulated that the strong inhibition of HSV pol and formation of a “dead end suicide complex” is only observed when the next incoming nucleotide is bound to the acyclovir monophosphate-terminated DNA duplex (36). The side chains of conserved residues in region II (Y722) and region III (T887) are suggested to limit modifications permitting incorporation of acyclovir monophosphate (35). Therefore, mutations in regions II and III that confer resistance to acyclovir are likely to prevent incorporation of the acyclovir monophosphate and block the formation of the “dead end complex” that terminates DNA chain elongation.

4 Penciclovir and Famvir

Penciclovir is a guanosine analog with a broken sugar ring similar to acyclovir. The oxygen at the 2 position in the broken sugar ring has been replaced by a carbon, and two CH_2OH groups are attached at the end of the broken ring, instead of only one in acyclovir (Fig. 3). The mechanism of action of penciclovir is very similar to that of acyclovir (37). The CH_2OH group on the broken sugar ring is phosphorylated by the HSV TK. Cellular enzymes add additional phosphate groups to form penciclovir triphosphate. Penciclovir triphosphate binds to the viral DNA polymerase and is a competitive inhibitor for the incorporation of guanosine triphosphate into elongating DNA. Penciclovir monophosphate is incorporated into elongating DNA, and penciclovir is not a chain-terminating drug. Since penciclovir

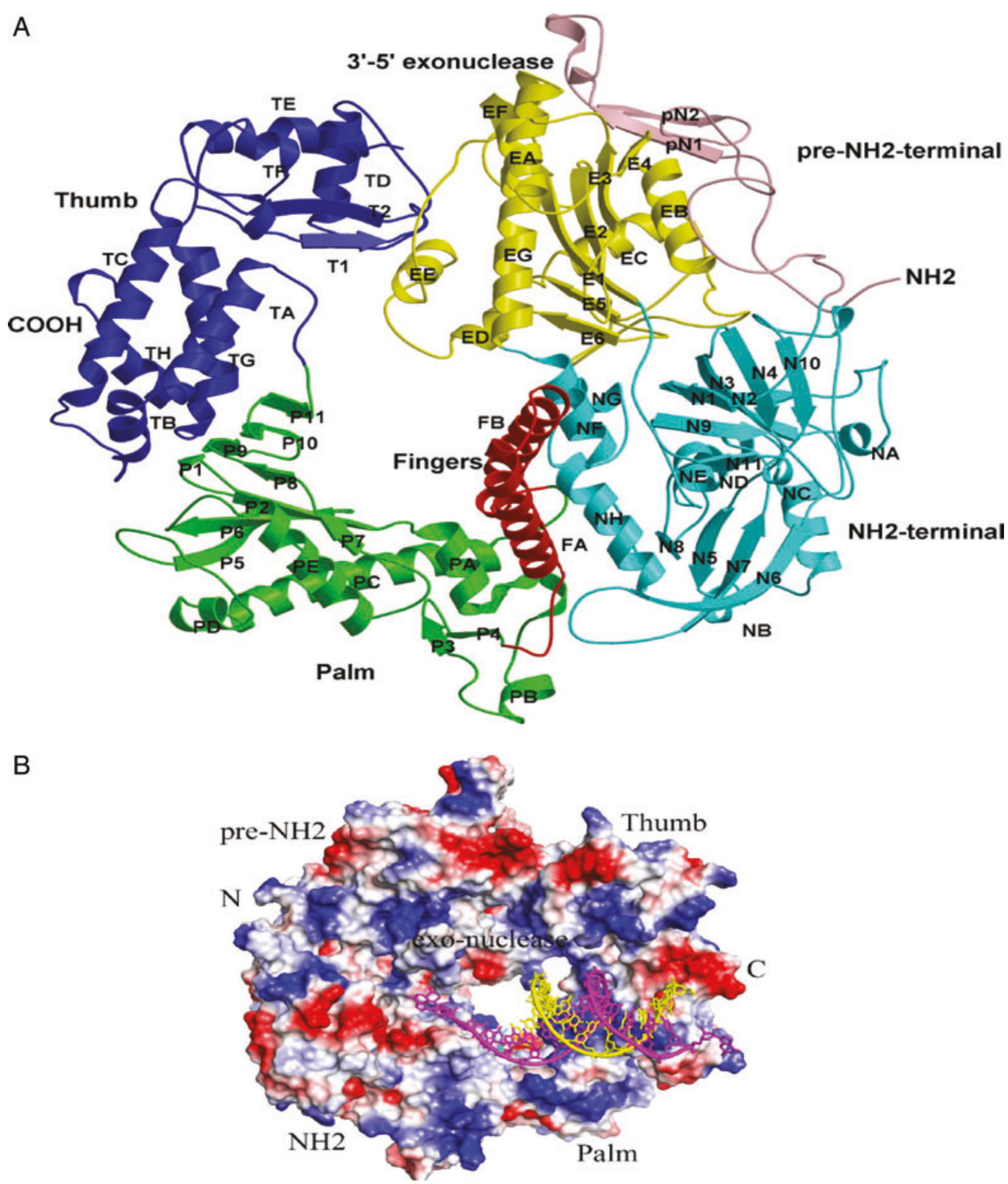

Fig. 1 Structure of HSV-1 DNA polymerase in ribbon diagram (**a**) showing the six domains of the polymerase. The finger subdomains comprise amino acids 767–825 and include conserved regions III and VI. Palm sub-domain comprises amino acids 701–766 and 826–956 and includes regions I, II, VIII. Thumb subdomain comprises amino acids 957–1,197 and includes region V, diagram (**b**), which is at the back of Fig. 1a (35) (*See Color Plates*)

requires a competent viral TK for phosphorylation, the most common mechanism of resistance is by selection of TK-deficient mutants that are not able to phosphorylate penciclovir. There is almost complete cross-resistance of TK-deficient mutants of HSV to acyclovir and penciclovir. Since the final target of penciclovir triphosphate is the viral DNA polymerase, resistance mutations in the viral DNA polymerase also confer resistance to penciclovir. These resistance mutations in the viral DNA polymerase confer almost complete cross-resistance to both acyclovir and penciclovir, with rare exceptions. Therefore, resistance to acyclovir and penciclovir exhibits a high degree of cross-resistance owing to both TK and DNA polymerase mutations. Penciclovir is not orally bioavailable, but when complexed with two acetate esters, it becomes readily bioavailable to 68% (34, 38). This compound is called famciclovir (Famivir) and is the oral form of penciclovir. It is readily converted to penciclovir in the plasma by the action of the two esterases, one in the intestinal mucosa of the human small intestine and the other in the liver (37). Following absorption in the small bowel and one pass through the liver via the portal vein, Famivir results in high blood levels of penciclovir. The resistance mechanisms for Famivir are identical to penciclovir and are mediated by the viral TK and DNA polymerase. One potential antiviral advantage of penciclovir over acyclovir is the high intracellular concentration of penciclovir triphosphate. This concentration persists longer than acyclovir triphosphate and the half-time ($T_{½}$) of penciclovir triphosphate is 8.5 h compared to 2.5 h for

acyclovir triphosphate (37). The clinical advantage of this persistent high concentration is not clear.

5 Human Cytomegalovirus

The human cytomegalovirus (HCMV) is the largest virus to infect humans and is a significant cause of disease in immunocompromised patients. The virus encodes a DNA polymerase enzyme, like all herpes viruses, and has functional domains similar to HSV. Ganciclovir, a nucleoside analog of guanosine, is the mainstay of treatment for CMV. Ganciclovir is the only orally useful drug to treat CMV. Ganciclovir is phosphorylated by a viral protein kinase (UL97), and cellular kinases convert this to ganciclovir triphosphate, the active inhibitor of CMV DNA synthesis. Ganciclovir monophosphate is incorporated into elongating CMV DNA, but unlike acyclovir it is not a chain terminator. CMV DNA synthesis continues at a slow rate and small fragments of CMV DNA encoding the origin of replication in ORIL continue to be made, but the synthesis of full-length CMV DNA is greatly inhibited (39, 40).

6 Resistance to Ganciclovir

Resistant mutations conferring resistance to ganciclovir are found in two viral genes: the viral protein kinase (UL97), which phosphorylates ganciclovir, and the viral polymerase (UL54), which is inhibited by the ganciclovir triphosphate as a competitive inhibitor for nucleotide incorporation into the growing CMV DNA strand. Multiple incorporations of ganciclovir monophosphate near the origin of viral replication greatly slow the action of the viral DNA polymerase. Unlike acyclovir, ganciclovir is not a chain terminator, and CMV DNA elongation does not stop completely with ganciclovir monophosphate incorporation (39, 40). Mutations in the UL97 protein kinase gene in a region of the gene extending from codons 590–607 and in two other regions of the gene encoded by the codon 460 or 520 introduce amino acid changes that confer resistance to ganciclovir by blocking phosphorylation (41). These regions of the protein kinase are probably where ganciclovir binds and is in close approximation to where ATP binds, enabling a phosphate moiety to be transferred from the ATP to the nucleoside analog ganciclovir. Most of the resistance with the clinical use of ganciclovir occurs owing to mutations in the UL97 gene, which are either single amino acid mutations at codon 460 or 520 or short deletions in codons from 590 to 607. This results in a protein kinase that does not effectively phosphorylate ganciclovir. The UL97 protein is an important protein in CMV replication. Laboratory-derived mutants of HCMV with substantial deletions in the UL97 gene are severely growth attenuated (50). It is able to participate in phosphorylation of other viral proteins and phosphorylates the UL 44 product, a processivity subunit of the CMV DNA polymerase complex (51, 52). The CMV UL97 protein also phosphorylates cellular proteins. It has been shown to have activities similar to cellular cyclin-dependent kinases (CDK), and UL97 phosphorylated and inactivated the retinoblastoma tumor suppressor (53).

7 Maribavir

The UL97 protein is also the target for the antiviral drug Maribavir, an L-ribofuranosyl nucleoside, which is a potent inhibitor of CMV replication by inhibiting CMV DNA synthesis (Fig. 3). Maribavir strongly inhibits the kinase activity of the viral UL97 (43). Maribavir also inhibits phosphorylation and accumulation of EBV early antigen D, an essential co-factor in EBV replication (44).

When CMV develops resistance to Maribavir, mutations are found in the UL97 protein but in regions that are distinct from mutations conferring ganciclovir resistance. Passage of laboratory strains of HCMV in the presence of Maribavir resulted in a mutation L397R in UL97, which was associated with high-level MBV resistance. Recently, passage of two clinical HCMV isolates in the presence of Maribavir, beginning at 0.3 μM and increasing to 15 μM, resulted in two Maribavir-resistant viruses with mutations at T409M and V353A of UL97 and a 20-fold increase in the IC50 concentration needed to inhibit CMV replication (45). When the T409M and V353A mutations were transferred to a CMV laboratory strain, the recombinant viruses also showed 15- and 80-fold increase, respectively, in Maribavir resistance. Experiments with an error-prone strain of CMV resulted in confirmation of a new Maribavir mutation at codon 411 (H411L, HLHY, or H411N). V353A and T409M are the most commonly selected UL97 mutations in vitro. Combinations of UL97 mutations at codon 353 with those at codons 409 or 411 result in very high levels of MBV resistance (>150-fold increased IC50), which is similar to that observed by L397R alone (49).

The known MBV resistance mutations (codons 353, 397, 409, 411) are located in the vicinity of the kinase ATP binding domain and are upstream of known GCV resistance mutations. Structural models of UL97 kinase strongly suggest that MBV is an ATP competitive kinase inhibitor (49). The mutation at L397R has been suggested to affect MBV binding either by affecting a contact point or altering spacing between residues 353 and 409–411 (49). A lack of cross-resistance between GCV resistant isolates of CMV and those that are resistant to Maribavir is generally reported. Recently identified MBV-resistant UL97 mutants have been tested against GCV and found to be susceptible to GCV (49)

8 CMV DNA Polymerase

The human CMV DNA polymerase is the target of all the licensed drugs for the treatment and prevention of HCMV infection. The crystal structure of the HCMV DNA polymerase has not been determined, but it is probably highly similar to the recently elucidated structure for the HSV DNA polymerase. This is because the HCMV DNA polymerase has similar regions of highly conversed amino acids, arranged in a strictly similar relationship to each other, as are observed in the DNA polymerase of the HSVs. Following the initial phosphorylation of ganciclovir to ganciclovir monophosphate by the UL97 enzymes, cellular enzymes convert this to ganciclovir triphosphate. Ganciclovir triphosphate is the active competitive inhibitor of the CMV DNA polymerase (UL54).

In the presence of ganciclovir, CMV elongation is greatly slowed, but short fragments of CMV DNA from the origin of replication (ORIL) continue to be synthesized (39, 40). Ganciclovir monophosphate is incorporated into these short segments and a slow rate of replication continues. The site of binding of ganciclovir monophosphate to CMV DNA polymerase is not clear, but it appears to be distinct from the binding site of PAA. In studies on recombinant HSV DNA polymerase, it was observed that ganciclovir and PAA were synergistic against drug-resistant mutants, indicating that these two drugs were able to bind to different regions in the HSV DNA polymerase (38). Synergistic activity of ganciclovir and foscarnet against CMV has been shown in vitro (46).

The mutations in the CMV DNA polymerase, which confer resistance to ganciclovir, are, with one exception, located in the highly conserved regions of the polymerase enzyme. This is also true for resistance to cidofovir and PFA, the two other polymerase inhibitors approved for treatment of CMV disease. To assess whether resistance to antiviral drugs is only associated with mutations in these regions or if the mutations in these conserved regions might be attributed to genetic polymorphisms in these regions, a series of 40 clinical isolates of HCMV, all sensitive to ganciclovir, were analyzed by nucleotide sequencing of the CMV DNA polymerase gene (UL54) (26). The results showed that there was only a 4% variation in the nucleotide sequence of the CMV polymerase gene. No mutations were detected in the highly conserved regions of the CMV DNA polymerase. Therefore, although very small amounts of genetic polymorphisms are observed in the CMV DNA polymerase, they were not observed in the highly conserved regions of the enzyme. If a mutation is detected in one of the highly conserved regions following use of an antiviral drug, the mutation is almost certainly associated with resistance to the antiviral drug. Therefore, in the use of nucleotide sequence analysis of the CMV DNA polymerase genes to detect drug resistance mutations, a strategy of nucleotide sequencing, which is focused on direct sequencing of these highly conserved regions, provides a rapid approach to detecting drug resistance mutations in human specimens. Cross-resistance to several antiviral drugs that act on the CMV DNA polymerase protein can occur with a single mutation in one of the conserved regions of the polymerase peptide. Specifically, this has been noted with resistance to ganciclovir and cidofovir with mutations in conserved regions VII in the CMV DNA polymerase (34, 47). Cross-resistance to several antiviral drugs can have clinical significance and requires phenotypic assays of resistance to reliably determine which alternate antiviral drug should be employed in patients who develop primary resistance to an antiviral drug (Fig. 2). Cross-resistance between ganciclovir and foscarnet has been observed infrequently. One report described GCV-PFA cross resistance conferred by mutations in conserved region III (54).

In the clinical use of foscarnet (PFA) to treat CMV retinitis in AIDS patients, resistance to PFA has been associated with clinical failure. Resistance mutations to PFA were observed in the clinical isolates from these patients in nucleotides located

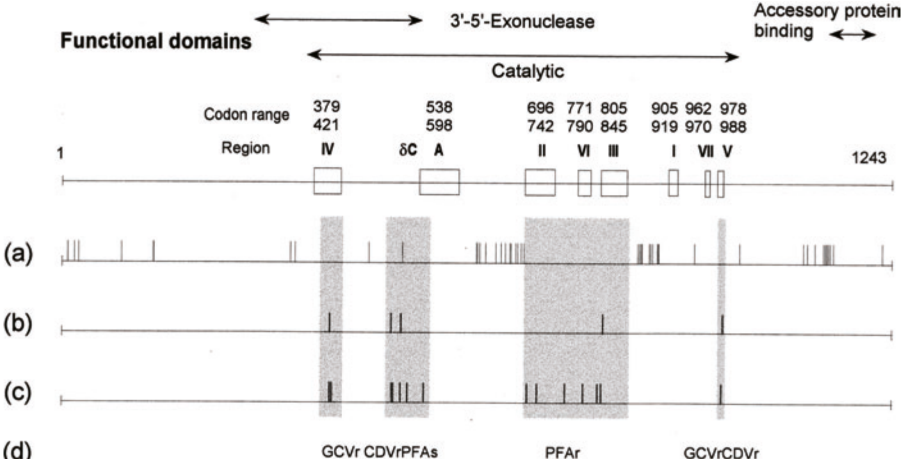

Fig. 2 Map of cytomegalovirus (CMV) DNA polymerase.
The CMV DNA polymerase showing functional domains and highly conserved regions of DNA nucleotide sequence (I-VII). Loci of amino acid changes are mapped below. **A**) Codons showing variations in wild type drug sensitive clinical isolates (26); **B**) Codons mapped to drug resistance in laboratory strains; **C**) Codons mapped to resistance in clinical isolates; **D**) Drug resistant mutants according to region. Regions associated with drug resistant mutants are shown as bars.
Abbreviations: CDV – Cidofovir; GCV – ganciclovir; PFA – Foscarnet (26)

Fig. 3 Chemical structure of acyclovir, penciclovir, ganciclovir, the nucleoside deoxyguanosine, foscarnet, cidofovir, and Maribavir

in region II, VI, and III of the CMV DNA polymerase (48). This included foscarnet resistance mutations E756Q (region VI) and V787L (region VI), which were confirmed by marker rescue. All the foscarnet resistance mutations occur in the shaded region marked PFA_R in Fig. 2. Resistance mutations were also observed that conferred resistance to ganciclovir and cidofovir, but not to foscarnet. When a clinical isolate of CMV is highly resistant to ganciclovir (ID50 > 30 µM) and contains mutations in both UL97 and the polymerase genes, cross-resistance to cidofovir may also be observed (42, 47). These isolates remain sensitive to foscarnet.

9 Conclusion

The nucleoside analogs that inhibit replication of the human herpes viruses are able to utilize viral-encoded kinases to phosphorylate the nucleoside analog to the monophosphate. This is most notable with acyclovir and the TK of HSV, VZV, and EBV as well as ganciclovir and the protein phosphokinase (UL97) of CMV and Kaposi's sarcoma herpes virus. This viral-specific kinase provides a great deal of specificity for these nucleoside analogs and prevents cellular toxicity. Cellular kinases convert the monophosphate to the triphosphate of acyclovir or ganciclovir, and the triphosphates are the active inhibitors of viral DNA polymerase. In the clinical use of these nucleoside analogs, the most common mechanism of resistance is the selection of mutants that are defective in the function of HSV viral TK or the protein phosphokinases (UL97) of the CMV.

The analysis of the crystal structure of the herpes simplex viral DNA polymerase at 2.7 Å resolution provides new insights into the mechanisms of resistance to acyclovir. The HSV DNA polymerase has a structure with finger and palm domains, remarkably similar to the HIV-1 RT p66 subunit in the "right-hand model of HIV-1 RT" (34). Acyclovir resistance mutations are found in both the finger subdomains (region III), similar to the resistance mutations to the nucleosides (AZT, ddI, d4T) in the HIV-1 RT; and in the palm subdomain (region II), similar to the non-nucleoside RT inhibitors (Efavirenz and Nevirapine) against HIV-1.

Therefore acyclovir monophosphate appears to bind in a significant way to both the fingers and the palm domains of the HSV DNA polymerase. A clear function associated with the resistance mutations to acyclovir, such as the enhanced excision of AZT monophosphate with K215Y mutation in HIV RT, has not been shown for acyclovir resistance mutations. The solution of the HSV DNA polymerase crystal structure and the mapping of the acyclovir resistance mutations on the structure reveal the remarkable similarities between the α-family of DNA polymerases found in all the human herpes viruses and the HIV-1 RT structure mutations. Ganciclovir and foscarnet resistance in the CMV DNA polymerase occur in different regions of the CMV DNA polymerase, indicating that these drugs bind to different regions of CMV DNA pol. Cross-resistance to ganciclovir and foscarnet has not been observed in clinical treatment of CMV disease.

Resistance to the new drug to prevent CMV disease in the transplant population, Maribavir, is located in the ATP-binding region of CMV UL97 (45). Maribavir inhibits CMV DNA synthesis by preventing phosphorylation of the CMV pol accessory protein UL44 and blocks the kinase function of UL97.

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Chapter 31
Influenza M2 Ion-Channel and Neuraminidase Inhibitors

Margaret Tisdale

1 Introduction

1.1 Influenza Viruses

There are two main types of influenza viruses, influenza A and B, that cause mild to serious respiratory disease in humans, which is associated with increased deaths every year, and referred to as seasonal influenza. In addition, influenza A viruses which infect several different animal species, are able to undergo genetic reassortment and mutate to produce new antigenic sub-types which are capable of causing pandemics of serious influenza infections in humans, associated with high mortality. Influenza A viruses are divided into sub-types based on the surface glycoproteins that project through the lipid membrane of the virus, the haemagglutinin (HA) or virus receptor and neuraminidase (NA), an enzyme that cleaves terminal sialic acid from glycoproteins/glycolipids. There are a total of 16 HAs (H1–16) and 9 NAs (N1–9) which together form the antigenic sub-types. Within the lipid envelope of the virus are also found virus M2 ion channels. Lining the inside of the lipid membrane is the M1 matrix protein which encloses the ribonucleoprotein complexes. Influenza viruses have a segmented negative-strand RNA genome, consisting of eight RNA segments which produce a total of 10 (A viruses) and 11 (B viruses) viral proteins.

1.2 Influenza Virus Replication

Knowledge of how the virus replicates in cells is important for understanding the mechanism of action of antiviral agents (see Fig. 1). First, the virus has to enter the cell, and this occurs through binding of the surface haemagglutinin to the terminal sialic (neuraminic) acid containing cell receptors. Once binding has occurred the virus is endocytosed into the cell. The haemagglutinin undergoes an acid-induced conformational change within the endosome which triggers fusion of the virus lipid membrane at low pH with the cellular lipid membrane of the endosome. The M2 ion channels facilitates entry of hydrogen ions into the virus from the acidified cellular endosome and the low pH inside the virus particle triggers M1 protein uncoating and release of the ribonucleoprotein complexes into the cytoplasm. Transport of the ribonucleoprotein complexes to the cell nucleus occurs followed by primary virus transcription of the vRNA, by the polymerase present in the virus, into positive sense mRNA ready for production of virus proteins within the cytoplasm. Later a switch to synthesis of full-length complementary RNA occurs from which vRNA will be produced for the progeny virus. New virus products are transported to the cell membrane where assembly and packaging of the ribonucleoprotein complexes occurs. Insertion of the viral glycoproteins into the cell membrane is followed by budding through the cellular membrane to form new virus particles. Finally, to allow release of virus from the cell and to aid spread of virus, the neuraminidase enzyme, which functions extracellularly, removes terminal sialic acid from the surface of the virus and surrounding glycoproteins and glycolipids.

2 M2 Ion-Channel Inhibitors: Amantadine and Rimantadine

Amantadine (1-adamantanamine hydrochloride, Symmetrel™, Lysovir™, Symadine™) and its close analogue rimantadine (α-methyl-1-adamantanemethylamine hydrochloride, Flumadine™, Roflual™) (see Fig. 2) were first shown to possess potent anti-influenza A activity in 1964/1965 in cell culture and in ferret and mouse animal models (1, 2). Later amantadine was approved for the prophylaxis and treatment

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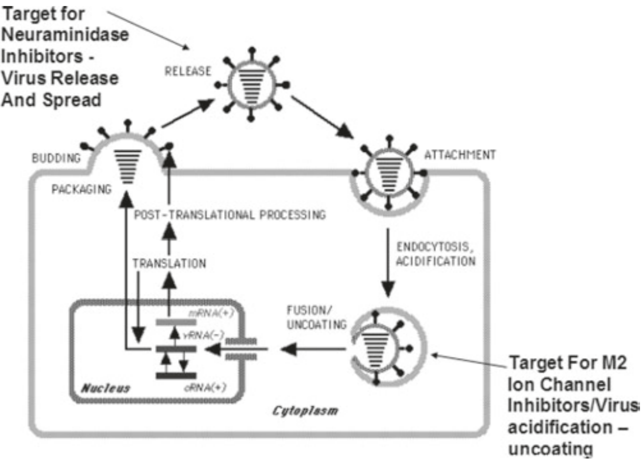


Fig. 1 Influenza virus replication cycle taken from: www.tulane.edu/~dmsander/WWW/335/Orthomyxoviruses.html, showing targets for inhibition by the M2 inhibitors, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir

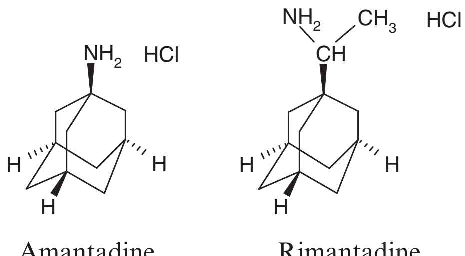


Fig. 2 Chemical structures of the M2 ion-channel inhibitors: amantadine and rimantadine

of influenza A (H2N2, Asian) virus infections in humans in the USA in 1966, and after further clinical evaluation for all influenza A infections in 1976. Rimantadine, which was first widely used for influenza prophylaxis in the USSR, was only later approved for use against influenza A infections in the USA in 1994 after demonstrating similar potency but reduced side effects to amantadine (3, 4).

2.1 Antimicrobial Mechanisms of Action

The anti-influenza A activity of amantadine and rimantadine was discovered empirically during random large-scale screening of molecules for activity against influenza either in cell culture or in ovo (4, 5). In vitro, rimantadine is up to eightfold more active than amantadine with activity (50% inhibitory concentrations by plaque reduction) at <5 µM or <1 µg/mL against the most susceptible strains in cell culture (6). Both amantadine and rimantadine were used in the clinic long before the target protein, the M2 protein, was identified and consequentially before the more detailed molecular mechanism of inhibition could be elucidated. These inhibitors became valuable tools using resistance studies to help identify the target protein and to later help understand the function of this protein as the first viral ion channel to be discovered. Today viral ion channels have been discovered in many viruses including HIV, HCV, influenza B, rhinoviruses (7, 8) and are a target for antiviral chemotherapy, although still a relatively difficult target to discover inhibitors using specific ion-channel assays.

2.1.1 Direct Studies on the Mechanism of Action in Cells

After the fortuitous discovery of these clinically potent influenza A inhibitors, amantadine and rimantadine, work was rapidly initiated to try to understand further the mechanism of action in cell culture. The majority of this work has been undertaken with amantadine but appears to apply equally to rimantadine. Early studies were rather limited by the technology available but it was shown that an early stage in the replication of human influenza viruses, during virus entry into the cell, was blocked. Under single cycle conditions, with influenza A/WSN/33(H1N1) and A/Japan/305/57(H2N2) the inhibitor was only active when administered within 10 min of infection (9). Adsorption of the virus to the cell surface was not affected, since a considerable drop in the virus titre of the supernatant fluid occurred within 1 h of infection in the presence of amantadine hydrochloride. The above workers suggested that penetration of the virus through the cell membrane into the cell was inhibited by amantadine, by demonstrating that infectious virus remained sensitive to neutralisation by antisera. Later it was shown using fowl plague virus that virus penetration was not affected but that amantadine inhibited uncoating of virus as demonstrated by a block in the loss of photosensitivity of virus labelled by neutral red (10). More detailed understanding of the mechanism of inhibition of uncoating by amantadine came several years later with detailed studies of the structure and function of the M2 protein.

2.1.2 General Structure and Function of the M2 Protein

The M2 protein consists of just 97 amino acids, which is an integral membrane protein present as a homotetramer channel in the virus membrane (23–36 copies per virion) and virus-infected cell membranes (11). The M2 channel includes a 24-residue N-terminal extracellular domain, a 19-residue highly conserved hydrophobic transmembrane domain and a 19-residue cytoplasmic tail (12).

The M2 channel was later demonstrated to be an acid pH-activated ion channel and the passage of ions could be blocked by amantadine and rimantadine (13–15). The channel is closed at physiological pH and is activated at pH

≤6.2. The M2 protein is therefore involved with uncoating of the virus during endocytosis by mediating the passage of protons from the acidic medium of the endosome into the virion to induce low pH mediated dissociation of the RNP/M1 complex and release of the RNP complex into the cytoplasm for transport to the nucleus. Based on viral resistance studies and the observation that inhibition of virus uncoating mediate by M2 occurs at relatively low inhibitor concentrations (<5 μM or <0.75 μg/mL), this is considered the clinically relevant mechanism of inhibition.

The M2 ion channel plays a second role in virus replication in that the ion-channel activity of M2 can increase the pH within vesicles of the trans-Golgi network and protect the structure and function of the acid sensitive HA of some avian A/H7 viruses during transport to the cell surface. Thus, the virus was able to bud from the cell surface with intact HA ready to infect further cells. In the presence of amantadine the pH of the trans-Golgi network decreased and cleavage of HA into HA1/HA2, the low pH form, occurred, and release of infectious virus was inhibited (16, 17). Again, studies with amantadine helped to determine the second role of the M2 ion channel in replication of some influenza viruses.

2.1.3 Structure/Function/Inhibitor Binding to the M2 Protein

Further studies have attempted to understand the detailed mechanism of the block in M2 function. Kinetics of inhibition suggest irreversible binding of one molecule of inhibitor per channel (15, 18). It was postulated that amantadine/rimantadine binds to the M2 protein at an allosteric site which triggers a conformational change in the pore region which interferes with proton transfer through the ion channel across the membrane of the virus or endosome (14). However, neutron studies and resistance studies suggested interaction of the inhibitor with the region between residues 22 and 46 of M2 which would have a direct effect on the pore region (19, 20). Molecular modelling of the ion channel using molecular dynamics calculations (21) or based on mathematical analysis of the functional properties of a series of mutants (22) produced similar three-dimensional structures of the trans-membrane region. The predicted structure consists of four parallel trans-membrane α-helices around a central channel. It was proposed that amantadine binds to hydrophobic groups lining the pore which form a widening near the centre of the bilayer (22).

Analysis of the structure and function of the M2 protein have been undertaken using site-directed mutants. Residues 25–44 in the transmembrane domain were individually replaced by cysteine and it was shown that A30, G34, H37 and W41 line the pore (23). Further, H37 is important in the conduction mechanism of the channel and is believed to form a hydrogen-bonded interaction with the ammonium group of amantadine (22). The indole side chain on tryptophan, W41 in the transmembrane domain acts as a gate that opens and shuts the pore and H37 acts selectively on transport of protons (24). This reveals the simplicity of the mechanism of the M2 ion channel in that only two residues are responsible for the functions of selectivity and activation. Transient exposure to low pH outside the membrane will result in lasting acidification of the virus because the protons are retained by the tryptophan gate. The channel is believed to transport protons by way of a proton wire using a continuous water molecule file (25, 26).

Further structural studies of the M2 channel using solid-state NMR and site-specific Fourier transform infra-red dichroism analysis have identified a helix tilt and determined the rotational pitch angles of the helices within the functional channel, and refined the backbone structure of M2 (27–31).

2.1.4 Other Effects of Amantadine/Rimantadine on Virus Replication

At high inhibitor concentrations (>100 μM, >15 μg/mL), amantadine and rimantadine block cell fusion by directly raising the pH of the cellular endosome and preventing the acid-induced conformational changes in the cleaved HA which is required for fusion of virus with the endosomal cell membrane. This non-specific effect is also observed with other weak bases (32), but is not considered to be clinically relevant due to the high concentrations of inhibitor required.

2.2 Mechanism of Drug Resistance

2.2.1 Genetics–Mutations Associated with Resistance

Understanding the mechanism of drug resistance and the mutations responsible for resistance has been key to unravelling the clinically relevant mechanism of action of amantadine and rimantadine. Immediately after the discovery of amantadine it was shown that resistant virus could be readily isolated after one or two passages of virus with amantadine in cell culture (2, 33). Similarly, it was possible to isolate resistant virus from virus stocks at an estimated frequency of 1 in $10^3/10^4$ (34), and some early isolates such as A/WSN/33(H1N1) and A/PR8/8/34(H1N1) were naturally resistant to amantadine before ever being exposed to the drug (35). Later drug-resistant strains were also isolated in vivo in the mouse model after one pass in lung tissue (36), and may be readily isolated within 6 days in the ferret model for

human influenza (37). In humans naturally resistant isolates have been observed sporadically before exposure to the drug (38, 39), and during treatment or prophylaxis with amantadine and rimantadine (6, 40–47). More recently, the avian H5N1 viruses circulating in South East Asia in 2004/2005 have been reported to be resistant to amantadine/rimantadine (48), and there has been a substantial increase in the number of adamantine-resistant viruses circulating world-wide (49).

Initial studies with genetic reassortments, using dual infections with an early resistant strain A/Bel/42 (H1N1) and an amantadine sensitive strain A/Japan/57(H2N2) demonstrated that resistance was linked to RNA segment 7 (34). Influenza segment 7 codes for the M gene which produces two products, a co-linear truncated product, the M1 matrix protein and a spliced product, the M2 protein. Other reassortment studies with avian strains implicated that the neuraminidase (NA), the nucleoprotein (NP) (50) and HA genes (51) may contribute to drug resistance. The later observation may be explained by the effect of M2 on transport of avian HA to the cell surface.

Further studies with resistant viruses, selected after passage in cell culture or by plaque selection in the presence of amantadine or rimantadine, and then analysed by NA sequencing, confirmed that the M gene was linked with resistance. These studies further defined that the M2 protein was the drug target due to the presence of mutations in the M2 region downstream of the M1 termination site (52). Analysis of in vitro derived resistant virus revealed that single amino acid substitutions were sufficient to produce total resistance to amantadine or rimantadine. This conflicts with some of the early passage studies which implied varying degrees of resistance to amantadine developed in cell culture (2, 33). However, this probably reflects that virus mixtures of sensitive and resistant virus were present in early studies, whereas later studies generally used cloned viruses.

Initially, from in vitro studies, mutations at four amino acid residues, and later a mutation at a fifth amino acid residue were identified in the M gene, at L26H, V27A/G/D or I27S/T/A/N, A30T/P/S, S31N or G34E all within the transmembrane domain of the M2 protein (51, 53). Based on these findings, analysis of amantadine/rimantadine resistance in humans included NA sequencing of the M gene and susceptibility analysis using ELISA assays (40). The first study in humans revealed the presence of mutations at residues V27A, A30V and S31N consistent with observations made in cell culture (40). Later studies have confirmed that these mutations may arise after treatment or prophylaxsis with amantadine or rimantadine, (41, 43–47) and identified a fourth mutation, at residue L26F (42). Similar studies in chickens identified mutations at residues I27S/T, A30S/T and S31N, (54) and in ferrets at residues L26F, V27A, A30S/T/V, S31I/N (37). Further details of the changes observed are shown in Table 1.

In all these studies changes in the M2 have consistently been linked to abolition of susceptibility to amantadine and/or rimantadine, showing that this is the specific mechanism of inhibition of these inhibitors in vivo for both human and avian strains of influenza virus. The amantadine-induced changes in HA of avian influenza viruses could be abrogated by M2 mutations alone which would affect the pH of the trans-Golgi network (16). Thus, HA mutations which change pH stability of avian strains have relatively minor effects on amantadine/rimantadine susceptibility compared to the total loss of drug susceptibility with M2 mutations, and do not appear to be clinically important (55). Analysis and mutagenesis of the M2 sequences of the Rostock and Weybridge avian strains revealed that residue 44 alone mapped to the trans-Golgi pH modulation whereas changes in residues 27, 38 and 44 were required to switch the activation characteristics of the Weybridge M2 to those of the Rostock M2 (56).

2.2.2 Effect of Mutations on Function and Structure of the M2 Ion Channel

Functional studies examining effects of amantadine mutations in the M2 protein on ion-channel activity have confirmed that this is the specific mechanism of virus inhibition by amantadine. When the influenza virus M2 protein was expressed in Xenopus oocytes it was shown to possess ion-channel activity with selectivity for monovalent ions, the transport of which could be blocked by amantadine (13). Further when amantadine-resistant mutants were expressed in Xenopus oocytes it was shown that amantadine no longer blocked the ion-channel activity of these mutant M2 proteins. Similarly, studies using deuterium-labelled amantadine and influenza M2 peptides suggested that amantadine bound 0.5 nm from the centre of the bilayer in an area between V27 and S31, a location consistent with the formation of a steric block within the ion channel (20). Similar studies with an amantadine-resistant mutant peptide revealed no interaction with amantadine. Based on solid-state NMR structural studies the amantadine mutations at residues 27, 30 and 34 were positioned facing the pore of the channel, and residue 31 was positioned partially in the protein-protein interface and partially in the pore (57). Fig. 3 shows amantadine bound within the ion channel of wild-type influenza virus.

Binding analysis of amantadine to M2 peptides from different viral strains showed that the virus developed two processes of overcoming the amantadine block (57).

1. The channels mutate so that amantadine can no longer bind.
2. A novel mechanism which retains binding of amantadine but the mutation maintains the function of the pore.

In this second process the pore size is increased thus allowing protons to move through the channel in the presence of the inhibitor. It was shown that mutations that introduced a

Table 1 M2 Mutations observed from in vitro, in vivo and clinical studies with amantadine and rimantadine

			Selected (reference)			
Inhibitor	Virus/sub-type/source	Mutation (no. of isolates)	In vitro	In vivo	In clinic	Transmission in humans
Amantadine	H3N2 (human)	L26F (2)			Yes (42)	NR
Amantadine	H7N1 (avian)	L26H (1)	Yes (53)			
Amantadine	H3N2 (human)	L26F (1)			Yes (68)	NR
Amantadine	H3N2 (human)	L26F (1)		Yes (37)		
Amantadine	H2N2 (human)	V27A (2)	Yes (51)			
Amantadine	H7N7 (avian)	V27A (3)	Yes (51)			
Amantadine	H7N1 (avian)	V27A (1)	Yes (51)			
Rimantadine	H3N2 (human)	V27A (1)			Yes (40)	NR
Rimantadine	H3N2 (human)	V27A (1)			Yes (41)	NR
Amantadine	H3N2 (human)	V27A (3)			Yes (43)	Yes
Rimantadine	H3N2 (human)	V27A (1)			Yes (45)	NR
Amantadine	H3N2 (human)	V27A (1)		Yes (37)		
Amantadine	H3N2 (human)	V27A (1)	Yes (46)			
Amantadine	H3N2 (human)	V27A (8)			Yes (47)	Yes
Amantadine	H7N7 (avian)	V27G (2)	Yes (51)			
Amantadine	H7N7 (avian)	V27D (1)	Yes (51)			
Amantadine	H5N2 (avian)	I27S (1)		Yes (54)		Yes
Amantadine	H7N1 (avian)	I27S (17)	Yes (51)			
Amantadine	H7N1 (avian)	I27S (10)	Yes (53)			
Amantadine	H5N2 (avian)	I27T (3)		Yes (54)		Yes
Amantadine	H7N1 (avian)	I27T (8)	Yes (51)			
Amantadine	H7N1 (avian)	I27T (6)	Yes (53)			
Amantadine	H7N1 (avian)	I27T (1)	Yes (53)			
Amantadine	H7N1 (avian)	I27A (1)	Yes (51)			
Amantadine	H7N1 (avian)	I27N (2)	Yes (53)			
Rimantadine	H3N2 (human)	A30V (2)			Yes (40)	NR
Rimantadine	H3N2 (human)	A30V (1)			Yes (41)	NR
Amantadine	H3N2 (human)	A30V (1)			Yes (43)	NR
Amantadine	H3N2 (human)	A30V(2)			Yes (44b)	NR
Rimantadine	H3N2 (human)	A30V (1)			Yes (45)	NR
Amantadine	H3N2 (human)	A30V (3)			Yes (47)	Yes
Amantadine	H3N2 (human)	A30V (4)		Yes (37)		
Amantadine	H3N2 (human)	A30V (1)	Yes (46)			
Rimantadine	H3N2 (human)	A30T (1)			Yes (40)	NR
Rimantadine	H3N2 (human)	A30T (1)			Yes (41)	NR
Amantadine	H5N2 (avian)	A30T (1)		Yes (54)		Yes
Amantadine	H3N2 (human)[a]	A30T (1)			Yes (46)	No
Amantadine	H2N2 (human)	A30T (6)	Yes (51)			
Amantadine	H7N7 (avian)	A30T (7)	Yes (51)			
Amantadine	H3N2 (human)	A30T (1)			Yes (41)	
Amantadine	H7N7 (avian)	A30T (11)	Yes (53)			
Amantadine	H3N2 (human)	A30T (1)			Yes (67b)	
Amantadine	H3N2 (human)	A30T (3)		Yes (37)		
Amantadine	H7N7 (avian)	A30P (2)	Yes (51)			
Amantadine	H5N2 (avian)	A30S (1)		Yes (54)		Yes
Amantadine	H7N1 (avian)	A30S (1)	Yes (53)			
Rimantadine	H3N2 (human)	S31N (10)	Yes (5)[a] (40)		Yes (5)[a] (40)	NR
Rimantadine	H3N2 (human)	S31N (14)			Yes (41)	Yes
Amantadine	H3N2 (human)	S31N (1)			Yes (43)	NR
Amantadine	H3N2 (human)	S31N (2)			Yes (42)	NR
Amantadine	H5N2 (avian)	S31N (1)		Yes (54)		Yes
Amantadine	H2N2 (human)	S31N (8)	Yes (51)			
Amantadine	H7N1 (avian)	S31N (4)	Yes (51)			
Rimantadine	H3N2 (human)	S31N (5)			Yes (6)	Yes
Amantadine	H7N1 (avian)	S31N (5)	Yes (53)			
Amantadine	H3N2 (human)	S31N (5)			Yes (39)	Possible
Amantadine	H3N2 (human)	S31N (2)			Yes (67b)	
Amantadine	H3N2 (human)	S31N (4)			Yes (44b)	
Rimantadine	H3N2 (human)	S31N (6)			Yes (45)	Yes
Amantadine	H3N2 (human)	S31N (16)	Yes (9)[a] (46)		Yes (7[a]) (46)	Yes
Amantadine	H3N2 (human)	S31N (5)		Yes (37)		
Amantadine	H3N2 (human)	S31I (1)		Yes (37)		
Amantadine	H7N7 (avian)	G34E (29)	Yes (51)			
Amantadine	H7N1 (avian)	G34E (4)	Yes (53)			

[a] = number of isolates, NR - not recorded. [b] = immunocompromised

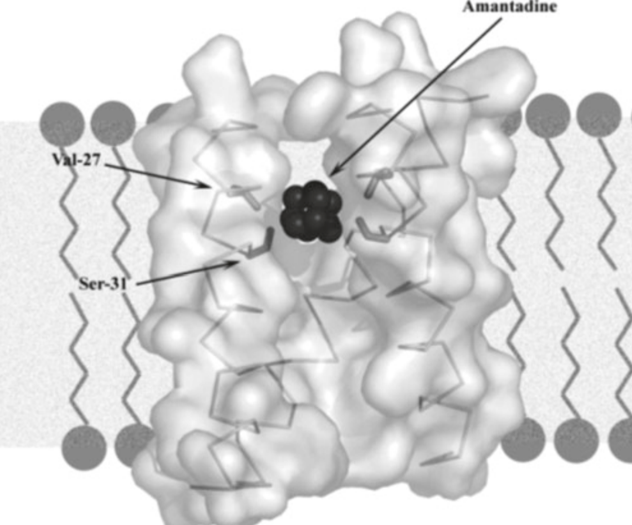


Fig. 3 Cross-section of the tetrameric A/M2 ion (proton) channel within the viral lipid membrane showing binding of amantadine. The hydrophobic adamantane moiety is associated with the pore-lining residues of the amantadine-sensitive proton channel. The two most common amino acid residues associated with amantadine resistance, S31 and V27, are highlighted. Kindly prepared and provided by Dr Alan Hay, MRC National Institute for Medical Research, Mill Hill, London, UK

larger amino acid either S31N or A30T blocked binding of amantadine possibly by reductions in pore size (S31N), or changes in chemical nature (A30T), i.e. steric hindrance or chemical incompatibility due to changes in hydrophobicity. Mutations that introduce a smaller amino acid either V27G/S/T/A retain amantadine binding but the pore is larger in size so that the drug does not block the pore. Based on these studies amantadine appears to be located near residues 30 and 31, whereas the water molecule file required for the H^+ wire is located in the vicinity of residue 27 (57). Mutants that lose binding of amantadine due to reductions in pore size also have reduced proton transfer, whereas mutations that increase pore size have increased proton transfer.

2.3 Cross-Resistance

Amantadine and rimantadine are structurally very similar (see Fig. 2) and it is assumed that binding within the M2 ion-channel pore would be identical for both molecules. Data from cross-resistance analysis would confirm this because selection of resistant variants and the total resistance observed for mutations at each of the five different residues selected appear the same. Cross-resistance has been reported for other related molecules such as cyclo-octylamine (58), cyclononane (59) and BL-1743 (60) (see Sect. 2.5).

2.4 Mechanism of Spread of Resistance

The majority of studies on emergence of resistance to amantadine or rimantadine whether in vitro or in vivo, including clinical studies, demonstrate the relative ease with which resistance can develop to the M2 ion-channel blockers. In vitro or in vivo resistant isolates are stable during replication in the absence of inhibitor. This fits with the data obtained with functional studies on amantadine-resistant M2 ion channels. Selective proton ion-channel activity is retained, although mutations may result in some impairment or enhancement of activity, but sufficient activity must be retained to result in acidification of the interior of the virus and in uncoating of the RNP complexes. Comparison of amantadine-resistant and parent virus from studies with avian viruses showed no differences in replication capacity, on transmissibility or in pathogenicity (54, 61). Similarly, for human influenza viruses no differences were observed for amantadine or rimantadine selected drug-resistant influenza A viruses compared with the drug-sensitive progenitor strains in replication in cell culture, or in ovo (36, 40). When isolates from a rimantadine clinical study, containing single mutations at either V27A, A30V or S31N, were compared with parent virus in the ferret model, no differences in replicative capacity or virulence were observed between matched isolates, although the parent isolates differed in pathogenicity (62). Clinical studies in paediatrics and in family studies or nursing homes have shown that resistant isolates appear to have the same ability to cause illness and may be readily transmitted (6, 41, 43, 45, 63, 64). There is no evidence either that human infections caused by resistant viruses are any more severe. Overall the data show that mutations have no apparent deleterious effect on the virus, but also do not confer any advantages on the virus in the absence of drug.

Prevalence of the different mutants selected during prophylaxis and therapy does appear to vary (see Table 1). The S31N is the most frequent mutant isolated from the clinical setting and has been shown to be transmitted the most during therapy (6, 41, 45). Similarly, surveillance studies, although reporting that isolation of amantadine/rimantadine resistance was rare, the mutation most frequently detected was the S31N virus (65, 66). Analysis of H1N1 virus isolates from the 1930s also detected S31N mutants (35), and the Avian H5N1 strains circulating in South East Asia in 2004/2005 have the S31N mutation. Switches from one resistance mutation to another have also been found suggesting that the S31N may have some advantage over other mutants (67, 45). In vitro studies with human H2N2 virus also showed increased frequency of selection of the S31N mutant over the V27A and A30T (51). In contrast, with avian strains other mutations may be dominant including I27S or G34E suggesting there may be some variation with sub-type (see Table 1).

From clinical studies of H3N2 influenza, amantadine- and rimantidine-resistant isolates have been observed in up to 30% of patients including children and adults (68), and more recently in up to 80% of patients (69). Children have been shown to secrete resistant virus for longer periods of time than wild-type although this did not appear to affect resolution of symptoms but would potentially increase the risk of spread (63). This high potential for resistance development and transmission of resistant virus has led to discussion of how best to use these M2 inhibitors to limit development of resistance. Interestingly, early clinical studies in families with amantadine indicated that prophylaxis of contact cases was 100% protective whereas in a later study treatment of both index and contact cases lead to only 20% protection (70, 71). A later study with H1N1 influenza showed 69% protection with prophylaxis without treatment of index cases (72). Although no clinical studies have been run comparing these different treatment strategies directly, the clinical data indicate that the increased problem of resistance development when both index and contact cases are treated seriously compromises efficacy of the M2 inhibitors (73). Studies with the newer neuraminidase inhibitors indicate this may not be an issue with inhibitors with lower thresholds of resistance development (74, 75). Despite the high potential for resistance development with amantadine/rimantadine, surveillance studies had not reported high levels of circulating resistant virus from year to year. This may have been due to relatively low rates of use of these inhibitors in the community. However, it was suggested that this may be linked with the seasonal epidemiology of influenza where new strains under antigenic pressure tend to arise each year (6, 76). Since 2003 the number of resistant isolates has increased substantially initially in China (77) and the Far East where it is believed there was increased use of amantadine for respiratory conditions as a result of increased awareness of influenza due to the Avian H5N1 outbreaks. Similarly in other parts of the world with increased use of these inhibitors high levels of resistance have been observed circulating worldwide (49). In the USA the high levels of resistance observed has prompted the CDC to issue warnings not to use the M2 inhibitors for treatment of influenza infections (78, 79).

2.5 Alternative Agents

Over the years since the discovery of amantadine there has been tremendous effort and resource put into the synthesis and evaluation of diverse chemical series, many with cage-like structures which resemble amantadine (5, 80–84). Several active series have been identified but only three compounds have progressed into clinical trials. One of the best of these was spiroadamantadine (1′-methyl-spiro (adamantine-2,3′-pyrrolidine) maleate) which in clinical studies at 70 and 120 mg/day had only modest prophylactic (85) and therapeutic efficacy (86) and was not developed further. Similarly a cyclononane was developed at ICI in the UK and after initial promise at 100 or 200 mg/day in experimental prophylaxis studies showed minimal efficacy in experimental therapeutic studies (87). Finally, cyclo-octylamine hydrochloride, a cyclic amine which was administered intranasally as a 0.4% solution every 2 h had only marginal efficacy (88). Despite all this effort amantadine and rimantadine still remain the only two M2 ion-channel inhibitors available in the clinic.

The more detailed structural studies of amantadine binding to the M2 ion channel have renewed the interest in developing further novel M2 ion-channel inhibitors. More recently an inhibitor developed using an M2 expression yeast system (89) was shown to have specific M2 ion-channel inhibitory activity in Xenopus oocytes (60). The inhibitor BL-1743[2-(3-azaspiro (5,5)undecanol]-2-imidazoline] is a reversible inhibitor of the M2 ion-channel activity in the M2 oocyte model, compared to amantadine which is an irreversible inhibitor in the assay system used. This molecule must have similarities in binding the M2 channel to amantadine since all the amantadine-resistant isolates were also resistant to BL-1743. However, one mutant selected by BL-1743 had >70-fold resistance to BL-1743 and only tenfold resistance to amantadine indicating some differences in binding in the ion channel. Although BL-1743 was not developed these studies do show that new approaches may yield M2 ion-channel inhibitors with different characteristics and possibly increased potency over amantadine/rimantadine.

Further discovery and development of inhibitors to block the M2 ion channel would greatly benefit from improved assay design to measure ion-channel activity with higher throughput. Detailed structural information is also required to help design new inhibitors to this proven anti-influenza target. Suggestions made to exploit other areas of the pore include targeting the key amino acid residues H37 and W41 involved with ion-channel activity which appear conserved in an HXXXW motif in both influenza A and influenza B ion channels (90). There may be the potential to design inhibitors to this target in the future which are active against both influenza A and B and to amantadine-resistant isolates and where there may be more constraints on resistance development.

3 Neuraminidase Inhibitors: Zanamivir and Oseltamivir

Zanamivir [4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acety-neuraminic acid, or 4-guanidino-Neu5Ac2en, Relenza™] (see Fig. 4) was first described in 1993 as a potent and selective influenza A and B inhibitor designed to inhibit the influenza virus neuraminidase enzyme, and which inhibited virus

R = H
R = Et

Oseltamivir Zanamivir

Fig. 4 Chemical structure of the neuraminidase inhibitors: zanamivir and oseltamivir, R = H parent active molecule, R = Et pro-drug converted by liver esterases to active parent

replication in both cell culture and in animal models (91). After clinical evaluation, zanamivir, given by oral inhalation, was approved for therapeutic use against influenza A and B infections in the USA and in Europe in 1999, for prophylaxis use in 2006, and for both prophylactic and therapeutic use in many countries from 1999 to 2007. In addition, an IV formulation of zanamivir, at 600 mg BID, was evaluated in experimental infection in humans and was shown to be highly efficacious in preventing infection with A/Texas/36/91(H1N1)(92).

A further series of carbocylic sialic (or *N*-acetylneuraminic) acid analogues were reported in 1997 and an ethyl ester derivative, oseltamivir phosphate [(3*R*, 4*R*, 5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid, GS4104, Ro 64-0796, Tamiflu™] (see Fig. 4) was developed as an orally available prodrug which is converted by liver esterases to the active form oseltamivir carboxylate [GS4071, Ro 64-0802] which is a potent inhibitor of influenza A and B viruses. Like zanamivir, oseltamivir was active in cell culture and the prodrug active in vivo in different animal models (93–95). Oseltamivir was approved for prophylactic and therapeutic use against influenza A and B infections in the USA, Europe and in many other countries in 1999/2000. Both oseltamivir and zanamivir have been shown to be active, in vitro and in the murine model, against avian strains of virus isolated from the Far East, including the H5N1 strain, A/HongKong/156/97, which caused lethal infections in humans (96–98). Further evaluation of oseltamivir against a highly pathogenic A/Vietnam/1203/04 (H5N1) strain in mice showed that more prolonged dosing (8 days) and higher doses of oseltamivir produced a more beneficial antiviral effect (99). Similar studies in the ferret model examined post-exposure prophylaxis and treatment started 4 or 24 h after infection, using low dose $10–10^2$ EID_{50} of either highly pathogenic A/Vietnam/1203/04 (H5N1) or low pathogenic A/Turkey/ 15/06 (H5N1). Again higher doses of oseltamivir were required to protect the ferrets from lethal infection with the more pathogenic virus and if treatment was delayed for 24 h with the low pathogenic strain (100).

3.1 Antimicrobial Mechanisms of Action

3.1.1 Function of the Neuraminidase in Viral Replication

The neuraminidase of influenza A and B functions by cleaving terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides. Specifically, the viral enzyme catalyses the hydrolysis of α-(2, 3) or α-(2, 6) glycosidic linkages between the terminal sialic acid and the adjacent carbohydrate moiety. It has also been called receptor-destroying enzyme because it cleaves the sialic acid residues that are bound to the viral receptor HA, which is a sialic acid specific receptor, and thus causes release of virus from the cell surface. The functions of the NA and HA appeared to be in opposition, and from studies with neuraminidase inhibitors it has become clear that there is a fine balance between the affinity of binding of the HA to the sialic acid-containing cell-receptor and entry into the cell and release of virus from the cell surface through receptor cleavage by the NA.

The function of the NA is, therefore, in the release of virus particles from the cell surface, in preventing aggregation of virus particles which occur through the virus/virus HA sialic acid interactions after budding from the cell, and in vivo, in aiding spread of virus through the mucus layer in the respiratory tract by removing sialic acid from mucin. Blocking of neuraminidase function using neuraminidase inhibitors in vitro has shown aggregation of virus particles at the cell surface (101). In cell culture neuraminidase function is not essential for replication of virus (102), however, in vivo the function appears critical for spread of virus within the respiratory tract and to aid release and transmission of virus to the next host.

3.1.2 Structure of the Neuraminidase and Enzyme Active Site

The development of the clinically active neuraminidase inhibitors, which occurred roughly 30 years later than the M2 inhibitors amantadine and rimantadine, benefited from all the advances that had been made in molecular virology over this time. However, screening to look for neuraminidase inhibitors was also initiated in the 1960s (103) and later the transition-state analogue Neu5Ac2en (DANA) and the more potent trifluoro-acetyl analogue (FANA) were shown to be potent inhibitors of neuraminidase in vitro but not in vivo (104, 105). In this 30-year period extensive studies were undertaken to try to understand the structure and function of the surface glycoproteins of influenza, the haemagglutinin and the neuraminidase, and their high antigenic variability.

Large quantities of proteins were produced for structural studies and in 1981 the crystallographic structure of the H3 haemagglutinin was published (106), and 2 years later in1983 the structure of the N2 neuraminidase was presented (107) together with a detailed description of the invariant catalytic pocket (108). Later, further NA structures followed with the avian N9 in 1987 (109) and influenza B in 1992 (110). Comparison of these three NA structures further supported the high conservation of the NA active site and the suitability of this as a target for drug design. In 2006 further crystal structures of avian N1, N4 and N8 were solved by molecular replacement (111). Comparison of these structures further confirmed overall similarities of the active site but also revealed significant differences in the form of a cavity close to the active site in these three NAs. Based on phylogenetic analysis the neuraminidases of influenza A have been divided into two groups, Group 1 contains N1, N4, N5 and N8 and Group 2 N2, N3, N6, N7 and N9 (111).

Influenza virus neuraminidase is a tetrameric glycoprotein with a total molecular weight of 240 kDa. It characteristically has a mushroom shaped morphology with a hydrophobic stalk peptide which anchors the molecule in the cell membrane, and a globular head in which each monomer contains a deep pocket, the conserved enzyme active site, surrounded by the highly variable antigenic sites. Each enzyme monomer consists of six β-sheets of four anti-parallel strands arranged like the blades of a propeller (107). Accumulation of sequence data from different neuraminidase sub-types plus studies on binding of inhibitors to the enzyme identified 24 key active site residues which have been numbered here based on the original N2 structure (107, 108, 112). The 24 conserved residues identified contain a large number of potentially charged amino acids including six basic arginine residues, R118, R152, R156, R224, R292, R371, and a basic histidine H274 and asparagine N294, six glutamic acid residues E119, E226, E227, E276, E277, E425 and five aspartic acids D151, D198 (N198 in N7, N9 sub-types), D243, D293, D330, plus four hydrophobic residues, tryptophane W178, tyrosine Y406, leucine L134 and isoleucine I222, plus a hydrophilic residue, serine S179.

3.1.3 Binding of Substrate and Inhibitors to the Active Site

The detailed structural data on the Group 2 (N2 and N9) neuraminidase enzyme, particularly the highly conserved active site, allowed the development of rational drug design based on the understanding of the structure of the target molecule and its interaction with the substrate, sialic acid. Sialic acid (Neu5Ac) is bound within this pocket in the α-anomer in a half-chair configuration. The carboxylate moiety of the sugar lies between R118, R292 and R371 residues and the glycerol side-chain is H-bonded to G276, the 4-hydroxy interacts with E119, and the glycosidic oxygen interacts with the carboxylate oxygen of R151. The 5N-acetyl side chain is in a hydrophobic pocket formed by W178 and L134, with the N–H group interacting with a bound water molecule in the floor of the active site, and the oxygen is hydrogen bonded with R152.

Using computer modelling and based on this detailed understanding of the molecular binding of sialic acid to the neuraminidase enzyme, two potent inhibitors, were designed (91). These inhibitors were substrate analogues with modifications at the 4-hydroxy group to produce, 4-amino Neu5Ac2en and 4-guanidino-Neu5Ac2en (zanamivir). These substitutions were sufficient to increase the dynamics of binding and stability of the molecules to result in inhibition of virus in both cell culture and intranasally in vivo in the mouse and ferrets models, which was a major advance in developing neuraminidase inhibitors to be active in the clinic. Substitution of the 4-hydroxyl group by an amino group produced a significant increase in the overall binding interaction due to a salt bridge formation with the side chain carboxylic acid group of E119. The replacement of the 4-hydroxyl group with the more basic guanidino group produced a tighter affinity due to lateral binding through the terminal nitrogens of the guanidino group with E119. Binding of zanamivir to the NA of different influenza sub-types including A (Group 1 and 2) and B strains is considered to be similar (91, 111, 113). In neuraminidase enzyme assays both inhibitors were potent competitive inhibitors with inhibition constants of 5×10^{-8} M for the 4-amino derivative and 2×10^{-10} M for the 4-guanidino derivative (91). Further enzyme kinetics studies showed the 4-guanidino-derivative, zanamivir to be a slow-binding inhibitor of both influenza A (A/Aichi/ 2/68(H3N2)) and B (B/Beijing/1/87) viruses, with association and dissociation constant almost identical for both enzymes (114). This slow binding is suggested to occur due to slow release of a tightly bound water molecule by the guanidinium group (115). Further studies showed zanamivir to possess similar potency to all nine NA sub-types including different human sub-types (116) and against different sub-types of avian origin (117, 118). Later surveillance studies conducted by the Neuraminidase Inhibitor Susceptibility Network (NISN) against large numbers of virus isolates have recorded mean enzyme susceptibility for zanamvivir against the H3N2 isolates of 2.17 nM ($n = 664$), for H1N1 isolates of 0.61 nM ($n = 139$) and for influenza B isolates of 2.57 nM ($n = 148$) (119). Activity against the neuraminidase of avian strains isolated in 1997 and 2005 with the potential to infect humans have been recorded. The IC_{50} values for these H5N1 viruses were 0.5–5 nM (96, 97, 120), and for H9N2 isolates

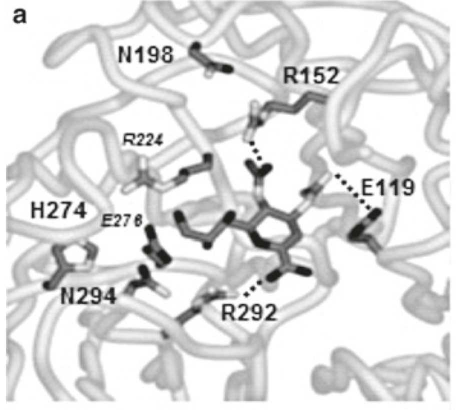
7–10 nM (97). This broad-spectrum activity is believed to derive from the structural design of the inhibitor to bind to only highly conserved residues within the active site.

The rational chemistry/drug design approach lead to the development of a second series of potent inhibitors of the neuraminidase enzyme, the carbocylic sialic acid inhibitors which culminated in the development of the orally active drug, oseltamivir (121). Oseltamivir differs from zanamivir in having a cyclohexene ring structure, a hydrophobic substitution replaced the glycerol moiety at the 6-position, and the 4-guanidino group was replaced by a 4-amino group. When binding to the active site, due to the presence of the hydrophobic substitution at the 6-position, oseltamivir causes a small conformational change in the active site of the NA to accommodate binding of the inhibitor. The residue E276 forms a salt link with R224 (see Fig. 5) and this conformational change results in the formation of a hydrophobic pocket for the substituent at the 6-position (122). From enzyme kinetic studies oseltamivir is also reported to be a slow binder of influenza A (A/Tokyo/3/67(H3N2)) and B (B/Memphis/3/89) viruses due to slow rate of dissociation of the compound from the neuraminidase (123). From surveillance studies by NISN, the mean IC_{50} values for oseltamivir against H3N2 isolates was 0.62 nM ($n = 767$), for H1N1 isolates 0.92 nM ($n = 139$), and for influenza B was 5.21 nM ($n = 148$) (119). Activity against the neuraminidase of avian strains isolated from 1997 to 2005, with a potential to infect humans, have been recorded. The IC_{50} values for these H5N1 viruses were 0.08–7.0 nM (98, 120, 124), and for H9N2 10–15 nM (98). Again broad spectrum activity was observed which is characteristic of the NAIs.

3.2 Mechanism of Drug Resistance

3.2.1 Development of Resistance to the NAIs

One rational for developing the neuraminidase inhibitors which bind only to highly conserved residue in the active site of the NA was that there would be major constraints on the development of resistance to these drugs (91, 107). Following on from the discovery of zanamivir, the first NA active in vivo, there was a flurry of activity to look at the potential for resistance development in cell culture with zanamivir. Overall it was found that passage of virus in increasing levels of zanamivir did result in reductions in susceptibility of the virus to zanamivir and drug resistance mutations were characterized (125–134). Fewer cell culture passage studies have been undertaken with oseltamivir, but the results also showed that it was possible to generate resistant variants to oseltamivir (135). However, unlike amantadine and rimantadine, it was found that many passages in cell culture at relatively high drug concentrations were required before resistant variants to the NAIs were selected. Only one study has been undertaken in vivo, in the ferret model to look at development of resistance to zanamivir in comparison with amantadine. Whereas resistance to amantadine developed rapidly within 6 days, similar to that observed in the clinic, no resistance to zanamivir was detected after two passages over 18 days' treatment (37). No comparative passage studies using in vivo models appear to have been done with oseltamivir and amantadine, although prophylaxis and treatment in the mouse model did not give rise to resistance to either drug (136). A later study in an immunocompromised murine



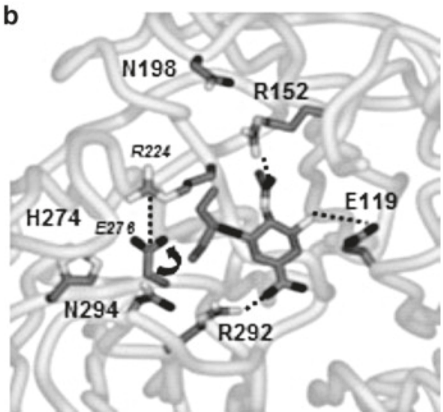


Fig. 5 (**a**) Binding of zanamivir within the active site of N9 NA without change in shape of the active site. (**b**) Binding of oseltamivir within the active site of N9 NA showing a conformational shift of the E276 residue to form a salt bridge with R224 to make a pocket to accommodate binding of oseltamivir. Some oseltamivir-resistance mutations (R292K, H274Y, N294S) prevent this conformational change blocking binding of oseltamivir but not zanamivir nor the natural sialic acid receptor. Residues associated with NAI resistance development E119, R152, N198, H274, R292, N294 are highlighted. Kindly prepared and provided by Dr Jennifer McKimm-Breschkin and Dr Mike Lawrence, CSIRO, Melbourne Australia

model infected with A/Japan/305/57(H2N2), oseltamivir was compared with another neuraminidase inhibitor A-32278. Resistance development was monitored by clonal analysis and one oseltamivir resistant virus but no resistance to A-32278 was detected (137).

Extensive surveillance studies on influenza viruses circulating worldwide have been undertaken by the NISN to look at NAI susceptibility using the NA enzyme assay. No naturally occurring resistant isolates were observed in more than 1,000 isolates circulating between 1996 and 1999 before the introduction of the NA inhibitors into the clinic (119). Similar studies have also been conducted by the CDC and WHO Australia, and no natural resistance detected at this time (138, 139). These data are consistent with these drugs targeting the highly conserved active site of the NA enzyme. Monitoring for resistance development during treatment and prophylaxis studies has been undertaken for the clinical development of both NAIs, using the NA enzyme assay and sequencing of the NA and HA genes. No emergence of zanamivir-resistant mutants has been detected during treatment of more than 5,000 immunocompetent patients with zanamivir (140). For immunocompetent patients treated with oseltamivir, resistance has been detected in viruses isolated from 1 to 2% of adults (140, 141), and from 5 to 6% of children (142), and more recently in Japan from 16 to 18% of children infected with H1N1 and H3N2 virus respectively (69, 143).

The potential for development of resistance in immunocompromised patients is expected to be higher than for immunocompetent subjects because of the higher levels of virus produced and the prolonged virus replication times. Although few immunocompromised subjects have been included in studies after treatment with NAIs, resistance has been observed to develop in at least six subjects, one (influenza B infection) after treatment with zanamivir, five (four A and one B infections) after treatment with oseltamivir (140, 144–147). Two of these subjects (both A infections) were later treated with zanamivir and only wild-type virus was isolated (141, 146, 147).

NISN has undertaken further worldwide monitoring of NAI susceptibility of 2,287 isolates of influenza A and B, circulating during the first 3 years (1999–2002) of NAI use (119, 148). These studies revealed eight isolates (two B, six A) with reduced susceptibility (>10-fold shift) to oseltamivir of which two (one A and one B) also had reduced susceptibility to zanamivir. Drug use at this time was relatively stable except for a tenfold increase in the use of oseltamivir in Japan in 2002. It was concluded that the frequency of isolation of variants did not increase significantly over this time (1, 0.22% in 1999/2000; 3, 0.46% in 2000/2001; and 4, 0.41% in 2001/2002). Later local surveillance studies within Japan have shown marked reduced susceptibility of influenza B isolates circulating within the community to oseltamivir (median IC_{50} values 55.8–85.1 nM) and slight reductions to zanamivir (median IC_{50} values 7–15.8 nM) (149). In addition, six pretreatment isolates had high level resistance to oseltamivir with IC_{50} values from 204.2 to 513.8 nM, and four of these with moderate reductions in susceptibility to zanamivir (IC50 values 29.5–61.7) and one other B isolate with an IC_{50} of 191.3 nM to zanamivir (149). This data has led to concerns that increased clinical treatment with NAIs in Japan, possibly may have selected for natural influenza B variants with reduced NAI susceptibility (150) but the magnitude of these changes may partly relate to differences within NA assays methods used (151). In the 2007/2008 season, the WHO/ECDC and CDC reported that worldwide drug susceptibility monitoring had identified widespread transmission of oseltamivir resistance in H1N1 viruses, which was not associated directly with drug treatment.

Susceptibility monitoring of highly pathogenic A(H5N1) avian influenza viruses circulating in poultry in various regions of South East Asia between 2004 and 2006 showed most strains to be highly sensitive to both zanamivir and oseltamivir. However, two isolates had reduced susceptibility to oseltamivir and one of these also showed a significant reduction in susceptibility to zanamivir (152). A second study of H5N1 isolates in poultry in the Far East revealed that while all isolates were sensitive to both oseltamivir and zanamivir there was up to 30-fold reduction in susceptibility to oseltamivir between some of the clade 1 isolates and the clade two isolates from Indonesia (153).

3.2.2 Genetic Analysis of Resistance to the NAIs

HA Variants (Mutations Based on H3 Sub-type Numbering)

Analysis of virus variants from some of the earliest passage studies with zanamivir in cell culture revealed mutations in the HA gene only, generally in residues close to those involved with receptor binding (see Table 2). However, the structure of the sialic acid binding site for HA is very different from that for NA and these inhibitors based on the design strategy should not bind to the HA site. The mechanism of drug resistance was considered to be due to decreases in affinity of binding of HA to the cell receptor such that the virus was less dependent on neuraminidase function for virus release. Mutations in the HA which resulted in weaker binding of the virus to the cell appeared not to compromise virus replication in vitro, but it was not known if this was relevant to the in vivo situation where neuraminidase function is essential. However, when one of these HA variants was used to infect ferrets and treated with zanamivir the virus was fully susceptible to zanamivir (133). Similar variants may occur naturally in the clinic, and although showing resistance in cell-based assays, are not resistant in the ferret model (154). This difference in susceptibility between in vitro and in vivo assays may reflect differences in receptor usage between cell culture (primarily α-(2–3)

Table 2 NA and HA mutations observed from in vitro, in vivo and clinical studies with NAIs

Inhibitor	Virus sub-type	NA mutations N2 numbering	HA mutations[b] H3 numbering	In vitro	In clinic
Zanamivir	A/H1N9 (human/avian recombinant)	N346S[a]	T155A	Yes (126, 127)	
		None	R229S		
		G90Q[a]	V223I/R229I		
Zanamivir	A/H1N9 (human/avian recombinant)	None	S165N,S186F	Yes (128, 129)	
		None	S186N,K222T		
Zanamivir	A/H2N2 (human)	None	E135A	Yes (133)	
		None	R137Q		
		None	A138T		
Zanamivir	B/(human)	None	L226Q,V93A	Yes (134)	
Zanamivir	A/H4N2	R249K[a]	G75E	Yes (130–132)	
		None	Y234L/A35T/K68R		
Oseltamivir	A/H3N2 (human)	None	A28T/R124M	Yes (135)	
Oseltamivir	B/ (human)	None	H103Q	Yes (216)	
Peramivir	A/H3N2 (human)	None	K189G	Yes (217)	
Zanamivir	A/H1N9 (human/avian recombinant)	E119G	None	Yes (128, 129)	
		E119G	S186F		
Zanamivir	A/H1N9 (human/avian recombinant)	E119G	None	Yes (125)	
Zanamivir	B/ (human)	E119G	N145S	Yes (125)	
		E119G	N150S		
Zanamivir	A/H4N2 (avian)	E119G	None	Yes (130–132)	
Zanamivir	A/H4N2 (avian)	E119A/R249K[a]	G75E	Yes (130–132)	
Zanamivir	A/H4N2 (avian)	E119D	None	Yes (130–132)	
Zanamivir	B/(human)	E119G	L226Q, V93A	Yes (134)	
Oseltamivir	A/H3N2 (human)	E119V(1)	None		Yes (161)
Oseltamivir	A/H3N2 (human)	E119V(3)	None		Yes, (141)
A-315675	A/H1N9 (human/avian recombinant)	E119D	None	Yes (162)	
		E119D	R233K,S339P		
Oseltamivir	A/H1/N9 (human/avian recombinant)	E119V/R305Q	H154Q	Yes (161)	
		E119V/R292K	None		
Oseltamivir	A/not defined (human)	E119V	None		Yes (142)
Zanamivir	B/(human)	E119A	Q218K	Yes (216)	
Oseltamivir	A/H3N2 (human)	E119V (2)	None		Yes (69)
Oseltamivir	A/H3N2 (human)^	E119V	A142G,Y195F,I239R		Yes (146)
Oseltamivir	A/H3N2 (human)[c]	E119V	V226I		Yes (146)
Oseltamivir	A/H3N2 (human)[c]	E119V,I222V			Yes (147)
Oseltamivir	A/H3N2 (human)	E119V			Yes (151)
Zanamivir	B (human)[c]	R152K (1)	T198I		Yes (144)
Oseltamivir	A (H1N1)	H274Y	None	Yes (165)	
Oseltamivir	A (H1N1)	H274Y (2)	None		Yes (164)
Oseltamivir	A (human) not defined	H274Y (1)	None		Yes (142)
Oseltamivir	A/H1N1 (human)	H274Y (1)	None		Yes (141)
Oseltamivir	AH1N1) (human)[c]	H274Y (7)			Yes (145)
Oseltamivir	A/H1N1 (human)	H274Y (7)			Yes (143)
Oseltamivir	A/H5N1 (avian)	H274Y (2)			Yes (166)
Oseltamivir	A/H5N1 (avian)	H274Y (1)			Yes (120)
Peramivir	B	H274Y		Yes (189)	
Peramivir	A/H1N1 (human recombinant)	H274Y	None	Yes (218)	
Oseltamivir	A/H1N1 (human)	H274Y			Yes (151)
6-Carboximide derivative of zanamivir	A/H1N9 (human/avian recombinant)	R292K	N199S	Yes (155)	
Zanamivir	A/H4N2 (avian)	R292K	Y234L/T267K/D304N/K68R	Yes (130–132)	
Oseltamivir	A/H3N2 (human)	R292K	A28T, R124M,	Yes (135)	
Oseltamivir	A/H3N2 (human)	R292K (8)	None		Yes (142)
Peramivir	A/H2N2 (human)	R292K	G130D	Yes (196)	
Oseltamivir	A/H3N2 (human)	R292K (10)	None		Yes (141)
Oseltamivir	A/H3N2 (human)	R292K (6)	S262N (1)		Yes (69)
Oseltamivir	A/H3N2 (human)	R292K			Yes d(137)
Oseltamivir	A/H3/N2 (human)	R292K			Yes (151)
Oseltamivir	B/ (human)[c]	D198N (1)	None		Yes (140, 146)
Oseltamivir	A/H3N2 (human)	N294S (1)	None		Yes (69)
Oseltamivir	B/ (human)	G402S			Yes (149)
Zanamivir	A/H1N1 (human recombinant)	Deletion 92–362	A200T	Yes (218)	
Oseltamivir	A/H3N2 (human)	Deletion SASG245–248			Yes (151)

[a] NA mutations recorded outside the NA active site, probably due to natural variation

[b] HA mutations are included when reported but for some clinical studies the HA may not have been sequenced

[c] Immunocompromised patients

[d] in vivo murine model

receptors) and in vivo (primarily α-(2–6) receptors). Variations in HA binding probably explain the characteristic larger variation in susceptibility to NAIs seen with different influenza strains in cell culture assays (95, 116).

NA Variants and Effects of HA Mutations (NA Mutations Numbered Based on the N2 Sub-type Numbering)

Later studies in cell culture revealed that mutations could arise within the active site of the NA, although generally also accompanied by changes in HA. The mutations observed in the NA active site are selected presumably due to direct effects on drug binding. A number of NA mutations have been observed after cell passage in vitro and in the clinic, and have been listed in Table 2, together with any accompanying HA mutations. The HA mutations observed in further cell culture studies with either zanamivir or oseltamivir have been scattered on the HA molecule, but some do appear to reduce receptor binding in cell culture (155) thus reducing virus dependence on NA. Some HA and NA mutations appear to work synergistically increasing the levels of resistance detected in cell culture (129, 156).

From the clinical studies with oseltamivir, NA-active site mutations have been linked with treatment, but HA mutations, although observed are probably natural variants not associated with drug treatment and have not shown altered drug susceptibility (157). However, with zanamivir the one clinical isolate with zanamivir resistance had both an NA-active site mutation, and an HA mutation with altered cell-culture binding properties. The HA mutation in cell-based assays totally masked the change in susceptibility resulting from the presence of the NA mutation as observed in enzyme-based assays (144). The cell-based assay results with this resistant variant plus the data from selection of HA variants in cell culture confirmed that cell-based assays were unreliable in monitoring susceptibility to NAIs (158). To overcome this problem, the MDCK cell line has been modified to overexpress human α-2,6-sialyltransferase(SIAT1), such that these cells have twofold increased expression of α−(2,6) receptors and twofold-lower α−(2,3) receptors (160). This improved the consistency of susceptibility recorded for NA mutations between the cell-based and enzyme-based assays (160). There must be constraints on reductions in affinity of HA binding that still allow efficient entry of virus into cells in vivo. This means that HA mutations alone will probably not compromise NAI therapy in the clinic. However, it may be possible that some HA variants may predispose the virus to the development of NA resistance mutations. Currently, NISN recommends the use of NA enzyme assays for monitoring susceptibility to NAIs in the clinic together with sequencing of the NA gene.

NA Variants Selected During In Vitro Passage or During Treatment in the Clinic

A total of seven NA residues within the enzyme active site region have been linked with selection of NAI resistance to date and are listed in Table 2. However, some mutations have only been observed in vitro, and some only in vivo. Interestingly some differences in the mutations observed have been seen between the different NA sub-types, and with the two inhibitors used, which relate to differences in binding within the active site.

Four different mutations have been observed at residue E119, three in vitro with zanamivir (E119G/A/D) in influenza A (H1N9), A(H4N2) and influenza B strains (125, 127, 129, 130–132, 134) and one (E119V) with oseltamivir in influenza A (H3N2) isolates from the clinic (69, 141, 142, 161) and in vitro (162). Despite the frequent isolation of E119 mutants by zanamivir in vitro, no E119 variants have been selected by zanamivir in the clinic. Based on studies with H3N2 viruses generated by reverse genetics, it has been suggested that these viruses may not readily arise in vivo due to their poor viability (163).

The most frequent mutation observed (see Table 2) in the clinic with oseltamivir treatment of influenza A(H3N2) viruses, is at the catalytic residue R292K (69, 141, 142, 157). This mutation was also selected by oseltamivir in vitro (135) and has also been selected in vitro by zanamivir in an avian influenza A strain (H4N2) (132).

A novel mutation within the NA active site was detected at residue H274Y in a volunteer study using experimental infection with influenza A/Texas/36/91(H1N1) to evaluate the efficacy of oseltamivir in humans (164). Subsequently, in vitro studies with an H1N1 strain were described where an H274Y variant was isolated suggesting that there were some differences structurally between the N1 and N2 enzymes which influenced selection of resistant variants to oseltamivir (165). This mutation has since been detected in H1N1 viruses isolated from two further subjects during clinical trials with oseltamivir in adults and children (141, 142), in 16% (7/43) children in an oseltamivir clinical study in Japan (143), and in H5N1 infections in the Far East (120, 166).

Limited studies have also been undertaken in immunocompromised subjects to evaluate the risk of development of resistance where virus clearance is more difficult and treatment courses are longer. The mutation R152K has been isolated once in an influenza B strain in an immunocompromised child after 12 days treatment with zanamivir (144) and similarly a mutation D198N in an influenza B isolate has been obtained from an immunocompromised subject treated with oseltamivir (140). For influenza A, resistant isolates have been detected in four immunocompromised patients treated with oseltamivir. Mutations included those already observed in

immunocompetent subjects, that is, the E119V (three patients) (140, 146, 147) and the H274Y (one patient) (140, 145). One patient with the E119V had a second mutation I222V (147) which is a highly conserved framework residue in all influenza A and B strains and has been observed previously from in vitro studies with oseltamivir together with the E119V or H274Y mutations (148). This I222V mutation enhanced the resistance observed with the E119V mutations. In addition, two patients treated with both oseltamivir and amantadine/rimantadine developed resistance to both inhibitors (M2 – S31N, plus NA H274Y or E119V) (140, 145, 147).

Finally, a new mutation was recorded in influenza A (H3N2) at residue N294S within the NA active site from one paediatric patient in Japan treated with oseltamivir (69). This mutation has since been observed in H5N1 virus isolated from one patient in Vietnam (120), and at least two patients in Egypt (151).

The degree of resistance that all these different mutations may confer against the selection drug, as evaluated in the enzyme assay, range from approximately 10- to >10,000-fold with the exception of the I222V which only produces low-level resistance (approximately twofold) to the selection drug. Susceptibility data will be discussed in detail in the following section on cross-resistance. Although susceptibility of some isolates, particularly in vitro isolates, have also been evaluated using cell culture assays, these data will be influenced by the presence of HA mutations, and cell receptor specificity, and therefore may be unreliable (158). Such data has been reviewed previously (155).

Variants Detected in Untreated Subjects During Surveillance Programmes

One important function of the NISN surveillance programme was to evaluate if resistance mutations selected during treatment may circulate within the community. Early studies did not detect any known NAI resistance mutations circulating (119) and during the first 3 years of NAI use only one H1N1 isolate contained an NA mutation previously found in clinical trials to be associated with oseltamivir resistance (H274Y) (148). However, later studies by NISN of annual surveillance of influenza viruses circulating in Japan reported further low level isolation of viruses with known oseltamivir resistant mutations, in 2003/2004, 1 × E119V (0.16%) and 2 × R292K (0.08%) in H3N2 viruses and in 2005/2006, 4 × H274Y (2.2%) in H1N1 viruses (159). This suggested that transmission of resistant virus may be possible. In other surveillance studies in Japan both the A198N and I222T oseltamivir resistance associated mutations were observed circulating in influenza B viruses in untreated subjects, with some reduced susceptibility to both oseltamivir and zanamivir (149). Surveillance in the 2007/2008 season revealed widespread circulation in man of H274Y variants in H1N1 virus in many countries (WHO/ECDC and CDC web-sites).

In addition to mutations observed during treatment with NAIs, some natural variants have been identified by the members of NISN surveillance programme that have given rise to some NAI resistance (148). Mutations observed include Y155H in A/Hokkaido/15/2002(H1N1) which is a natural variant with the Y155 conserved in all human N1 viruses, and H155 found in some swine and avian N1 viruses and some earlier N2 viruses which are susceptible to NAIs. Interestingly the A/Hokkaido variant gave resistance to both oseltamivir and zanamivir, indicating that the NA background in this isolate must be having secondary conformational effects on the active site which affect drug binding and influence susceptibility. One double natural variant, G248R/I266V, in an N1 background was also associated with resistance to both oseltamivir and zanamivir. Other mutations of unknown significance include E41G and Q226H in an N2 background which gave rise to low level oseltamivir resistance. In influenza B isolates, natural variants were observed at D198E and in I222T. Both of these residues, but with different amino acids, have previously been observed associated with development of oseltamivir resistance (140, 147, 148). In Australia, another natural variant N198E in an influenza B isolate was associated with low level resistance to oseltamivir and zanamivir (167). Finally, one isolate with a S250G mutation showed reduced susceptibility to zanamivir (149).

Susceptibility monitoring of highly pathogenic A(H5N1) avian influenza viruses circulating in poultry in various regions of South East Asia between 2002 and 2004 revealed two isolates with mutations associated with reduced susceptibility. Mutations identified included I117V plus I314V in a dual resistant virus and V116A in a variant with reduced susceptibility to oseltamivir. All three mutations are close to residues which are within the active site (152).

Other NA variants from in vitro studies

Finally, in some studies site-directed mutagenesis has been used to look at further mutations within the active site not seen by selection, either in vitro or in the clinic (168–170). Mutations at the E119 residue in the N9 enzyme background which were shown to produce reduced susceptibility to zanamivir but were not tested against oseltamivir included E119Q, E119T and E119L (168). Mutations at the H274 residue in the N1 enzyme background which produced reduced susceptibility to oseltamivir included the large substituent H274F, whereas some zanamivir resistance was observed with the mutants H274N, H274G, H274S, H274Q. In the third study mutations constructed in an N2 virus background (A/Wuhan/359/95(H3N2) and tested for susceptibility to zanamivir and oseltamivir included R118K, R371K, E227D, R224K, E276D, D151E. The R224K, E276D and R371K mutations conferred resistance to both zanamivir and oseltamivir, and the D151E mutation gave reduced

susceptibility to oseltamivir. However, based on genetic stability and replication efficiency data, they concluded that only the E276D variant might be viable in vivo (170).

3.2.3 Enzyme Functional Studies

Mutations observed in the vicinity of the neuraminidase active site may be divided into two types, those affecting the structural scaffold, the framework residues (E119, H274, N294, D198) and those affecting catalytic residues which interact directly with the substrate (R152, R292) (69, 119, 140, 146, 171). All the mutations studied have been reported to reduce enzyme activity and/or enzyme stability. Ideally, for accurate determinations of enzyme activity in comparison to wild-type, quantitation of native NA protein using conformational specific NA antibodies is important.

The framework mutation E119G affects stability of the enzyme but not the enzyme-specific activity (172), whereas E119A the most conservative mutation at residue 119 has a greater effect on reducing susceptibility and on specific activity of the enzyme (132). Kinetic analysis of zanamivir binding demonstrated that the E119G variant did not exhibit slow binding characteristic of that seen with the wild-type enzyme (128). All three 119 residue mutations in the avian (H4N2) virus have altered stability as determined using thermal and pH effects (132). In an H3/N2 virus plasmid rescue system the different 119 mutations were compared for enzyme activity, E119G had just 4.1% activity, E119A 5.2%, E119D 15% and E119V 45% NA activity compared to wild-type, allowing for different levels of native enzyme protein (163). This suggests that the 119V enzyme, the only 119 variant observed in the clinic has the greatest stability of all these 119 variants. This E119V variant has also been reported to have twofold greater binding affinity for the enzyme substrate than the E119 wild-type (161). Similarly, analysis of enzyme activity of the framework mutant H274Y in the N1 background (A/Texas/36/91) showed that this mutant had substrate affinity and enzymatic activity equivalent to wild-type (173). A later study using an in vitro derived mutant AWS/33(H274Y), enzymatic activity of the mutant (H274Y) was reported to have 30% activity of wild-type (169).

In contrast, the mutations in the catalytic residues (R152, R292) would be expected to have major effects on the enzyme activity with the natural substrate. The R292 residue is one of three arginines within the catalytic triad of the NA active site which are all highly conserved. Studies from in vitro-generated R292K mutants showed that all the variants in different N2 backgrounds had reduced specific activity from 2 to 44% activity, but high enzyme stability in contrast to the other variants described above (132, 135, 156). There was a reduction in affinity of the R292K enzyme for substrate of about fivefold and the pH optimum of the mutant enzyme had reduced to pH 5.0/pH 5.3 (132, 135). Further direct comparisons of the effect of the R292K mutation and the E119V mutation generated by reverse genetics in the same N2 virus background revealed that the R292K caused a greater reduction in enzyme activity and thermostability than the E119Vmutation (170).

Characterization of the NA enzyme activity of the R152K mutant virus isolated from an immunocompromised subject also showed substantial reductions in enzyme activity, 3–5% of parent virus (144).

3.2.4 Mutant Enzyme Structural Studies

Crystallographic analysis of the mutant G119 NA indicated that reduced binding of zanamivir was due partly to loss of stabilizing interactions between the guanidino moiety and the carboxylate at residue 119, and partly from alterations to the solvent structure of the active site (128). The carboxylate of wild-type E119 is involved with binding to the 4-guanidino group in zanamivir, but in the G119 mutant the carboxylate is replaced by a water molecule (128). Structural data on other 119 mutants has not been published. Although the E119 does not interact with the 4-amino-group of oseltamivir directly (91), increase in size of the amino acid substituents at the 119 residue, such as with 119V, may lead to increased displacement of oseltamivir resulting in greater resistance (155). Why the 119V retains susceptibility to zanamivir is not understood without structural data on this mutant.

Structural studies with the R292K mutant enzyme revealed that this mutation affects the binding of both substrate and NAI substrate analogues through the carboxylate group on the sugar (122). This correlates with reduced enzyme activity of the variant and would account for reduced binding to zanamivir. For oseltamivir this mutation has much greater effects because the lysine at 292 prevents the formation of a salt bridge with A224, and the more stable E276 residue does not move to form a hydrophobic pocket to accommodate the bulky pentyl ether group, resulting in a substantial decrease in the binding of oseltamivir. However, interactions with the 2-carboxylate, the 4-amino group and the 5-acetamido group of oseltamivir are retained.

Detailed structural analysis has not been published for either the R152K mutant or the H274Y mutant. The wild-type R152 has been shown to form a hydrogen bond to the acetamide of the substrate, sialic acid, bound in the active site (109), and would be expected to affect the binding of all NAI substrate analogues. The H274Y mutant in H1N1 viruses is reported to act similarly to the R292K in the H3N2 viruses in that it forms a salt bridge stabilising the E276 residue preventing re-orientation and formation of the hydrophobic pocket to accommodate the pentyl ether group (136). The D294E mutations in the N2 background also affects the formation of the salt bridge preventing the conformational change to allow oseltamivir binding (174).

3.3 Cross-Resistance

Zanamivir and oseltamivir target the same region, the active site of the NA molecule, and therefore it may be expected that both drugs would show high levels of cross-resistance. However, because the two molecules bind in different ways within the active site not all mutations show cross-resistance, or the levels of resistance observed are different. These differences could be important in the clinic for use of the two drugs if resistance should become a problem (174). Generally tenfold shifts in susceptibility from wild-type are classified as resistant, however since wild-type strains may vary in susceptibility, care must be taken when interpreting shifts in susceptibility which should relate to IC_{50} values and drug levels achieved in the clinic. To date neither in vitro shifts in susceptibility nor IC_{50} values have been related to clinical efficacy to produce meaningful guidelines on clinical cut-off levels. However, recent clinical efficacy data with oseltamivir against influenza A and B virus infections in Japanese adults and children has shown reduced efficacy and susceptibility of influenza B strains (175, 176). This was also linked to longer virus shedding in influenza B than influenza A patients treated with oseltamivir. In contrast, zanamivir has shown similar clinical efficacy against influenza A and B strains (177). Further comparison of the effectiveness of zanamivir and oseltamivir in the treatment of influenza A and B infections in Japan showed comparative efficacy of the two inhibitors against influenza A but increased efficacy of zanamivir over oseltamivir against influenza B. These efficacy findings may relate to slightly greater susceptibility of influenza B strains to zanamivir compared with oseltamivir and possibly to higher local respiratory levels of zanamivir (178, 179) (see Fig. 6). Use of higher doses of oseltamivir may be required to increase efficacy against influenza B (151).

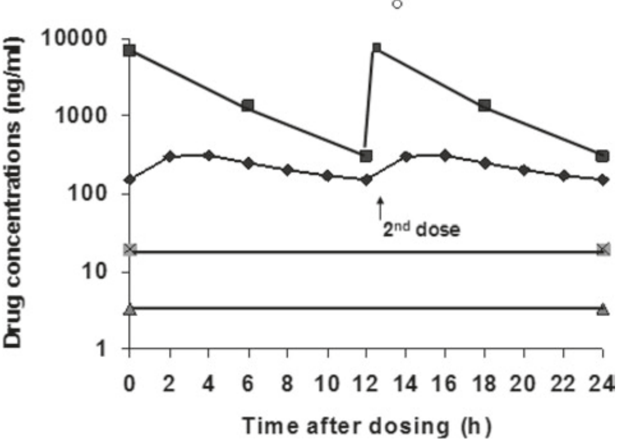


Fig. 6 Zanamivir (sputum) and oseltamivir carboxylate (plasma) q12h steady-state drug levels compared with median IC_{50} values for influenza B viruses isolated from Japanese subjects in 2004/2005 season. Information taken from publications by Hatakeyama S, Peng AW and He G (149, 178, 179). Zanamivir sputum drug levels (*filled square*); zanamivir median IC_{50} value (*filled triangle*); oseltamivir carboxylate plasma levels (*filled diamond*); oseltamivir carboxylate median IC_{50} value (*times symbol*)

3.3.1 Cross-Resistance Analysis with NA Variants Obtained from In Vitro Passage or Clinical Studies with NAIs

As stated earlier, the enzyme assay is the most suitable direct assay to study cross-resistance due to the NA mutations, since cell-based assays are influenced by receptor usage and changes in HA binding. Cross-resistance analysis has been undertaken with both in vitro derived and clinical isolates, with comparisons made using different assay conditions in the fluorescent NA assay (140, 180–182). Further comparisons of NAI cross-resistance have been carried out by the global NISN who have used the clinically derived NAI resistance isolates as controls in monitoring susceptibility of circulating influenza strains (183). Three NA assays have been compared in this study, two using the fluorescent substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUN or MUNANA), and one using a chemiluminescent substrate, 2′-(4 –NA Star)α-*D*-*N*-acetylneuraminic acid (NA-Star). Overall, the results obtained from all three assays were similar but the chemiluminescent assay was the more consistent. In Table 3, the published fold changes in NA susceptibility have been compared.

From this Table it may be seen that the most common NA mutant observed in the A/N2 sub-type in the clinic after treatment with oseltamivir, the 292K, shows high level resistance to oseltamivir >1,000- to 16,000-fold resistance, whereas cross-resistance to zanamivir is a relatively low 3.7- to 24.5-fold. The 274Y first seen in the A/N1 sub-type selected by oseltamivir in experimental infection and later in natural A/H1N1 infection in clinical trials and more recently in the highly pathogenic avian A/H5N1 infections in humans has lower resistance to oseltamivir than the 292K mutant with a shift of approximately 225- to 1,000-fold to oseltamivir but no apparent resistance to zanamivir. These differences in susceptibility for the two NAIs for these two mutants are consistent with the structural studies which indicate that both these mutations cause a block in the conformational change required for binding of oseltamivir. Zanamivir binds in a similar manor to natural substrate not requiring a conformational change.

The residue 119 group of mutations are interesting because differences in susceptibility are observed with the two inhibitors depending on which substituent is present. For the 119G selected in vitro by zanamivir but not seen in the clinic, high levels of resistance to zanamivir in the A/N2 sub-type are observed, from 40- to 333-fold shift in susceptibility but no significant or low level <10-fold resistance to oseltamivir (180–182), and in the B sub-type, 4,218- to 7,830-fold change to zanamivir and 35- to 119-fold change to oseltamivir (184). For the 119V in the A/N2 sub-type isolated from

Table 3 Cross-resistance analysis using NA enzyme assays, of neuraminidase variants obtained from in vitro passage studies and clinical studies with NAIs

NA mutations	Sub-type	Resistance to oseltamivir (fold-shift)[a]	Resistance to zanamivir (fold-shift)[a]	References
R292K	N2 avian	R (9,375)	I (8)	(180)
	N2 avian	R (>1,000–16,666)	I-R (8–12)	(181)
	N2 avian	R (15,000)	I (8)	(182)
	N2	R (>8,000–11,440)	I-R (3.7–24.5)	(183)
	N2	R (>1,600)	I (5)	(182)
R152K	B	R (187.5)	R (28.5)	(180)
		R (67–338)	R (20–94)	(184)
		R (>25–174)	R (30–3125)	(181)
		R (9.6–147.8)	R (12.5–36)	(183)
		R (100)	R (70)	(182)
H274Y	N1	R (1,000)	S (0.6)	(180)
		R (225 > 500)	S (1.1–1.3)	(181)
		R (353.5–634.8)	S (1.4–1.6)	(183)
		R (>700)	S (1)	(182)
	N1 avian	R (1,271–1,813)	S (1.4–3.4)	(120)
	B	I (2.4–6.6)	S (0.5–0.6)	(189)
	N1 recombinant	R (617)	S (1.3)	(218)
E119G	N2 avian	S (0.8)	R (40)	(180)
	N9	S (1–2)	R (249–984)	(184)
	B	R (35–119)	R (4,218–7,830)	(184)
	N2 avian	S (0.8)	R (40–333)	(181)
	N2 avian	S (2)	R (200)	(182)
E119A	N2 avian	S (2)	R (20)	(180)
		R (2.8–27)	R (20–417)	(181)
		I (9)	R (100)	(182)
E119D	N2 avian	S	R (60)	(180)
		R (1.3–9)	R (60–3,333)	(181)
		S (4.5)	R (323)	(182)
E119V	N2	R (52–335.4)	S	(183)
		R (130)	S (1)	(182)
		R (277)	S (3)	(182)
		R (276)	S (2.7)	(146)
D198N	B	I (9)	I (9)	(182)
		I (8.2)	R (10.7)	(146)
N294S	N2	R (300)	Not tested	(69)
	N1 (avian)	R (11.8–20.8)	S/I (3.2–6.2	(120)
G402S	B	R (3.9[b])	R (7)	(149)

[a] Fold shift = S < 5-fold difference from reference wild-type, I ≥ 5 < 10-fold difference from wild-type, R ≥ 10-fold difference from wild-type. In vitro fold-shifts have not been related to clinical efficacy

[b] Baseline virus high IC_{50}, resistant virus IC_{50} 281 nM, highly resistant – shift an underestimate

the clinic with use of oseltamivir, shifts in susceptibility for oseltamivir range from 50- to 335-fold, whereas zanamivir shows no shift in susceptibility (181, 182).

3.3.2 Cross-Resistance Analysis Using NA Variants Derived by Reverse Genetics or Recombinants Expressed in HeLa, 293T, or Insect Cells

Further cross-resistance analysis has been undertaken with neuraminidase variants prepared by reverse genetics or with recombinants expressed in insect cells, HeLa, or human kidney 293T cells (163, 168–170, 185–188). In this way the known resistance mutations have been studied and susceptibility to NAIs compared in different neuraminidase sub-types. The results of this cross-resistance analysis have been summarised in Table 4.

From this analysis it became apparent that the same mutations engineered into different NA sub-types may show marked differences between sub-types in the levels of susceptibility to oseltamivir and zanamivir. For the R292K mutation resistance to oseltamivir was highest in N2 but was also observed in the B background, but for zanamivir the resistance was much lower but variable in the N2 and B

Table 4 Cross-resistance analysis using NA enzyme assays of neuraminidase variants in different NA sub-types, derived by reverse genetics or from recombinants expressed in HeLa, insect cells or human kidney 293T cells

NA mutations	Sub-type	Resistance to oseltamivir (fold-shift)[a]	Resistance to zanamivir (fold-shift)[a]	References
R292K	N2	R (>10,000)	R (134)	(187)
	N2	R (>1,580)	S (2.5)	(163)
	N2	R (>60,000)	R (7)	(170)
	B	R (>300)	R (28.5)	(186)
R152K	N2	S (2.7)	I (5.5)	(163)
	N2	S (1)	S (1)	(170)
	N9	S (1.9)	R (9.6)	(163)
	B	R (252)	S (4.7)	(186)
H274Y	N1	R (200)	S (3)	(169)
	N1	R (427.8)	S (1)	(185)
	N1	R (754)	S (1)	(187)
	N1 (H5 avian)	R (292)	S (1)	(188)
	N1 (H5avian)	R (1,672)	S (2)	(188)
	N2	S (2.5)	ND	(169)
	N2	I (7)	I (5)	(187)
	N2	S (2.6)	S (3.7)	(163)
	N2	S (0.8)	S (1.2)	(163)
	N9	R (80)	S (2.7)	(163)
E119G	N1	I (8.74)	S (4)	(185)
	B	R (31.1)	R (>560)	(186)
E119A	B	R (>300)	R (>560)	(186)
E119D	N2	S (3.16)	R (32)	(163)
	B	R (>300)	R (>560)	(186)
E119V	N1	R (1,727)	R (2,144)	(187)
	N2	R (1,028)	I (7)	(187)
	N2	R (14–18)	S (0.8–1)	(163)
	N9	ND	R (145)	(168)
	B	R (>300)	S (1.9)	(186)
N294S	N1	R (197)	I (5)	(187)
	N1 (H5 avian)	R (83)	S (3)	(188)
	N1 (H5avian)	R (21)	S (3)	(188)
	N2	R (1,879)	I (8)	(187)

[a]Fold shift = S < 5-fold difference from reference wild-type, I ≥ 5 < 10-fold difference from wild-type, R ≥ 10-fold difference from wild-type. NAs used were B/Beijing/1/87 (186); A/WSN/33 (N1), A/Sydney/5/97(N2) (185, 187); A/Sydney/5/97 (N2), A/Tokyo/67 (N2), A/G70c N9 (163); A/G70c (N9) (168); A/WS/33 (N1) (169), A/Wuhan/359/95(N2) (170); A/Vietnam/1203/04(N1) (188).

variants. For the R152K selected during treatment with zanamivir in an influenza B infection, only a small shift in susceptibility to zanamivir (fivefold) was observed when introduced into B/Beijing1/87 background compared to a 250-fold change for oseltamivir. When introduced into N2 or N9 NA only small shifts in susceptibility were observed in both inhibitors (≤10) but were marginally greater for zanamivir. The H274Y only showed high level resistance to oseltamivir in the N1 and avian N9 backgrounds suggesting some similarity between these two sub-types in contrast to the N2 NA. The B NA was not constructed with the H274Y mutation, but previous data from in vitro passage with peramivir and influenza B virus has shown that this mutation also confers oseltamivir resistance in B virus (189).

Interesting results were observed with some of the 119 mutations, particularly in the N1 background. The in vitro mutation most frequently selected by zanamivir (E119G) and which gave high level resistance in the N2, N9 and B background was shown to remain sensitive to zanamivir in the N1 NA. Conversely, the E119V which was selected in the clinic by oseltamivir was sensitive to zanamivir in the N2 and B NAs but was highly resistant to zanamivir in the N1 background. These differences are not understood but may reflect structural differences between the Group 1 (N1) and Group 2 (N2, N9) NAs (111). The E119V mutation gave high level resistance to oseltamivir in all three NAs studied (N1, N2 and B).

For N294S resistance to oseltamivir was observed in both N1 and N2 constructs but the level or resistance was higher in N2 consistent with clinical data (69, 120), but for zanamivir only low level shifts were observed less than tenfold. As indicated in the structural studies these variations in

susceptibility suggest that there must be structural similarities and differences around the active site between the different sub-types (111).

3.4 Mechanism of Spread of Resistance

Based on functional studies, all the NA mutant enzymes show either substantial reductions in stability or enzyme activity, that is they all have compromised enzyme fitness. When replication kinetics were examined in cell culture, reductions in replication rates compared to wild-type strains have been detected for H274Y (173), and for R292K in two out of three studies (132, 135, 156), but not for E119G (125, 129), E119A (132) or E119V (161). However, kinetic studies in cell culture are not straightforward for NA mutants, because the presence of HA mutations, in addition to NA mutation, may result in increased growth of virus (156). In vivo studies using either the mouse or ferret models have also shown reductions in infectivity and pathogenicity, for the E119A (132) and E119V mutants (161, 190) but not the E119G mutant (129), and reductions for the R152K mutant (144), the R292K mutant (131, 132, 135) and the H274Y mutant (173). Again some of these studies were undertaken with HA and NA double mutants, where the HA background may have resulted in increased growth. Detailed studies in the mouse have indicated that HA mutations may play some role in resistance in vivo, although this has not been demonstrated in the ferret model (155). To overcome this problem the E119V and the R292K mutations were introduced into the N2 background and the same virus background using reverse genetics to allow direct comparisons of viral fitness in vitro and in vivo (191). This study confirmed the differences between these two mutations.

In addition, reverse genetics studies using two different H5N1 highly pathogenic virus backgrounds revealed that introduction of the H274Y or N294S mutations retained the high level pathogenicity in mammalian species (188).

Studies on fitness of NA variants gave rise to the theory that NAI variants would not be transmitted in humans. Although NA mutations may be detected during acute infection their presence has been reported to not affect resolution of symptoms (142), but virus shedding may be prolonged, particularly in children and the immunocompromised subjects increasing the risk of transmission (69, 140). Clinical trial studies and surveillance studies by the global NISN have revealed some potential for circulation of resistant viruses to date, with five H1N1 isolates with the H274Y mutation and two H3N2 isolates with the R292K mutation and one H3N2 isolate with the E119V mutation observed from untreated subjects (148, 159). Widespread surveillance studies are ongoing to determine how much of a problem transmission of resistant virus may be, in particular, transmission of the H274Y mutant in H1N1 in untreated subjects, observed in 2007/2008.

A model for influenza transmission in ferrets has been developed and used to study the potential for transmission of oseltamivir resistant clinical isolates from immunocompetent subjects (190, 192). This model which involves infecting four ferrets per group with mutant or wild-type virus and then housing these infected ferrets with three uninfected ferrets should help determine the potential for clinical transmission of the different influenza variants. The first studies with the R292K mutant showed no transmission for mutant virus but all the uninfected controls became infected showing transmission of wild-type virus. In addition, some contacts from the mutant virus group became infected with wild-type virus due to reversion to wild-type in the originally infected ferrets. Similar studies with the H274Y and E119V variants revealed under these experimental condition that these variants grew to high titre and could be transmitted, although the 274Y mutant had reduced infectivity and a 100-fold higher dose of virus was required to infect the ferrets (173). On transmission the virus variants remained stable and did not revert to wild-type. The E119V had similar infectivity to wild-type, and grew to similar titres as wild-type in both the donor and recipient ferrets (173, 191). Based on these studies the E119V variant in the H3N2 background appeared to have the highest potential for transmission (173, 191). However, the frequency of isolation of this N2 variant is very low compared to the more debilitating R292K mutant.

From all the clinical studies it appears that NA resistance arises due to single mutations within the highly conserved region of the active site of NA all of which have some effect on virus fitness. Interestingly there is one report from in vitro selection studies with oseltamivir of isolation of a double active site mutant (E119V/R292K), but at very low levels <10% of the virus population suggesting this virus was substantially compromised (162). Apart from the H274Y variants in man there is limited evidence that compensatory mutations in either the NA or HA could overcome the fitness deficit of the active site mutations. Further in a self-limiting disease where virus is normally cleared within 6–10 days there is a time limit during treatment on development of multiple mutations to produce fit virus. Even in immunocompromised subjects where virus shedding may be prolonged, and where both HA and NA mutations have been observed the virus fitness was still compromised (144). Currently it is not clear if different HA variants circulating in the community as described (154) may predispose virus to developing NA resistance and which may result in fitter virus with increased ability to transmit. From the NISN susceptibility surveillance studies and recent studies with H274Y transmission there are indications that differences in NA background may influence susceptibility and transmission of some mutations ((148), WHO/ECDC and CDC web-sites). However, limited cross-resistance between the NAIs may prove valuable. Compared

with amantadine/rimantadine, the potential for drug resistance to be a problem for the NAIs in treating influenza infections appears much reduced.

3.5 Alternative Agents

Zanamivir and oseltamivir are the only two neuraminidase inhibitors that are licenced for use against influenza infections in humans, with similar potency in both treatment and prophylaxis of seasonal influenza infections (193, 194), but with zanamivir showing greater potency against influenza B in vitro and in the clinic (177). Further substrate analogues have been designed and shown to be highly potent in vitro against influenza A and B viruses (195–198). These included a cyclopentane analogue [(4-acetylamino)-3-guanidinobenzoic acid, RWJ-270201, BCX-1812, peramivir] discovered at Biocryst and which showed oral efficacy in vivo in animal models (199, 200), including highly pathogenic H5N1 viruses (201), and was further evaluated in clinical studies by R.W Johnson Pharmaceuticals. In phase I studies, peramivir was shown to be effective in lowering virus titre but at relatively high drug concentration of 400–800 mg/kg/day (202). In phase 2 and 3 clinical trials, the primary end point of time to relief of symptoms did not reach statistical significance, probably due to the low oral bioavailability of peramivir (≤3%) (203). The clinical development of oral peramivir was halted in 2002. However, with the continued spread of the highly pathogenic avian H5N1 virus there is an increased need for influenza drugs to treat life-threatening influenza. Alternative formulations of peramivir, intramuscular and intravenous are being evaluated in clinical studies with seasonal influenza (204). In the mouse model it was shown that a single intramuscular injection of peramivir significantly reduced weight loss and mortality in mice infected with A/NWS/33(H1N1) or A/Victoria/3/75(H3N2) comparable to 5-day treatment with oral oseltamivir (203). The efficacy of a single intramuscular dose is explained by the tight binding of peramivir to the active site of neuraminidase. For N9 NA the peramivir $t_{1/2} > 24\,h$ compared with a $t_{1/2} = 1.25\,h$ for oseltamivir and zanamivir (203). In vitro passage studies of an A/H3N2 virus with peramivir selected for the mutation R292K in NA with a 10- to 20-fold reduction in NA susceptibility (196) and of H274Y in influenza B with a 16- to 31-fold reduction in susceptibility (189).

A second inhibitor, a novel pyrrolidine-based compound, [5-(1R,2S)-1-(acetylamino)-2-methoxy-2-methyl pentyl-4-[(1Z)-1-propenyl]-(4S,5R)-D-proline, A-135675, and isopropylester pro-drug A-322278] was discovered by Abbott Laboratories (197, 198), but despite showing good efficacy in vitro has not been developed further. In vitro passage studies of influenza A/N9 G70 virus in the presence of A-135675 selected E119D with a 162-fold reduction in NA susceptibility (162).

The development of resistance to oseltamivir, in the treatment of both seasonal influenza and avian H5N1 infections in humans, has renewed interest in drugs that will treat resistant virus. A detailed comparison between oseltamivir, zanamivir, peramivir and A-135675 susceptibility of resistant isolates obtained from both in vitro and clinical studies with oseltamivir and zanamivir has been undertaken (182). Interestingly A-135675 had the lowest cross-resistance profile, followed by zanamivir. This probably relates to some similarities of binding of these two inhibitors in that they do not cause a conformational change in binding to the NA active site whereas both oseltamivir and peramivir cause a conformational change due to the presence of the bulky hydrophobic substituent at the sixth position (162, 205). Against the H274Y variant, which has been isolated from some patients infected with H1N1 and H5N1 virus treated with oseltamivir, both A-315676 and zanamivir were active but peramivir showed a 100-fold reduction in susceptibility (182). In addition, the isopropylester pro-drug of A-135675 (A-322278), was shown to be effective against an oseltamivir selected B variant (D198N, N2-numbering) in the mouse (182). The additional structural studies undertaken with the N1, N4 and N8 of the Group 1 NAs have revealed a cavity close to the active site that closes on ligand binding which may be exploited in further drug design of NA inhibitors (111).

In addition to structural design, further approaches to development of more potent inhibitors has been that of the study of large hydrophobic pro-drugs of close analogues of zanamivir and multivalent zanamivir molecules by Sankyo in Japan, and Biota in Australia (206–208). These approaches were reported to prolong deposition of drug within the respiratory tract after oral inhalation with the potential for much reduced dosing frequencies, of possibly one prophylactic treatment per week, or once only treatment (209, 210). One inhibitor CS8958 in co-development by Daiichi-Sankyo and Biota is in phase II/III evaluation against seasonal influenza A and B.

The increased awareness of the potential for a new influenza A pandemic has stimulated research into the development of new influenza inhibitors. The rapid development of widespread clinical resistance to the M2 ion-channel inhibitors plus the emergence of some resistance to the NAI, oseltamivir, emphasises the need for new agents. These new influenza inhibitors may be developed to the same targets (NA and M2 ion channel) to cover resistant isolates as described above, or inhibitors to other targets within the influenza replication cycle should be developed.

Inhibitors in development to alternate targets include a sialidase fusion protein (DAS-81, Fludase) which is a recombinant fusion protein containing a sialidase catalytic domain derived from *Actinomyces viscosus* fused with a respiratory epithelium-anchoring domain. DAS-81 works by tethering to the respiratory epithelium and cleaving both α(2,6)-linked and α(2,3)-linked sialic acid receptors which are recognised by human and avian strains of influenza viruses,

and therefore blocks virus attachment (211). The molecule shows potent activity in vitro (IC_{50} values 0.04–0.9 nM) against both influenza A and B viruses and efficacy when given intranasally to mice before or after infection with H1N1 and H5N1 viruses (211, 212) DAS-81 is undergoing Phase 1 studies in humans.

A second inhibitor of interest is T-705 which is a substituted pyrazine 6-fluoro-3-hydroxy-2-pyrazinecarboximide which inhibits influenza virus RNA polymerase after conversion to the triphosphate (213). This inhibitor has activity against influenza A and B viruses in vitro with IC_{50} values in the range of 0.013–0.48 μg/mL (214), and in vivo efficacy against an A/Duck/MN/1525/81(H5N1) virus at 30–300 mg/kg/day, and was more effective than oseltamivir administered at 20 mg/kg/day (215). In 2007 T-705 entered clinical development in Japan to determine its efficacy and safety in humans.

Development of new inhibitors either to the proven targets, M2 ion channels and neuraminidase or to new targets and which may be active against resistant virus should improve treatment options for controlling influenza in the future. Some of these inhibitors may prove suitable for use in combination therapy which may limit the chances for resistance to develop.

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Chapter 32
Molecular Mechanisms of Resistance to Nucleoside Reverse Transcriptase Inhibitors

Marleen C.D.G. Huigen and Charles A.B. Boucher

1 Introduction

The viral enzyme reverse transcriptase (RT) is essential for viral replication. The enzyme RT is unique for retroviruses and transcribes the viral genomic RNA into a complementary DNA (cDNA) copy. Reverse transcription is a very complex process and depends on two distinct enzymatic activities of RT; a DNA polymerase that can use either RNA or DNA as template and a nuclease (Ribonuclease H or RNase H) specific for the RNA strand of RNA:DNA duplexes (1–3).

HIV-1 RT is a stable heterodimer consisting of two subunits of 66 (p66) and 51 kDa (p51) (4–6). The p51 subunit is generated by proteolytic cleavage of the p66 subunit by viral protease and lacks the C-terminal RNase H domain. Although the overall folding of the two subunits is similar, the spatial arrangement of the two subunits is completely different. The p51 subdomain adopts a closed formation and only plays a structural role, whereas the p66 subunit is organized to form a cleft into which the primer template binds and represents the polymerase active site. Crystallographic studies show that the p66 subunit resembles a right hand grasping the primer-template complex (7, 8). On the basis of this 3D structure, the enzyme has been divided into five distinct domains. These are the fingers (residues 1–90, 110–160), palm (90–110, 160–240), thumb (240–310), connection domain (310–430) and the RNAse H subdomain (430–565) at the carboxy terminus. The latter subdomain cleaves the template RNA strand and degrades the transcribed RNA. The palm domain harbors the polymerase active site, located in a cleft formed by the flanking fingers and thumb subdomain, which play a role in positioning the template. The active site of RT contains three aspartic acids at amino acids 110, 185 and 186, which are involved in metal-ion ligation and interact with the phosphates of the DNA primer and the incorporated nucleotides. These aspartates are highly conserved and required for the proper function of reverse transcription. The connection subdomain, as the name already implies, connects the polymerase and the RNAse H domain (1, 4, 9, 10).

Interestingly, unlike most polymerases, RT lacks a 3′-5′ exonuclease activity, which means that it is not able to identify and excise inappropriate nucleotides once they are incorporated in the growing DNA chain. As a consequence, RT is able to incorporate dNTP analogues in addition to the natural substrates (11, 12).

In 1987, the Food and Drug Administration (FDA) approved the first anti-HIV drug, Zidovudine (AZT), that was directed against RT. On the basis of their site of binding and mode of action, RT inhibitors can be subdivided into two classes: nucleoside and non-nucleoside inhibitors. This chapter will focus on nucleoside RT inhibitors (NRTIs) and the mechanisms of resistance to these drugs.

2 Nucleoside Reverse Transcriptase Inhibitors

The first RT inhibitor Zidovudine or AZT is a thymidine analog with an azido group at the 3′ position of the ribose (13). Subsequently, in the following years many other nucleoside analogs, dideoxynucleotides, were developed and introduced in the clinic. Nucleoside analogs or nucleoside reverse transcriptase inhibitors (NRTIs) are analogs of the normal dNTP substrates of DNA polymerase with important modifications. These nucleoside analogs are administered as precursor compounds or prodrugs, which have to be tri-phosphorylated by the host cellular kinases to their active form. After binding to the polymerase active site of RT, they compete with the natural dNTPs for recognition as substrate (binding) and incorporation into the nascent DNA chain. Since nucleoside analogs lack hydroxyl moiety on the ribose group, they prevent further DNA synthesis, once they are incorporated. Thus, they inhibit viral replication via two mechanisms (14, 15).

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Fig. 1 Structures from FDA-approved nucleoside reverse transcriptase inhibitors (NRTIs)

Currently, eight NRTIs are approved by the FDA to inhibit HIV-1 reverse transcription and thereby viral replication (Fig. 1). Two thymidine analogs: Zidovudine (AZT or 3′-azido-3′-deoxythymidine) and stavudine (d4T or 2′,3′-didehydro-2′,3′-dideoxythymidine), three cytosine analogs: zalcitabine (ddC or 2′,3′-dideoxycytidine), lamivudine [3TC or (−)-β-L-2′,3′-dideoxy-3′-thyacytidine] and emtricitabine (FTC), the adenosine analog didanosine (ddI or 2′,3′-dideoxyinosine) and the guanosine analog abacavir (ABC). Tenofovir (PMPA) is already phosphorylated once and hence is a nucleotide analog. Since both nucleoside and nucleotide RTIs act by the same mechanisms, the abbreviation NRTIs is used for both classes of compounds (14, 16, 17).

3 Resistance Against Nucleoside Analogs

Unfortunately, soon after the introduction of AZT monotherapy it became evident that the HIV could develop an increase up to 100-fold in IC_{50} towards this drug in patients receiving 6 months of treatment (18). The rapid emergence and selection of virus variants harboring resistance-associated mutations is a result of several properties from the HIV-1 replication process. First, HIV-1 RT has a very high error rate. As opposed to DNA polymerases, HIV-1 RT lacks proofreading activities, making it unable to correct errors that are generated during DNA synthesis. Estimations are that HIV-1 RT generates 3×10^{-5} errors per base pair per replication cycle (19). Second, the HIV-1 genome consists of two single-stranded RNA copies. RT can jump from one to the other RNA template resulting in a high rate of recombinant viral DNA sequences (20, 21). Third, HIV has a very rapid replication rate and a high viral turnover in an HIV-infected individual (10^7–10^9 viral particles per day) combined with an in vivo half life of ~2 days (11, 22, 23). And fourth, host cells harbor the cytidine deaminase APOBEC3G that deaminates deoxycytidines (dCs) to deoxyuridines (dUs) in minus-strand DNA during reverse transcription. This results in a high-level G-to-A hypermutation of the proviral plus-strand cDNA and subsequent abrogation of the viral replication. The viral enzyme vif binds to APOBEC3G and suppresses its incorporation in the virion. In this way, vif can reduce the G-to-A hypermutation to a nonlethal level, but cannot prevent all substitutions (9, 24, 25).

This generates HIV-1 viral quasispecies; a pool of closely related HIV virions. Under drug pressure specific virus variants harboring mutations conferring a decrease in susceptibility will have a competitive advantage over drug-sensitive variants. Amino acid changes in the RT gene that confer nucleoside analog resistance are shown in Table 1. These mutations are all located in the palm and fingers subdomains of HIV-1 RT (5, 11, 26, 27). Some substitutions are able to confer resistance on their own. The M184V/I mutation confers resistance to 3TC, FTC, ABC and ddC, the L74V against ddI, ABC and ddC and the K65R change confers resistance to d4T, ddI, ddC, 3TC, FTC, ABC and TFV. The Q151M is a key mutation in one of the multi-drug resistance pathways and is usually accompanied by the A62V, V75I, F77L and F116Y changes (28). For some drugs, such as AZT, resistance requires two or more mutations from the group of M41L, D67N, K70R, L210W, T215Y/F and K219Q (18, 29, 30). These mutations confer resistance to d4T as well, although to a lesser extent than to AZT. Since AZT and d4T are both thymidine analogues, these mutations are referred to as thymidine analog mutations (TAMs). However, TAMs have also been shown to confer resistance against other nucleoside analogs as well.

4 Mechanisms of Nucleoside Drug Resistance

The mutations that are generated during nucleoside analog treatment can be subdivided into two classes based on their mode of action. One class of mutations gives HIV-1 RT the

ability to discriminate between the nucleoside analog and the natural dNTP, by either impairing the binding and/or incorporation of the nucleoside analog. The other class of RT mutations cause the excision of the incorporated nucleoside analog from the DNA chain by a pyrophosphorolysis-like mechanism. These mechanisms of nucleoside drug resistance are respectively called discrimination and excision and will be discussed in the following sections. An additional mechanism explaining resistance against nucleoside HIV drugs has been proposed (12, 31). This mechanism is the repositioning of the template/primer, which changes nucleoside-analog incorporation. Since altered incorporation of the NRTI implies that a distinction is made between the drug and the natural dNTP, it is generally considered as a discrimination mechanism.

Table 1 Overview of mutations in RT and mechanism of action against NRTIs

Discrimination	
K65R	All NRTIs, except AZT
69Insert-complex[a]	All NRTIs
L74V	ddl, ddC, ABC
V75T	d4T, ddl
Q151M[b]	Multi-NRTI resistance
M184V/l	3TC, FTC, ddC, ABC
Excision	
M41L[c]	AZT, d4T
D67N[c]	AZT, d4T
69insert-complex[a]	All NRTIs
K7OR[c]	AZT, d4T
V75T[d]	d4T, ddl
L21OW[c]	AZT, d4T
T215Y/F[c]	AZT, d4T
K219Q/E[c]	AZT, d4T
Diminished excision	
L74V	ddl, ddC, ABC
K65R	All NRTIs, except AZT
Al14S	Foscarnet (PFA)
Y181I/C	NNRTIs
L1 00I	NNRTIs

TAMs are selected primarily by thymidine analogs, but confer resistance to other NRTIs as well

NRTI nucleoside reverse transcriptase inhibitor; *NNRTI* non-nucleoside reverse transcriptase inhibitor

[a]Complex with at least M41L, L210W or T215Y/F

[b]Complex with A62V, V75I, F77L and F116Y

[c]TAMs (thymidine-associated mutations); usually two or more mutations are necessary to confer resistance

[d]Excision is only determined with PPi and not with ATP as phosphate donor

5 Discrimination

The simplest way to explain HIV nucleoside drug resistance is a mechanism by which RT is able to exclude the NRTI, but is still able to recognize the analogous dNTP. This was indeed the first mechanism of nucleoside-analog resistance that was discovered and is called discrimination or exclusion (Fig. 2).

The efficiency of incorporation of the NRTI into the growing DNA chain compared to its natural substrate, the analogous dNTP, is described by k_{pol}/K_d(dNTP). k_{pol} is the catalytic rate constant for the formation of the phosphodiester bond with the incorporated dNTP. K_d(dNTP) is the dissociation or binding constant of the nucleotide for RT. Discrimination of the nucleoside analog relative to the dNTP can be explained by an increase in K_d(dNTP) or a decease in k_{pol}. In other words, discrimination can be the result of respectively impaired binding of the nucleoside analog compared to the dNTP or an impaired incorporation of the nucleoside analog. Discrimination can also be a result of a combination of both decreased binding and incorporation (6, 32).

On a genetic level, this mechanism of resistance is caused by the amino acid substitutions as M184V/I, L74V, V75T, K65R and Q151M (Table 1). All these changes are located in or close to the substrate (dNTP) binding site, which make it plausible that these mutations can affect the initial binding and/or positioning of the NRTI (5).

The main difference between a nucleoside analog and its corresponding nucleotide is the absence of a 3′-OH group. At first sight, it may seem difficult to imagine that RT is able to discriminate in favor of the structurally more complex dNTP. Several examples will be used to demonstrate the influence of the described amino acid substitutions on the binding and/or incorporation efficiency of the NRTI (12).

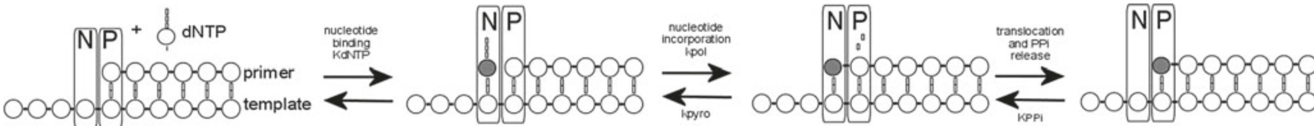

Fig. 2 Discrimination k_{pol} is the rate constant for creation of a phosphodiester bond, k_d is the dissociation constant. Discrimination can be explained by an increase in k_d (dNTP) or a decrease in k_{pol} (dNTP); impaired binding or subsequent positioning of the nucleoside analog (ddNTP)

5.1 The M184V/I

The discrimination mechanism is best described for the M184V/I mutation, which is located in the YMDD motif. This motif is highly conserved among all retroviral RT enzymes and contains two of the three catalytic aspartates of the DNA polymerase active site (33, 34). The initial change at this position during 3TC treatment is usually the isoleucine, which is rapidly replaced by the 184 valine (35–37). This mutation can emerge very soon and confers a more than 1,000-fold resistance for 3TC. It was already demonstrated that wild-type RT does not incorporate 3TC-TP very efficiently, but incorporation of the lamivudine triphosphate is decreased by the M184V change on a structural level (38, 39). Experiments using the M184V RT mutant demonstrated a 146- and 117-fold decrease in incorporation efficiency for respectively the DNA- and RNA-dependent DNA polymerization for 3TC-MP as compared with the wild-type HIV-1 RT (40). Discrimination of 3TC-TP by the M184V RT is probably caused by an effect on both k_{pol} and K_d (dNTP). The discrimination of 3TC-TP can be explained by the steric hindrance on a structural level. First, isoleucine and valine have a β-branched side chain compared to the wild-type methionine at position 184 (41). Second, 3TC-TP contains an oxathiolane ring, which replaces the deoxyribose present in normal dNTPs. This results in a sulphur group in the sugar ring, which is not present in the analogous substrate dCTP. Third, instead of the natural D configuration of the normal dNTPs, the L or opposite enantiomer of 3TC is used in the clinic to treat HIV-1 infection (42, 43). Considering these together, the extra side chain on the amino acid 184 makes an inappropriate contact with the sulphur group of 3TC-TP and prevents proper positioning of 3TC-TP, which is necessary for the formation of the phosphodiester bond. In conclusion, the M184V results in weaker binding and in a configuration that prevents proper incorporation of 3TC-TP (44).

5.2 K65R

The K65R amino acid substitution is located in the β3–β4 loop of the fingers domain of p66 from RT (45–47). First it was reported that the K65R mutation alters the binding affinity (48). However, the selectivity for the presence or absence of the hydroxyl group could not be explained by an effect on K_d(dNTP). Further experiments demonstrated that the presence of an intramolecular hydrogen bond between the 3′-OH group and an oxygen atom of the β-phosphate on the incoming nucleotide is critical for efficient catalysis. As has been described before, ddNTPs lack a 3′-hydroxyl group and as a result this intramolecular bond will not be formed when a nucleoside analog is incorporated. When K65R is present and the bond is absent, no correct alignment can occur, which is required for correct incorporation and subsequent catalysis. Thus, along with the effect on binding affinity, the K65R confers a decrease in k_{pol} as well (6, 12, 49). But, the K65R mutation confers no resistance against the nucleoside analog AZT. This is the result of the different effects caused by this substitution. The K65R can increase discrimination, but decreases excision as well. This results in no net effect on AZT susceptibility for the K65R mutant (50).

5.3 Q151M

A somewhat comparable mechanism is caused by the Q151M mutation located in the fingers subdomain at the active site. By itself, the Q151M confers low-level resistance to several NRTIs, which is increased by the selection of four other mutations: A62V, V75I, F77L and F116Y (51, 52). Resistance is caused by the decrease in the rate constant of the phosphodiester bond formation (k_{pol}).The Q151M change disrupts a critical hydrogen-bond network, through which particularly the alignment of ddNTPs is not possible any more (6, 53, 54).

5.4 L74V

When the L74V in the fingers subdomain emerges, the susceptibility for ddI, ddC and ABC is decreased. This mutation alters the position of these incoming ddNTP relative to its natural counterpart and thereby disturbs the attack of the 3′-hydroxyl group of the incorporated dNTP on the α-phosphate of the incoming ddNTP (5, 12, 55).

5.5 V75T

The V75T mutation is located in the fingers subdomain of the 66 kDa domain of RT. This substitution requires a two base-pair change and confers resistance to d4T and ddI (56). It has been demonstrated that the V75T mutation is able to discriminate d4T-TP. Repair of the d4T-MP-terminated primer (excision) has also been reported, but this could only be demonstrated with PPi and not with ATP as the phosphate donor (6, 57, 58). As will be described in the next section, there is still some debate about the in vivo phosphate donor used in the excision mechanism. Nevertheless, it is interesting to note that both the resistance mechanisms for d4T with the V75T change were demonstrated in vitro (6).

6 Excision

The other class of mutations is primarily selected by the thymidine analogs AZT and d4T and these changes are therefore called the thymidine-analog-associated mutations, abbreviated as TAMs. These mutations are the M41L, D67N, K70R, L210W, T215Y/F and K219Q in RT (18, 29, 30) (Table 1). They are all clustered around the dNTP-binding pocket suggesting a direct influence on AZT/d4T binding (Fig. 3). However, several studies showed that these mutations conferred only a very small increase in AZT discrimination, which could not explain the high levels of phenotypic resistance. Krebs et al. showed hardly any changes in the rates of TMP and AZT-MP incorporation (39). In 1992, it was found that AZT resistance was not associated with the TAMs when purified virions were used in endogenous RT assays (59, 60). This raised the question whether cellular factors were involved or that another viral resistance mechanism was responsible to explain the decreases in AZT sensitivity conferred by the TAMs.

First, it was found that AZT-resistant RT was able to bind AZT-terminated primers more tightly when compared to wild-type RT enzymes by Canard and colleagues (61). Subsequently, it was proposed that chain termination does not irreversibly block reverse transcription. Several researchers found that TAMs cause an increase in a pyrophosphorolysis-like mechanism (38, 62–64). Pyrophosphorolysis is the reverse of the polymerization reaction. The RT-primer/template complex binds pyrophosphate (PPi), which attacks the monophosphate group linking the two last nucleotides of the primer strand and regenerates a dNTP and a primer shortened by one nucleotide. In the situation that a nucleoside analog is incorporated in the DNA chain and prevents further DNA synthesis, pyrophosphorolysis means that the 3′-OH group on the primer will be free again when the nucleoside analog is excised. Forward direction DNA synthesis can then resume again (38). AZT-resistant RT was able to unblock the AZT-terminated primer using ATP as the PPi donor in contrast to wt RT (62, 65). This reaction, which is analogous to pyrophosphorolysis is named ATP-pyrophosphorolysis and is conferred by the TAMs. Initially, this reaction was discovered using DNA templates, but although less efficiently, it works on RNA templates as well (66, 67).

The difference between using ATP or PPi as donor is the generation of either AZTppppA or AZT-TP. The latter product can be incorporated directly into the viral DNA, while AZTppppA cannot be re-incorporated. Another difference is that the ATP-dependent pyrophosphorolysis is very inefficient with wild-type RT unlike pyrophosphorolysis. However, it is still not completely clear what the in vivo phosphate donor is, although several reports have found that the nucleoside excision reaction is ATP-dependent (12, 68–70).

ATP-dependent primer unblocking can be inhibited by the dNTP complementary to the next position on the template. When the primer is blocked with an NRTI missing the necessary 3′-OH group for the formation of a phosphodiester bond, this dNTP cannot be incorporated. The dNTP binding stabilizes the complex by decreasing the RT dissociation rate, thus trapping RT and the primer/template in a complex called a dead-end complex (DEC) (71, 72). Additionally, the binding of the dNTP causes the fingers to close making the complex more stable. There is relatively little excision of normal dNTPs that are incorporated by RT. The stability of the DEC depends on the chain terminator, the nature of the primer/template and the enzyme. DEC formation inhibits excision or unblocking reaction, which is beneficial for the action of the drug.

Boyer et al. have used all available data to develop a model explaining the ATP-dependent NRTI excision and the inhibition by the next complementary dNTP (Fig. 4). Crystallography studies have revealed two positions in the RT polymerase site where the 3′ end of the primer can be located. These sites are referred to as the P(priming)-site and the N(nucleotide binding)-site. The N-site is the site where the next complementary dNTP or NRTI can bind when the 3′ end of the primer remains in the P-site. As soon as this nucleotide has been incorporated, the (new) 3′ end of the extended primer translocates from the N-site to the P-site, probably after the PPi release. Excision of an NRTI by ATP-dependent phosphorolysis can only be carried out when the 3′ primer end is still located in the N-site. When translocation to the P-site has already occurred, the next

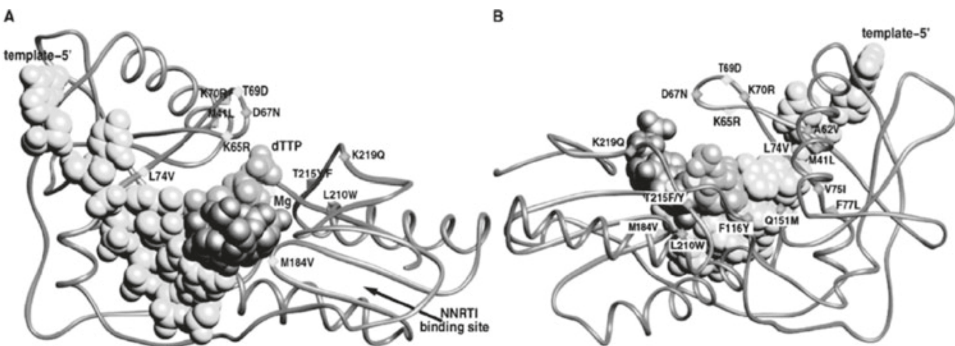

Fig. 3 Location of mutations associated with nucleoside analog resistance (from 5)

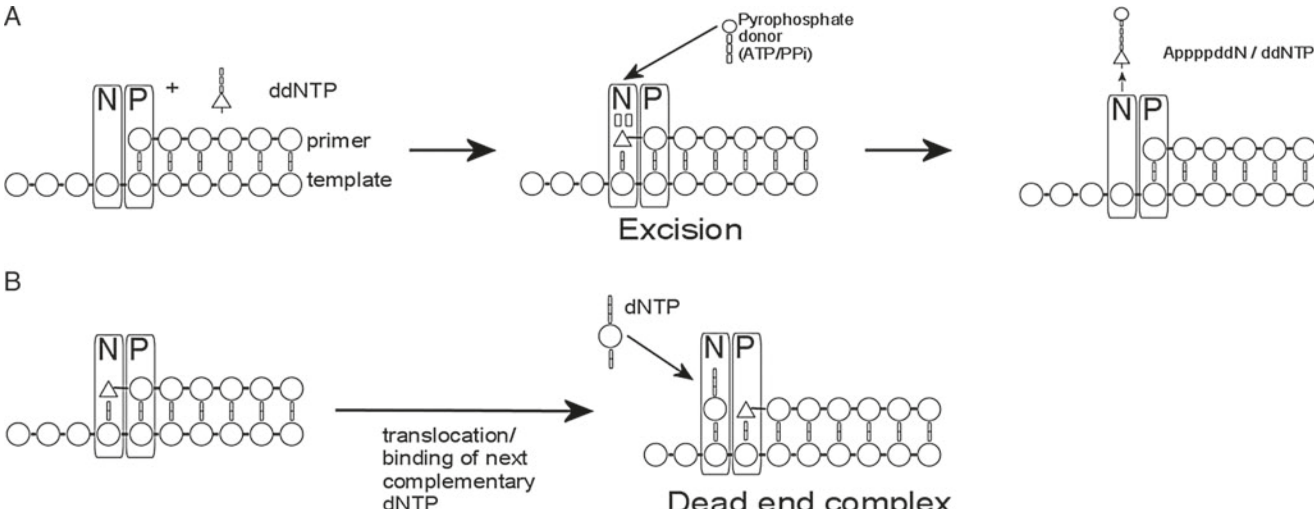

Fig. 4 Excision and dead-end complex (DEC) formation. Two positions at the polymerase site can be located; the N(nucleotide)-binding site and the P(priming)-site. (**a**) Excision of an NRTI can be carried out by a phosphate donor (ATP/PPi) when the 3′ end primer is located at the N-site. (**b**) After translocation to the P-site, the next complementary dNTP can enter the N-site and form a dead-end complex

complementary dNTP can enter the N-site and form a DEC. No excision reaction can be performed anymore because the NRTI is blocked at the P-site (69, 71, 73).

Any modification of the NRTI that increases the primer fraction in the N-site increases the excision mechanism. This will probably explain why the excision reaction is relatively specific for AZT. The nature of the AZT drug is unique because its 3′-OH group is replaced by a bulky azido group (NH_3, Fig. 1). AZT interferes with the formation of DEC, which can be explained by this large azido group of AZT (71). This group interferes with the ability to occupy the P-site and/or the ability of the next correct nucleotide to enter the N-site. This means that the AZT-terminated primer has good access to the N-site and subsequent excision. The other NRTIs do not have such a bulky 3′ group and consequently no good access to the N site. For all other ddNTPs than AZT, the overall equilibrium will shift towards DEC and away from the excision reaction. Removal of AZT is not inhibited at physiological dNTP concentrations causing DEC formation, while d4T-removal is inhibited at cellular concentrations of the complementary dNTP at the next position of the template. This might explain why TAMs confer a higher resistance to AZT than to d4T (74).

The main roles of the TAMs are to increase the affinity for ATP, the phosphate donor, or to optimize the alignment of the substrates for the ATP-pyrophosphorolysis and thereby increasing the rate of the unblocking reaction. Decreased sensitivity to the next correct nucleotide that can form a DEC is a third mechanism that has been demonstrated to increase resistance of RT mutants (2, 38, 75).

The 215/219 changes are located in the palm domain, while 67N/70R are located in the fingers domain. The 215/219 changes have been shown to increase the processivity of DNA synthesis by decreased template/primer dissociation from RT. They also reduced the inhibition of phosphorolysis by preventing the formation of a DEC.

The 67/70 mutations confer no increase in processivity, but increase the primer unblocking reactions. By the change of an aspartic acid into asparagine at position 67 (D67N), a negative charge is removed, while the lysine to arginine change at codon 70 (K70R) repositions the positive charge. The K70R, which is often the first mutation that appears under AZT monotherapy, confers a better interaction with the phosphate donor ATP or PPi,. Although several reports discussed that the 215 and 219 mutation did not have an effect on excision immediately, several researchers found that the T215Y/F mutations enhances the binding of ATP and PPi as well (76, 77). There is a large interplay between all the TAMs with the net result of the synthesis of full-length viral DNA in the presence of AZT.

7 Cross-Resistance and Synergy

Currently, many researchers are looking for new nucleoside analogs that diminish either the discrimination or excision reactions and therefore decrease the level of resistance.

As described before, specific mutations in HIV-1 RT are responsible for either the discrimination or excision mechanism. However, it has been shown that some changes confer nucleoside analog resistance via both the mechanisms. An example is the described mixed mechanism involving both the nucleotide selectivity (discrimination) and pyrophosphate-mediated, not ATP-, primer unblocking for d4T with the V75T mutation (6, 57, 58).

As described previously, single mutations in RT can confer resistance to multiple NRTIs. All nucleoside analogs bind to the substrate-binding site and as a result all the known resistance-associated mutations are located in or near this site. Therefore, it is not surprising to find that cross-resistance is a problem among HIV nucleoside analogs. Examples are

the two pathways conferring multi-nucleoside resistance (MNR). MNR can be the result of the Q151M complex or an insertion in the fingers domain of RT. Just like the accumulation of multiple TAMs, these pathways confer cross-resistance to many NRTIs (76, 78–80).

The Q151M complex starts with a glutamine to methionine change at codon 151 and increases resistance by the addition of A62V, V75I, F77L or F116Y which causes cross-resistance to AZT, ddC, ddI and d4T. For all these NRTIs, the mechanism of resistance is improved discrimination resulting from a decrease in the polymerization rate (k_{pol}). No effect on excision was demonstrated with the Q151M change, although it confers resistance against AZT (53, 54).

Insertions in the β3–β4 loop of the fingers domain can occur between S68 and T69 or T69 and K70. They are mainly polar dipeptides and are usually selected in the background of particular TAMs, especially M41L and T215Y/F. This insertion complex confers high-level resistance to AZT and mediate to high-level resistance to all the approved NRTIs (81–84). First, it was demonstrated that insertions in this loop increase its flexibility and confer a closer contact with the N-site. The subsequent more intense contact of the RT loop with the entering NRTI and primer/template affect the binding affinity and promote nucleoside analog discrimination (82, 85–88). Further experiments showed that accompanying TAMs are necessary for the resistance profile, since the insertion alone does not confer high-level MDR (82, 87, 89, 90). The tyrosine at position 215 plays a very important role by promoting the binding of the ATP as phosphate donor for the unblocking reaction. Another explanation is a less-stable DEC formation by the enhanced flexibility of the loop. Probably, the high-level multi-nucleoside drug resistance conferred by insertions in RT near codon 69 is caused by a combination of both discrimination and excision (76, 77, 85, 86).

The fact that the nucleoside resistance can be accomplished via two distinct mechanisms can be an advantage as well. Several studies have shown increased susceptibility for several anti-HIV drugs to AZT-resistant viruses.

First, it was described that AZT-resistant virus conferred no cross-resistance towards 3TC and the other way around the M184V change did not confer resistance against AZT (38). Instead, several reports demonstrated that the M184V increased susceptibility to AZT and other NNRTI in wild-type virus. Moreover, the M184V change resensitizes TAM-harbouring viruses to AZT by inhibiting the excision of AZT (69, 70, 91, 92). The precise mechanism is not completely clear, but valine alters the polymerase-active site either by repositioning the primer/template complex and move it from the ATP binding site or by increasing the fraction of AZT-terminated primer in the P-site by relaxing steric constraints (20, 69).

The L74V mutation conferring resistance to didanosine (ddI), another nucleoside analog, is able to resensitize the mutated virus to AZT as well (93). Two recent reports have demonstrated that this amino acid substitution is able to reduce the ATP-dependent removal of AZT-MP, although the precise mechanism is not completely clear yet (94, 95).

Another example has been given by foscarnet (PFA). PFA is a PPi analog and inhibits HIV-1 RT by competing with PPi for the PPi binding site, only when the primer is in the N-site. Several PFA-specific mutations are selected during the PFA treatment of cytomegalovirus infections in HIV-infected patients, but the K65R as well. As described before, the K65R mutation confers resistance via the discrimination pathway to all approved NRTIs, but confers no resistance against AZT (47). By affecting the interaction with PPi, ATP and PFA, this mutation not only creates a PFA-resistant RT but inhibits the unblocking reaction as well. Thus, the K65R mutation restores AZT sensitivity (96–98). The same is true for the A114S substitution, which confers resistance against PFA. This change diminishes the enhanced pyrophosphorolysis activity and processivity associated with AZT-resistant RT (75).

Besides these NRTIs and PFA, several non-nucleoside RT inhibitors, the second class of RT inhibitors, have shown high-level synergy with AZT in inhibiting the emergence of AZT-resistant viruses. These NNRTIs, such as nevirapine, TIBO and the thiocarboxanilide NNRTI UC781, inhibit excision and resensitize AZT-resistant polymerase to AZT-TP. Several mutations which are selected by NNRTIs, such as Y181I/C, L100I, have been shown to suppress AZT resistance by respectively decreasing the binding efficiency of the pyrophosphate donor ATP or increasing the discrimination (20, 99–103).

8 Anti-HIV Drugs that Overcome Resistance

Currently, many researchers are investigating new nucleoside analogs that diminish either the discrimination or excision reactions. To prevent discrimination, this new generation of nucleoside analogs should have a higher K_d (dNTP) by binding more tightly to RT and they should have a higher incorporation rate. An example is the dioxolane nucleosides, which have a decreased volume dioxolane pseudoribose ring instead of thioxolane or acyclic ring. The dioxolane moiety has been demonstrated to stabilize the binding between the mutant RT and the nucleoside triphosphate (104).

Another class of modified inhibitors is the alpha-boranophosphate analogues, which harbor a borano (BH_3^-) group on the alpha-phosphate of the NRTI. These BH3-dNTPs do not influence binding to the active site, but enhance k_{pol} and diminish discrimination (49, 53, 105). This latter class has also been shown to be excised less efficiently (105).

More research is being performed on the development of NRTIs inhibiting the excision mechanism. Several approaches have been made, such as interfering with the productive binding of ATP, the phosphate donor that is necessary for the unblocking reaction. New compounds should bind in or near the ATP-binding site on the RT enzyme. A problem, however,

is the fact that the ATP-binding site has no obvious role in the reverse transcription process and resistance to these new drugs will probably occur very rapidly (106). A biphosphonate inhibitor (BPH-218) has been identified which might compete with ATP/PPi for binding to RT (107).

Rigourd et al. have proposed another approach. They hypothesized that the unblocking resistance mechanism has only been demonstrated for DNA-dependent DNA elongation. Resistant RT was not able to unblock the AZT-terminated primer during initiation. Therefore, inhibitors should be designed which specifically target the initiation of reverse transcription (67).

Another approach is the use of analogs of dinucleoside tetraphosphate, the product of the excision reaction. They act as substrate for the DNA polymerization and were able to incorporate in TAM-bearing RT better than the wild-type RT (108).

Boyer has conducted a study using the so-called “delayed chain terminators”. These analogs have a 3′-OH moiety on a pseudosugar group locked in the north conformation. This allows extension of the primer with two or three additional nucleotides before chain termination and makes them relatively resistant to excision (109).

However, all these new nucleoside analogs are still far away from the clinic and other mechanisms and mutations conferring resistance towards nucleoside analogs may exist. Pathak and coworkers have shown that specific mutations in the C-terminal part (RNAse H domain) of RT enhance resistance against AZT and d4T alone or in combination with TAMs. These mutations reduce the rate of RNA degradation and thereby increase the time of excision (110, 111). Furthermore, novel mutations have been identified that confer an increase in NRTI resistance, such as the K70E that decreases TNV-TP, Carbovir-TP and 3TC-TP incorporation, whereas this mutation also impairs AZT-MP excision (112, 113).

In conclusion, in the last few years new insights have been generated unraveling the mechanisms of HIV-1 nucleoside resistance. Now, it is clear that (at least) two distinct mechanisms are responsible for the development of resistance against this class of RT inhibitors; discrimination and excision. This knowledge about the molecular mechanisms, but also about cross-resistance and synergy of RTIs, can help us to develop new strategies of treatment regimens. Besides enabling us to have more insight into specific drug combinations that should or should not be administered to the patients, this knowledge may lead to the development of promising anti-HIV compounds that seem to overcome the classical resistance.

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Chapter 33
Resistance to HIV Non-Nucleoside Reverse Transcriptase Inhibitors

Robert Elston and Pierre R. Bonneau

1 Introduction: HIV Reverse Transcriptase

The human immunodeficiency virus (HIV) is a member of the Retroviridae family (lentivirus genus), characterized by the presence of a virally encoded reverse transcriptase (RT) enzyme (1). Upon cell entry, the RT enzyme is responsible for the conversion of the single-stranded viral genomic RNA into a complementary double-stranded DNA copy (cDNA). The RT enzyme possesses both RNA-dependent and DNA-dependent DNA polymerase activities as well as RNase H activity, which is responsible for the degradation of the genomic RNA and tRNA primer (2, 3). The process of cDNA synthesis involves a complex of the RT enzyme, the template (either genomic RNA or single-stranded DNA), the tRNA primer and deoxynucleotides (dNTPs).

Crystal structures of HIV-1 RT either unliganded (4–6) or in complex with double-stranded DNA (7–10) have allowed for the three-dimensional description of the enzyme. RT is a heterodimer composed of the p66 and p51 subunits. The p51 subunit is generated through proteolytic cleavage of p66 by HIV protease and is composed of the first 440 residues of p66 (the remaining 120 residues of p66 forming the C-terminal RNase H domain of the enzyme). The dimerization interface between the subunits is quite extensive, involving several conserved areas (11). On the basis of the resemblance of the p66 subunit to a right hand, common regions within p66 and p51 have been identified and termed as the 'fingers', 'palm', 'thumb', and 'connection' subdomains. The folding of the individual subdomains is similar in p66 and p51, but their spatial arrangement differs significantly (Fig. 1). The p66 subunit has an open quaternary structure and contains the active sites for both the polymerase and RNase H activities while p51 adopts a more closed conformation and mainly plays a structural role inducing p66 to perform catalysis. RT is highly flexible and undergoes conformational changes during the course of the enzymatic process. In the absence of primer-template substrate, the p66 'thumb' domain is folded down and closes the DNA-binding cleft. Upon primer-template binding, the 'thumb' folds back in an upright position, opening up an area delineated by amino acids from the p66 'fingers', 'palm' and 'thumb' subdomains that precisely place the substrate for catalysis. These regions of p66 responsible for the correct placement of the substrate are commonly referred to as the 'primer' and 'template' grips. In this arrangement, the primer 3′-OH terminus lies near the three catalytic residues (D110, D185, D186) of the polymerase-active site, and is poised for attack on the incoming nucleotide while the rest of the duplex extends on the surface toward the RNase H domain.

2 The Non-Nucleoside RT Inhibitors

The HIV RT enzyme offered an opportunity to selectively inhibit an essential viral target prior to the irreversible integration of viral cDNA into the human chromosome. The first approved antiretroviral therapies (ART) were the nucleoside RT inhibitors (NRTIs). NRTIs, after intracellular conversion to their corresponding triphosphates, resemble the natural dNTPs, but lack a 3′-hydroxyl (OH) group and therefore result in premature chain termination of the cDNA after incorporation into the primer strand (12). NNRTIs represent a second class of RT inhibitors, which bind to the enzyme close to the active site causing disruption of reverse transcription. In contrast to NRTIs, NNRTIs are non-competitive inhibitors and do not require intracellular phosphorylation to be active (13).

Three NNRTIs are currently approved for use by the FDA therapy: nevirapine (NVP, Viramune® approved in 1996), delavirdine (DLV, Rescriptor® approved in 1997) and efavirenz (EFV, Sustiva™ approved in 1998) (Fig. 2). The Department of Health and Human Services (DHHS) guidelines for the use of antiretroviral agents in adults recommends NNRTI-based therapy for first-line use (14). EFV (600 mg, once daily) is the preferred NNRTI, and NVP (200 mg, twice

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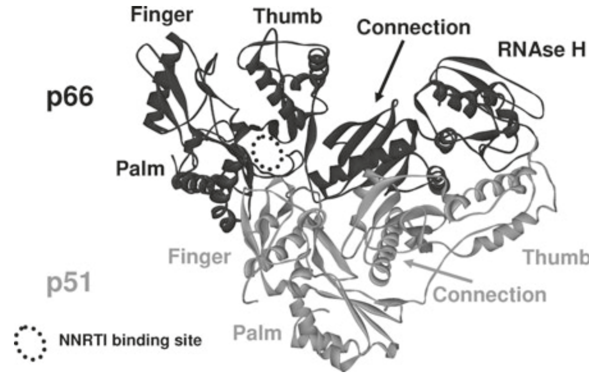

Fig. 1 Three-dimensional structure of HIV-1 reverse transcriptase

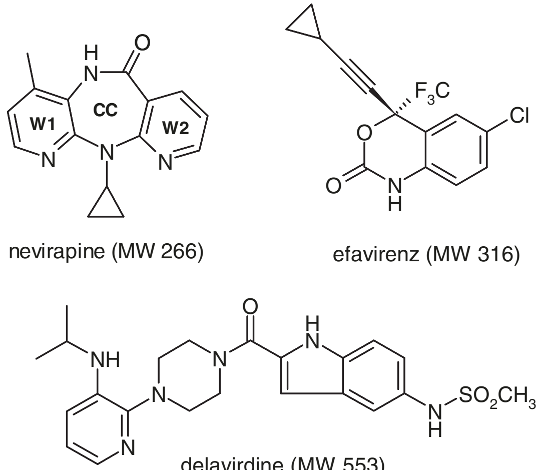

Fig. 2 Chemical structures of the three clinically approved NNRTIs (W1, W2, and CC respectively denote wing I, wing II, and central core of nevirapine)

daily) is listed as the alternative from this class, whereas DLV (400 mg trice daily) is not recommended because of inferior potency and dosing schedule (14). Clinical trials with NNRTIs in combination with NRTIs have demonstrated superior efficacy over triple-nucleoside regimens (15, 16) and unboosted PI-based regimens (15, 17, 18), and superior or equivalent efficacy to boosted PI-based regimens (18).

Although NNRTIs form a broad and chemically diverse class of inhibitors (19, 20), the available data on crystal structures have collectively shown that NNRTIs bind within the same area of the enzyme, in the so-called non-nucleoside inhibitor-binding pocket (NNIBP). The NNIBP is a flexible, allosteric site located in the palm subdomain of p66 (at the junction with the thumb subdomain) about 10Å away from the polymerase-active site itself and not overlapping with the DNA-binding site (consistent with the non-competitive mode of inhibition, generally characterizing NNRTIs).

Interestingly, the NNIBP is not present in the unliganded form of RT; the space to be occupied by the NNRTI is instead filled by a well-packed hydrophobic core mainly formed by residues L100, Y181, Y188, F227 and W229. The NNIBP becomes apparent only upon NNRTI binding through rotational movements of key amino acids delineating the site (mainly Y188 and Y181) as well as by relocalization of the short three-strand β-sheet containing F227 and W229 (4, 5). Two surface depressions in the unliganded structures of RT have been identified as possible entrances to the NNIBP (5). One entrance is located near K101, K103, V179, and E138 (the latter residue in p51). The second entrance is located between a connecting loop including K101 to K103 and a β-hairpin from P236 to K238. While the first entrance remains visible in NNRTI complexes, the second entrance is either closed following complexation of RT with small NNRTIs (e.g. nevirapine and efavirenz) or partially visible following complexation with larger NNRTIs (e.g. delavirdine) (21, 22).

Stabilization of the NNIBP by NNRTIs is believed to cause inhibition by either altering the precise geometry of the polymerase catalytic machinery and its bound substrates, or by preventing the movement of the p66 thumb subdomain necessary for translocation along the nucleic acid, or both (23–25). Overall, these effects combine to drastically slow down the rate of the chemical step for DNA synthesis (26, 27). In doing so, NNRTI binding may trap the enzyme in a conformation that is only transient in the polymerization process; this mechanism would be consistent with the fact that NNRTIs do not interfere with binding of the primer-template or the incoming nucleotide substrates. More specific mechanisms have also been advanced for NVP and EFV. In the case of NVP, additional contribution to the overall inhibition may result from a reduction of RT processivity (pausing) and in the alteration of the RNase H cleavage specificity (28). In the case of EFV, binding to the p66 subunit was observed to considerably tighten both the p66/p66 homodimer and p66/p51 heretodimer, potentially deregulating *gag-pol* precursor processing and interfering with late-stage viral replication (29, 30).

NVP was the first clinically available NNRTI to be crystallized with wild-type RT (24, 25, 31). The 'butterfly'-like shape of bound NVP is a convenient analogy to describe the mode of binding (Fig. 3). Analysis of the structure revealed that Wing I of NVP makes important hydrophobic contacts (π-interactions) with the side chains of Y181, Y188, and W229. Residue V106 makes contact with both the N-cyclopropyl group and the carbonyl oxygen of the 'butterfly' central core of NVP while Wing II makes weaker contacts with Y318. Residue L100 is located above the inhibitor and makes interactions with both wings.

Crystallographic binding details of EFV and DLV have also been reported (21, 32, 33). While both drugs bind in the same pocket as NVP and use comparable contacts, they also present notable differences. Thus, in the case of EFV, the smaller propynylcyclopropyl group leads to more limited

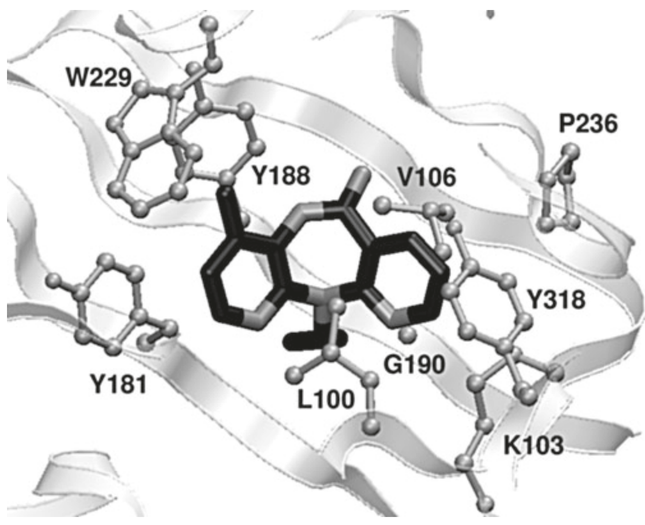

Fig. 3 Binding mode of nevirapine within the NNIBP of reverse transcriptase

interactions with Y181 and Y188 that are compensated for by the NH of EFV making a favourable hydrogen bond with the main-chain carbonyl oxygen of K101 (32). In the case of DLV, the larger and more flexible nature of the molecule causes it to explore regions beyond the original NNIBP and to project into the solvent. The pyridine ring makes the usual hydrophobic contacts with Y181 and Y188, while the indole ring makes extensive interactions with the cyclic side chain of P236 which remains in an 'open' conformation similar to that of the unliganded RT (21).

3 Mechanism of NNRTI Drug Resistance

Much of our initial knowledge relating to the identification of NNRTI-associated resistance mutations has come from in vitro and early Phase II clinical studies which often included exposure to NNRTI monotherapy. In the case of NVP, this knowledge has been supplemented by the genotypic analysis of virus from women receiving 'single dose' (sd) NVP for the prevention of mother-to-child transmission (MTCT). More recently, the resistance profile of virus from patients failing NNRTI-based HAART regimens has been described (34–38).

From these studies, it is apparent that the development of NNRTI resistance follows some general principles. NNRTIs have a low genetic barrier for the development of resistance. Single amino acid substitutions can cause large reductions in susceptibility, and these mutations are likely to pre-exist as minority quasi-species prior to NNRTI therapy (39). Many of these mutations also confer cross-resistance to the other NNRTIs. Viral replication during NNRTI therapy leads to the rapid amplification of these resistant quasi-species. Continuation of failing therapy results in the emergence of combinations of mutations that confer increased resistance and/or improved replication capacity (34–38, 40, 41).

A feature of NNRTIs believed to contribute to the development of resistance is their long plasma half-life. EFV and NVP have plasma half-lives of 40–55 h and 25–30 h, respectively, following multiple dosing, exceeding that observed with NRTIs and PIs (42, 43). Polymorphisms within the Cyp2B6 gene have been associated with extended EFV half-life (44). Following discontinuation of an NNRTI based HAART regimen, the slower decay of the NNRTI compared to NRTIs may result in functional monotherapy and increased risk of the development of resistance (45, 46). More recently, approved NRTIs (TDF and FTC), which have longer intracellular half-lives compared to older NRTIs, have been proposed to reduce the incidence of NNRTI resistance following virological failure (34)

4 Mutations Associated with Resistance to NNRTIs

4.1 Nevirapine Resistance

In vitro passage experiments demonstrated that the development of resistance to nevirapine was predominantly associated with the K103N, V106A and Y181C mutations (47). In 24 patients who were treated with NVP monotherapy, the Y181C mutation was most commonly observed (79%) and to a lesser extent K103N (33%), G190A (17%), V108I (8%) and Y188L (8%), but not V106A (48). The Y181C mutation conferred high levels of resistance to NVP (100-fold), and cross-resistance to DLV, but not to EFV (1.7-fold) (49, 50).

The incidence of the Y181C mutation is lower in patients failing NVP-based dual or triple ART regimens that include a thymidine analogue. Thus, in the presence of a thymidine-like NRTI mutations at residues K103N, Y188L/H, G190A/H/S and to a lesser extent at V106A were more commonly observed (48, 51). An explanation for the decreased incidence of the Y181C mutation in this population stems from the fact that introduction of the Y181C mutation into an AZT resistant virus significantly suppressed resistance to AZT (52). Interestingly, the capacity of AZT to suppress the development of the Y181C mutation is diminished if patients have already developed resistance to AZT. The most prevalent NNRTI mutations observed in thirty patients failing a NVP/AZT/DDI regimen after prior AZT failure was the Y181C (50%) and G190A (50%) mutations; with mutations at other positions (A98G, L101E, K103N, K103R, V106A, V108I, and Y188L) developing to a lesser extent (36).

In women and infants, who received sdNVP in the absence of other ART for the prevention of MTCT, recognized NVP resistance mutations were detected (predominantly K103N, Y181C and G190A); however, the incidence of specific mutations differed (53–55). The K103N mutation predominated in mothers (K103N 89% vs. Y181C 33%) while the Y181C mutation predominated in infected infants (K103N 18% vs. Y181C 82%) (55). The presence of the more resistant, but less 'fit' Y181C mutation was proposed to be related to higher NVP exposure in the infants (55). Interestingly, there was a trend toward greater incidence of Y181C mutation in mothers 1 week after prophylaxis, whereas the K103N mutation predominated 6–8 weeks later (56, 57).

4.2 *Efavirenz Resistance*

The resistance profile of EFV has been studied through in vitro passage experiments by a number of groups using different conditions (58–62). In the majority of studies, the K103N/L100I double mutant was selected with additional mutations at positions A98G, V108I, and V179D (58, 61). The single K103N and L100I mutations both confer reduced susceptibility to EFV (36-fold and 24-fold respectively), however the combination of L100I/K103N confers a significantly greater reduction in susceptibility (2,500-fold) (49). In other passage experiments, selection of the K103N was not detected; Winslow et al. (60) reported selection of the L100I/V108I mutations during passage in PBMCs, but L100I/V179D/Y181C during passage in MT2 cells and De Bethune et al. (59) reported selection of the G190E mutation.

The resistance mutations observed in NNRTI-naïve patients failing EFV in vivo largely mirrored those mutations observed to develop in vitro; however, differences in the incidence and combination of mutations was observed. Virus from patients failing EFV in the majority of cases (>80%) developed the K103N mutation. The K103N/L100I combination observed in the majority of in vitro passage studies was only observed in approximately 10–30% of virological failures (37, 49, 51). Interestingly, the L100I mutation was never detected in the clinic in the absence of the K103N mutation (49). Other NNRTI mutations (V106I, V108I, Y188C, Y188H, P225H, F227L) that were selected did not confer significant levels of resistance to EFV in the absence of other NNRTI mutations (49).

EFV resistance in the absence of the K103N mutation was observed at low frequency and often involved the selection of the Y188L or G190E/S mutation. The Y188L mutation, which was not detected in vitro, was observed to develop in vivo either alone or with other NNRTI mutations and conferred high-level resistance to EFV and other NNRTIs. Interestingly, the Y188L mutation requires a two-nucleotide substitution and this may explain the low prevalence in the general patient population (63).

4.3 *Delavirdine Resistance*

In vitro passage experiments with DLV resulted in the selection of the P236L mutation (50). This mutation conferred high-level resistance to DLV, but conferred no resistance or even increased susceptibility to EFV and NVP (49, 50). Clinical studies involving DLV monotherapy, however, revealed that resistance rarely developed through the P236L mutation in vivo, with the predominant mutations detected being the K103N (>80%) and Y181C mutations (10%) (64). The low prevalence of the P236L mutation is associated with a replicative defect (65). Similar to the selection of Y181C by NVP, selection of the Y181C mutation by DLV may depend upon the regimen. The K103N/Y181C mutations were frequently detected after failure of DDI + DLV therapy, whereas Y181C was not selected in two patients receiving DDI + DLV + AZT (38).

5 Cross-Resistance

In treatment-experienced patients with evidence of resistance, there is a widespread prevalence of NNRTI mutations (63). The most frequently observed NNRTI mutations present in 19,689 clinical isolates collected between 2004 and 2006 were: K103N 33%, Y181C 14%, V179I 11%, G190A 11% of isolates (63). Owing to considerable overlap between the resistance profiles of each NNRTI (Table 1) cross-resistance is commonly observed following virological failure. Although detailed investigation using laboratory viruses harbouring individual NNRTI mutations did identify a number of NNRTI specific mutations (Fig. 4), this analysis also demonstrated that combinations of two or more NNRTI mutations rarely retained susceptibility to any currently available NNRTI (49).

Table 1 Summary of the resistance mutations associated with resistance to NNRTIs in clinical use

NNRTI	NNRTI mutations associated with reduced susceptibility[a]
Efavirenz	L100I, K103N, V106M, V108I, Y181I/C, Y188L, G190A/S, P225H
Nevirapine	L100I, K103N, V106A/M, V108I, Y181I/C, Y188C/L/H, G190A
Delavirdine	K103N, V106M, Y181C, Y188L, P236L

[a]Based upon the IAS Guidelines Fall 2006 (155)

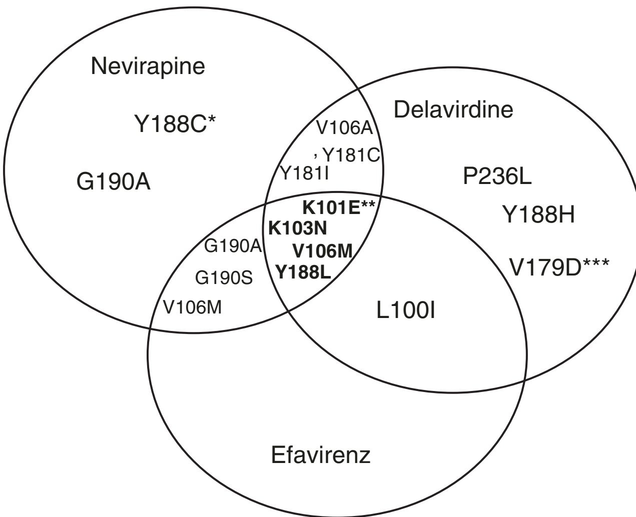

Fig. 4 Impact of individual NNRTI mutations on phenotypic Susceptibility to Nevirapine, Efavirenz and Delavirdine (based upon phenotypic susceptibility reported for clinical isolates harbouring the mutation of interest in the absence of other NNRTI and NRTI mutations (Stanford University HIV Drug Resistance Database, Accessed Sept 2007. http://hivdb.stanford.edu)). Mutations G98S, H101Q/R, K103R, V106I, V108I, P225H have minimal impact on NNRTI susceptibility when present alone. *Low-level reduced susceptibility to EFV, DLV; **low-level reduced susceptibility to NVP, EFV, DLV; ***low-level reduced susceptibility to DLV

Studies investigating the prevalence of NNRTI mutations have shown that a majority of the isolates harbouring NNRTI mutations harbour more than one mutation, and are therefore likely to be resistant to all currently available NNRTIs (66–68). In a large (4,000 patient) analysis of the most prevalent NNRTI resistance patterns collected between 1997 and 2003 in California (66) over half of the RT sequences harbouring an NNRTI-associated resistance mutation had two or more NNRTI mutations. The Y181C mutation, in the absence of other NNRTI mutations, was present in ~5% of isolates and represents the most prevalent NNRTI resistance mutation that retains susceptibility to a second NNRTI (EFV only). The Y181C/V108I dual combination, present in less than 1% of the sequences, retained susceptibility to EFV, while no dual NNRTI combinations retained susceptibility to NVP.

Several studies have addressed whether EFV would be effective in salvage therapy for patients failing NVP (67–71). Antinori et al. (67) investigated the virological responses to EFV of patients failing NVP in the presence or absence of NNRTI mutations that cause cross-resistance to EFV. In this study, twelve patients failing NVP harboured virus with single NNRTI mutations not associated with EFV resistance (6 Y181C/I, 3 G190A, 2 V106A, 1 V108I). Only two of the twelve (17%) patients reached undetectable viral load after 3 months of EFV treatment, compared to 52% (35/67) of patients treated with EFV after failing a PI-based regimen.

A similar outcome was reported by Warmsley et al. (68), where only 3/16 patients achieved virological response with an EFV-based salvage regimen following NVP failure. Two of the responding patients had no NNRTI mutations detected at baseline, and the third harboured the Y181C mutation and only had a transient virological response. No virological response was observed in patients with decreased susceptibility to EFV. A further study did observe differences in the initial virological response depending upon baseline genotype; however, this difference was not sustained after 3 months (69). The strongest predictor of virological failure was the presence of NNRTI resistance mutations at any time point and NNRTI history. Of note, resistance analyses in a further study reported that the G190A mutation was preferentially selected in patients harbouring the Y181C mutation at baseline and failing an EFV-based salvage therapy (70). Although this study did not establish whether these newly emerging mutations existed as minority quasi-species after failure of the initial regimen NVP, it does indicate that the prior NNRTI therapy can influence the resistance pathway observed with subsequent NNRTIs.

In contrast to the previous reports, Requena et al. (71) investigated the correlation between EFV trough concentrations and virological response following failure of NVP therapy. Forty-eight patients experiencing early virological failure (two confirmed samples > 50 copies/mL) on a NVP-based regimen, had EFV substituted for NVP while maintaining the same NRTI regimen. At weeks 12, 24, and 48, an undetectable viral load (<50 copies/mL) was observed in 25, 40 and 42% of patients respectively.

Although the presence of EFV-resistance associated mutations at baseline was not found to correlate with virological response, genotype could only be amplified from 35 patients presumably because of low viral load (median 4,313 (72–45,148 copies/mL)). Six of 35 patients had no baseline NNRTI mutations. In contrast, EFV trough concentrations were found to be associated with virological response. At week 12, 75% of patients with EFV levels exceeding 3 μg/mL (measured ~12 h post dose) had a virological response (<50 copies/mL or > 1 $\log_{10}$copies/mL reduction in viral load) despite the presence of EFV resistance mutations at baseline. In contrast, only 22% of patients had a virological response if EFV levels were below 3 μg/mL.

In summary, these studies demonstrate that cross-resistance between EFV and NVP has in the majority of cases resulted in poor efficacy when NNRTIs have been used sequentially. Although virological responses have been observed in a limited number of patients, this has been generally in patients with no or minimal NNRTI resistance mutations and most likely with good EFV exposure. This is consistent with the data from the more recent clinical trials evaluating the next generation NNRTIs against NNRTI resistant viruses or evaluating HAART following the use of

sdNVP for the prevention of MTCT (discussed later); better virological responses were reported in patients with historic evidence of NNRTI mutations, but absent when commencing the second NNRTI regimen or when a significant time delay has occurred between the first and second NNRTI regimen.

6 Effect of NNRTI Mutations on Enzyme Activity and Viral Replication

In contrast to many PI or NRTI mutations, most in vitro studies have demonstrated that the most prevalent NNRTI-associated resistance mutations, such as Y181C and K103N, have minimal consequences on viral replication (41, 72). This perception has been supported by clinical data demonstrating that NNRTI mutations can persist for several months after cessation of NNRTI therapy or after transmission to ART-naïve patient (73, 74).

Even though the K103N and Y181C mutations are not associated with large decreases in replicative capacity, evidence that they do impact viral replication comes from mothers who received sdNVP for the prevention of MTCT. Following detection of mutations at 6–7 days, the incidence of the Y181C mutation decreased, while the incidence of the K103N mutation increased by week 8 (56) apparently because of reduced NVP exposure selecting the less resistant but more ‘fit’ K103N mutation. Subsequently, detection of any NNRTI mutation decreased over time so that at 12–24 weeks after treatment more sensitive sequencing approaches were needed to detect resistant viruses (75).

Accumulating evidence from in vitro replication studies indicates that not all NNRTI mutations impact viral replication equally. NNRTI mutations such as the V106A and P236L mutations have been demonstrated to affect viral replication to a greater extent than the K103N and Y181C mutations and are indeed less frequently detected in the clinic (41, 72, 76, 77). It is noteworthy that certain combinations of NNRTI mutations may have restored replication capacity compared to virus harbouring certain single or double mutants (41). The V106A mutation when combined with the Y181C mutation had improved replication, and this may provide one explanation for the continued accumulation of NNRTI-associated mutations despite the presence of high-level resistance.

The consequence on viral replication of combinations of NRTI and NNRTI mutations remains largely uncharacterized. Kleim et al. (78) reported the selection of NRTI-associated mutations L74V, V75L, and K219E in combination with NNRTI mutations during the in vitro selection of resistance to the NNRTI HBY097. The L74V and V75I mutations were later shown to compensate for the reduced replicative capacity of the G190E NNRTI mutation (79). More recently the L74V mutation has been demonstrated to compensate for the reduced replicative capacity of the L100I/K103N double NNRTI mutant (80).

The mechanism underlying the reduced replication capacity associated with the V106A and P236L mutations has been identified (65, 76). Both the mutations resulted in normal DNA polymerization; however, both had reduced rates of DNA 3′-end- and RNA 5′-end-directed RNase H cleavage.

7 Natural Resistance to NNRTIs

NNRTI-associated resistance mutations, reported to develop in HIV-1 group M viruses, occur as natural polymorphisms in HIV-2 (V106I, V179I, Y181I, Y188L, and G190A) (81, 82) and some HIV-1 group O (A98G, V106I, K103R, V179E, Y181C) clinical isolates (83, 84). As expected, some of these polymorphisms conferred high levels of resistance to NNRTIs and have negatively impacted the utility of NNRTIs for patients infected with these viruses.

Two large studies have been reported on investigations of the phenotypic susceptibility of predominantly HIV-1 subtype B clinical isolates derived from treatment-naïve patients (85, 86). Both studies reported greater natural phenotypic variability in drug susceptibility to NNRTIs than that for NRTIs or PIs, with the greatest variability being observed with DLV and least with EFV. Univariate analyses identified twelve polymorphisms associated with reduced DLV susceptibility, namely K49R, A98S, K101Q, V108I, I135L/T, I142V/T, I178L, and V179D/E/I (86). Leigh Brown (87) identified the I135L and L283I polymorphisms as being significantly associated with decreased susceptibility to NVP. This observation was confirmed with recombinant virus; virus encoding the I135L/L283I mutations had 4- to 5-fold decreased susceptibility to the NNRTI class. Gao et al. (88) also identified the V245T polymorphism in addition to the I135L polymorphism, as responsible for significantly decreased susceptibility to NVP and DLV in a sub-type D isolate.

Although a higher rate of NNRTI resistance-associated polymorphisms have been reported in non-subtype B virus (including positions A98S, K101E, K103R, and I135L (89, 90)), several reports have indicated no significant decrease of susceptibility of non-subtype B isolates. The phenotypic variability observed in approximately 200 subtype C viruses (South African origin) revealed no greater variation in NNRTI susceptibility (85). Similarly, biochemical analysis using RT derived from recombinant form RCF01_AE and subtype C clinical isolates, reported no significant difference in susceptibility (91). The clinical significance of low-level reduced susceptibility to NNRTIs remains to be established.

8 NNRTIs Hypersusceptibility

Phenotypic hypersusceptibility to DLV, EFV, and NVP was detected in virus from 5, 9, and 11% of treatment-naïve patients, respectively (92). Surprisingly, the incidence of hypersusceptibility increased to 21–29% in isolates derived from NRTI treatment-experienced patients (92). Although the mechanism behind hypersusceptibility has not yet been established, it is proposed that it depends on the locality of the NNRTI pocket in relation to the active site and a complex interplay between NRTI resistance-associated mutations and natural polymorphisms (92, 93).

Two studies have attempted to correlate the genetic *loci* associated with increased NNRTI susceptibility. The first study identified a series of NRTI-associated mutations and natural polymorphisms associated with hypersusceptibility, as well as two positions negatively associated with NNRTI hypersusceptibility (92). The second study identified amino acids 215 (predominantly Y), 208 (predominantly Y) and 118 (predominantly I) as being important for EFV hypersusceptibility, with 85% of patients harbouring virus containing mutations at positions 215 and 208 being hypersusceptible to EFV (93). Although single mutations 118I, 208Y and 215Y alone did not confer hypersusceptibility when introduced individually into recombinant virus, different combinations of these mutations did result in hypersusceptibility (94).

The clinical significance of NNRTI hypersusceptibility has been demonstrated in several retrospective analyses of clinical trials where the observed viral load reductions were superior in patients having virus with increased NNRTI susceptibility at baseline (95, 96). Although this could lead to treatment strategies utilizing the increased NNRTI susceptibility, current guidelines on antiretroviral therapy mean that the number of patients presenting with NRTI-resistant, but NNRTI-naïve virus, will be low.

9 Prevention of Mother-to-Child Transmission

In the resource-poor setting where access to HAART is limited, single-dose nevirapine is widely used in the prevention of mother-to-child transmission (MTCT). Without antiretroviral therapy intervention, the risk of MTCT is estimated to be 15–30% in non-breast-fed infants, rising to 20–45% in breast-fed infants (97). The use of sdNVP, provided to the mother at the onset of labour and the infant within 72 h of birth, has been reported to reduce the risk of transmission by half (98, 99). Unfortunately, the use of sdNVP in the absence of other ART can result in the selection of NVP-associated resistance mutations (55, 100, 101, 102). Using standard sequencing methodologies in the HIV NET 012 Study, NNRTI resistance was detected in 19% (21 of 111) mothers and 46% (11 of 24) of infected infants, 6–8 weeks after NVP administration (55). The rate of NVP resistance was similar in mothers whose infants were or were not infected. The incidence of resistance detection increases further when more sensitive methodologies for detecting resistance mutations are employed (103).

The rapid selection of resistance mutations after sdNVP is associated with the slow clearance of NVP, since this results in sub-optimal levels of NVP persisting for extended periods (104). The plasma half-life after sdNVP is longer than that after multiple dosing (45 h vs. 25–30 h) and detectable NVP plasma concentrations have been reported 2 weeks after receiving sdNVP (43). This has prompted investigation of strategies involving the short-term use of NRTIs to suppress virus immediately after receipt of sdNVP. Initial reports suggest that these strategies can suppress the development of resistance (105).

A major concern is that the presence of archived NNRTI resistance mutations may (1) reduce the efficacy of NVP-based prevention strategies in future pregnancies and (2) reduce the efficacy of NNRTI-based HAART. Although preliminary data indicated that the efficacy of sdNVP to prevent MTCT was reduced in women with prior exposure to sdNVP (106), this observation has not been confirmed in more recent reports (107, 108). It is postulated that the absence of effect is due to resistance levels waning between exposure to sdNVP, with transmission rates being higher in women whose interdelivery time was more than 12 months (108).

Jourdain et al. reported that even in the absence of detectable NNRTI mutations, mothers exposed to NVP prophylaxis had reduced response to NVP-based HAART regimen (53). More recent reports, however, indicate that response to NNRTI-based HAART following sdNVP may differ depending upon the time lapsed prior to initiation of HAART (109, 110).

10 The Influence of Subtype on Resistance Mutation Pathway

The NNRTI-associated resistance mutations reported to be selected in non-subtype B virus were similar to those reported for subtype B virus, however, the relative incidence of mutations may differ particularly at sites that are polymorphic, for example, A98S, K101E, V106I, and V179I (111–113). One well-characterized exception is the V106M mutation that rarely develops in subtype B virus, but is commonly selected by EFV (110, 114) and to a lesser extent by NVP (115) in patients infected with subtype C virus. Brenner et al. (116) demonstrated that different codon usage for valine between subtype B (GTA) and sub-type C (GTG) virus influenced the

selection of the V106M (ATG), with sub-type C virus requiring only a single-nucleotide substitution. The V106M mutation conferred resistance to all NNRTIs (92, 116).

11 Transmission of NNRTI Resistance

The increasing number of NNRTI-experienced patients harbouring resistance mutations has been reflected by an increased rate of transmission of drug-resistant virus (117). Transmission of drug-resistant virus can seriously impair the efficacy of combination therapy, especially within the NNRTI class where broad cross-resistance is associated with many of the most prevalent NNRTI mutations (66, 118, 119). The risk of transmission of NNRTI-resistant virus is increased because of a number of factors including (1) the widespread use of NNRTI therapy, (2) the low genetic barrier to the development of NNRTI resistance resulting in a relatively high incidence of mutations in the population and (3) the relatively benign effect common NNRTI mutations have on viral replication often leading to their persistence.

The overall prevalence of ART-associated resistance mutations in treatment-naïve HIV-infected patients in North America and Europe have been estimated at around 10% (120, 121). This value however is largely cohort dependent, with each cohort being influenced by pre- and post-HAART prescribing practices, adherence rates, and patient risk factors. Although the prevalence of transmitted NNRTI mutations has been reported in as many as 13% of treatment-naive patients (118), generally reported rates of transmission are lower, especially in Europe. In the largest European study (CATCH Study) involving 2,208 patients from 19 European countries, the overall prevalence of NNRTI mutations in ART-naïve patients was 2.9%, with the most common mutations being K103N (1.5%), V108I (0.5%), and Y181C (0.5%) (121). This drastically contrasts with the 27.8% incidence observed in the treatment-experienced patients. It is noteworthy that this study also reported that the incidence of NNRTI resistance in treatment-naïve patients increased over time from 2.3% in 1996–1998 to 3.1% in 1999–2000 to 9.2% in 2001–2002, largely mirroring the use of NNRTIs.

12 Structural Determinants of Resistance to NNRTIs

Resistance to NNRTIs tends to cluster around the NNIBP and often involves residues in close contact with the inhibitors, thus providing a rationale for the high level of cross-resistance observed with the currently available drugs within this class. X-ray structures of the Y181C mutant in the complex with NVP and EFV have provided insights into an important structural determinant of resistance (122). As expected, the loss of crucial aromatic–aromatic stacking interactions in the case of NVP accounts for most of the 100-fold decrease in antiviral potency caused by the Y181C mutation. EFV also suffers a substantial albeit more limited decrease in potency, in agreement with its lower dependency on interactions with Y181. The effects of the Y188C and Y188L mutations are similar, although they occur less frequently than at position 181 (122, 123).

A second mechanism of cross-resistance has been proposed for the K103N mutation, which is the most prevalent NNRTI-resistance mutation (63, 124). The naturally occurring K103 is located at the outer rim of the NNIBP and is generally not making significant interactions with NNRTIs. The extensive cross-resistance caused by the K103N mutation was therefore proposed to be independent of the nature of the NNRTI (6, 31). This hypothesis was later confirmed upon resolution of the X-ray structure of the mutant (5, 33) which revealed the presence of a key hydrogen bond between the side-chain nitrogen atom of N103 and the side-chain phenoxy oxygen of Y188. Molecular dynamic simulations have also added further support to the notion that this hydrogen bond effectively stabilizes the already well-packed hydrophobic core of RT and imposes a substantial energy barrier to the 'NNIBP-forming' rotation of the Y188 side chain (125, 126). Collectively, these studies have provided a rationale for the decrease in potency of most NNRTIs against the mutant K103N compared to wild-type.

The role of several other mutations has also been deduced from X-ray structures (127). While some mutations, like V106A and P236L, cause reduction in NNRTI potency mainly through weakening of van der Waals contacts with the inhibitor (127, 128), others, like the prevalent G190A, are believed to create steric compression (123, 129). Other mutations play a more indirect role, V108I for example, that causes small but important movements of the neighbouring Y181 and Y188 side chains leading to suboptimal stacking interactions with the inhibitor (127, 130).

13 New NNRTIs in Development

Although it is now over 8 years since the last NNRTI received regulatory approval from the FDA, there are continuing efforts to develop a next-generation NNRTI with improved efficacy against NNRTI-resistant viruses and/or improved genetic barriers to the development of resistance. The diversity of resistance mutations observed within the NNIBP makes the development of new NNRTIs particularly challenging. X-ray structures of NNRTI-bound wild-type and mutant RTs can be used to make general rules for the design

of superior NNRTIs with greater resilience toward mutations (23, 131, 132). Of prime importance is the introduction of inhibitor contacts with main-chain protein atoms as such interactions are usually much less sensitive to mutations. Contact with residues having low (or no) mutational rate in response to NNRTI treatment (such as W229, L234, and Y318) and lesser reliance on interactions with the side chain of Y181, Y188, and F227 are of potential advantage. Greater flexibility of the inhibitor may also enable better adaptation to the changing environment of the mutable NNIBP.

Several NNRTIs that meet to varying degrees these objectives are currently in clinical trials: TMC-125 (Etravirine, Tibotec (133)); TMC-278 (Rilpivirine, Tibotec (134)); UK-453,061 (Pfizer (135)); and BILR 355 BS (Boehringer Ingelheim (136)) (Fig. 5). The difficulties in developing a next-generation NNRTI are highlighted by the recent discontinuation of clinical development of Capravirine (CPV, Pfizer) (137). Development was discontinued after a Phase IIb study failed to demonstrate a statistically significant difference in virological response in favour of CPV compared to placebo when co-administered with LPV/r + 2NRTIs in the treatment-experienced patients (138). Encouragingly for next-generation NNRTI development, however, subanalyses did indicate benefit of the CPV therapy in patients with reduced susceptibility to concomitant ART demonstrating that in the absence of an active backbone, CPV did enhance clinical response (139).

TMC-125 (Etravirine, 200 mg twice daily) is currently in Phase III development for the treatment of NNRTI-resistant viruses. TMC-125 belongs to a broad family of diarylpyrimidines and diaryltriazepines from which potent NNRTIs have been derived including TMC-120 and TMC-278 (133). Although high-resolution complexes with wild-type RT have been elusive, a 2.9Å complex with the K103N RT mutant is available (140). While conformational heterogeneity of TMC125 within the NNIBP is likely the cause for the challenging crystallization, it is also suggested as the main source of its resilience to mutations. Indeed, the four rotatable bonds of TMC125 act as efficient swivels that allow for the exploration of a vast conformational space and facilitate adaptation to the mutating NNIBP environment (141). Thus, while the complex structure reveals typical hydrophobic contacts with residues such as Y181 and Y188, it is likely that flexibility compensates for the potential loss of these interactions (subsequent to mutation) by searching for other beneficial interaction partners.

Profiling of TMC-125 against an extensive panel of individual NNRTI mutations identified four single mutations that conferred more than tenfold decreased susceptibility, namely Y181I or V, F227C and M230L (142). In addition, the double mutant Y179F/Y181C and triple mutant L100I/K103N/Y181C conferred more than tenfold decrease in susceptibility (143). Analysis from Phase II (C223 Study) and Phase III (DUET 1 & 2) clinical trial data have demonstrated the benefit of TMC-125 compared to placebo in NNRTI experienced patients over 24 weeks (144, 145, 146). The benefit of TMC-125 was most evident in patients receiving few or no active concomitant antiretrovirals. Decreased virological response to TMC-125 was associated with the presence of three or more specific NNRTI mutations from V90I, A98G, L100I, K101E or P, V106I, V179D or F, Y181C or I or V, and G190A/S (147, 148).

Although the clinical results with TMC-125 are encouraging, certain limitations on NNRTI sequencing remain because of NNRTI cross-resistance. Virological responses were generally better in patients with no NNRTI mutations at baseline, but with historic evidence of resistance or with only one NNRTI mutation present at study entry highlighting the difficulties of treating patients with the most heavily mutated virus (147–149). Secondly, the early discontinuation of an additional study (TMC125-C227) comparing TMC-125 to a PI in NNRTI-experienced, but PI-naïve patients, demonstrated that the use of a protease inhibitor remains superior to TMC-125 in NNRTI experienced patients (150). Future analyses and clinical experience should provide clearer guidance on when it is appropriate to use TMC-125.

TMC-278 (Rilpivirine, 75 mg once daily) has recently completed Phase II development for the treatment of NNRTI-naïve patients (151). TMC-278 is a second-generation DAPY compound displaying improved potency against a panel of NNRTI-resistant isolates (152). The favourable pharmokinetic profile of TMC-278 allows for once-daily dosing and therefore potential for use as a first-line therapy. In this situation, the favourable resistance profile of TMC-278 may offer advantages over EFV in patients infected with NNRTI mutations. Following 48 weeks of treatment, TMC-278

TMC125 (etravirine) TMC278 (rilpivirine) UK-453, 061 BILR 355 BS

Fig. 5 Chemical structures of 'next-generation' NNRTIs currently in clinical trials

demonstrated comparable efficacy to EFV, with 80 and 81% of patients achieving <50 copies/mL at week 48; however, it may have advantages over EFV in terms of the CNS side-affect profile. Virological failure was associated with development of NNRTI mutations K101E, E138K, and M230L (151).

UK-453,061 is another NNRTI with potent activity against wild-type and clinically relevant NNRTI-resistant viruses. Following adequate demonstration of safety in healthy volunteers, UK-453,061 has recently completed a Phase IIa 7-day monotherapy study in treatment-naïve HIV-infected patients. Doses of 500 mg BID/QD or 750 mg QD resulted in the greatest viral load nadir (−1.91, −1.69, and −1.97 $\log_{10}$copies/mL decline) (135).

Finally, BILR 355 BS belongs to an extended family of dipyridodiazepines from which the first clinically approved NNRTI nevirapine was derived. Profiling of BILR 355 BS has revealed that many of the most prevalent single and double combinations of NNRTI mutations, namely, L100I/K103N and K103N/Y181C remain susceptible (153). Single mutations V106A or Y188L confer more than tenfold reduced susceptibility to BILR 355 BS while double mutations K103N/V106A and V106/E138K conferred more than 100-fold reduced susceptibility (154). Despite the presence of NNRTI mutations conferring resistance, the incidence of these mutations remains low. In Phase I studies with healthy volunteers, BILR 355 BS in combination with ritonavir, reaches plasma levels sufficient to suppress wild-type and several NNRTI-resistant viruses from treatment-experienced patients (155). X-ray structures of BILR 355 BS with wild-type and mutant RTs have recently been reported. Although BILR 355 BS binds within the NNIBP in a mode similar to that of NVP, its wing II makes additional interactions with Y318 (CH-π contact) as well as with the backbone of the K101-K103 loop. Moreover, the methyl group on the central core of BILR 355 BS (rather than on wing I as in NVP) confers superior adaptability upon mutation (or indirect disturbance) at Y181 by permitting a favourable rearrangement of Y188 (which is not possible in the case of NVP). Additional interactions made by the quinoline extension on wing II further explains the broad antiviral profile of BILR 355 BS (153).

14 Conclusion

Since their approval for clinical use, NNRTIs have been and continue to be important therapeutic options for the treatment of HIV-1. The major limitation associated with the NNRTI class is the low genetic barrier for the development of resistance, resulting in the rapid development of resistance. The next few years will be crucial in determining whether next generation NNRTIs will allow for a higher genetic barrier and superior durability compared to the first generation drugs.

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Chapter 34
Resistance to HIV-1 Protease Inhibitors

Louise Doyon, Robert Elston, and Pierre R. Bonneau

1 Mechanism of Action of Protease Inhibitors

The HIV-1 genome encodes an essential protease enzyme which is one of the major targets of antiviral therapy (1–3). Protease inhibitors (PIs) have been proven to be potent antiviral agents and their introduction in 1995 led to the era of highly active antiretroviral therapy, the most potent and prescribed treatment of HIV infections today (4, 5). Although resistance to HIV-1 reverse transcriptase inhibitors had been described in the late 1980s (6), it was originally thought that PIs would be much less prone to drug evasion because of intrinsic genetic and structural constraints. Contrary to these expectations however, a substantial number of patients in the initial studies with PIs experienced drug failure due to the accumulation of multiple mutations in the HIV-1 protease gene (7–15). To understand the mechanisms leading to PI resistance better, it is important to first briefly review the general structure of the enzyme as well as the interactions involved in inhibitor binding.

1.1 The HIV-1 Protease

The HIV-1 protease belongs to the aspartyl protease family. It is composed of 99 amino acids, encoded within the *pol* region of the viral genome. It is synthesized as part of a 160 kDa *gag-pol* polyprotein and its activation depends on dimerization and release from the precursor by autocleavage (16–18). As an active homodimeric enzyme, the HIV-1 protease can process the precursor proteins *gag* and *gag-pol*, thereby releasing all structural and enzymatic components of HIV (2). Inactivation of HIV-1 protease by mutation or by exposure to chemical inhibitors leads to the formation of immature, non-infectious virions, thus demonstrating the full potential of this enzyme as an antiviral target (19, 20).

Over 200 crystal structures of the HIV-1 protease either as a free enzyme or bound to various ligands have been resolved (21). These studies have demonstrated that the three dimensional structure of the enzyme is composed of nine β strands and a single α helix (22, 23) (Fig. 1). The active site of the enzyme is formed at the dimer interface by four antiparallel β strands with each subunit contributing a single aspartic residue to the catalytic triads Asp25-Thr26-Gly27 and Asp25′-Thr26′-Gly27′ (24). The active site is covered by two very flexible β-sheets, called the flaps, which have low structural stability when HIV-1 protease is in the unbound state. Upon substrate binding however, the flaps close access to the active site in a conformation that excludes water and creates a hydrophobic environment. Although the structure of HIV-1 protease dictates a symmetric active site in the enzyme, the binding pocket surprisingly accommodates nine different asymmetric cleavage sites within *gag* and *gag-pol* polyproteins as well as a cleavage site in Nef (Fig. 2) (25, 26). These cleavage sites not only share very little sequence homology but the amino acids surrounding the different scissile bounds vary both in size and charge distribution adding to the complexity of protein substrate interactions (25, 27). The exact mechanism by which HIV-1 protease can maintain specificity of cleavage when working with all these constraints is not completely understood (28, 29). Nevertheless, a number of distinct subsites in the protease active site accommodate the side chains of the substrate residues and upon binding, HIV-1 protease catalyzes the cleavage of the substrate by enabling nucleophilic attack of a water molecule at the carbonyl of the scissile peptide bond (3).

1.2 HIV-1 PIs

There are currently ten PIs approved for the treatment of HIV-1 infections; amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir,

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and tipranavir (Fig. 3) (5, 30, 31). Ritonavir is now mostly used as a PI boosting agent (see Sect. 2.6). All these inhibitors are competitive active site inhibitors, as they compete with natural substrates for the binding to the active site. Most inhibitors were developed by structure assisted (rational) design of molecules initially modeled on natural cleavage sites. Due to this optimization, inhibitors not only bind the active site of the enzyme with much higher affinity than the natural substrates, enabling them to bind preferentially in the presence of natural substrates, but also make them particularly sensitive to any change in the active site environment. The thermodynamic forces driving the binding of inhibitors to the protease active site are somewhat different depending on the inhibitors. Binding of the first-generation inhibitors (indinavir, ritonavir, saquinavir, nelfinavir) tends mostly to depend on hydrophobic interactions that cause a large favorable entropy change whereas for next-generation inhibitors which show very high affinity for HIV-1 protease (amprenavir, atazanavir, lopinavir, darunavir) both enthalpy and entropy changes contribute favorably to binding (24, 32). Yet another thermodynamic pattern was observed with tipranavir whose binding to HIV-1 protease is almost exclusively driven by favorable changes in entropy with virtually no change in enthalpy (32).

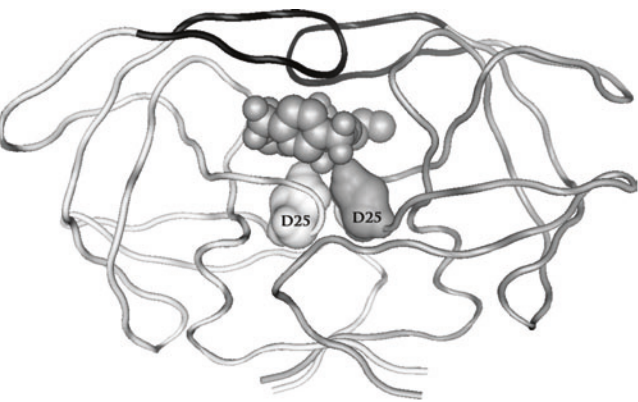

Fig. 1 Three-dimensional structure of the HIV-1 protease. HIV-1 protease is a homodimer composed of two identical subunits (shown here in *light grey* on the *left* and *grey* on the *right*). The D25 catalytic residues of each subunit are shown in close-packing representation. The flap regions of each subunit are shown in *black*. The non-peptidic inhibitor tipranavir occupies the substrate binding cleft

All but one approved HIV-1 PI and most of those currently in development are non-hydrolysable transition state peptidomimetics in which the cleavage site peptide linkage is replaced by transition state isosteres, such as norstatine, hydroxyethylene, hydroxyethylurea, or dihydroxyethylene (33). This strategy takes advantage of the fact that although there are a number of favorable interactions between the enzyme and the substrate in the enzyme-substrate complex, there are many more interactions between the enzyme and the substrate's transition state which results in tighter binging. Another potent design strategy is exemplified by the non-peptidomimetic inhibitor tipranavir, whose isostere is part of a 4-hydroxy-5,6-dihydro-2-pyrone system (34) and which includes a functional replacement of a conserved water molecule observed in protease-substrate co-crystal structures. This latter feature, coupled with the symmetrical nature of the protease binding site, is also utilized by urea-based inhibitors (33). Irrespective of the nature of the inhibitor, all PIs inhibit HIV-1 protease by competing for

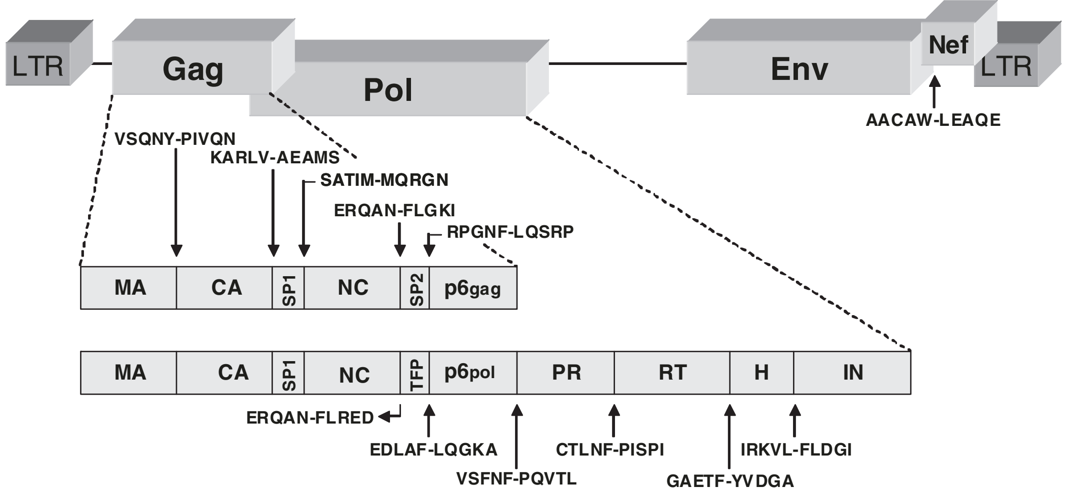

Fig. 2 Protease cleavage sites in HIV-1. The HIV-1 protease cleaves 11 cleavage sites in *gag* and *gag-pol* as well as 1 cleavage site in *Nef* (169). The biological function of the latter processing site is still unclear (170). Cleavage site sequences are indicated by *single letter* amino acid code with the scissile bound identified by a *hyphen*. *MA* matrix; *CA* capsid; *SP1* spacer peptide 1; *NC* nucleocapsid; *SP2* spacer peptide 2; *TFP* transframe peptide; *PR* protease; *RT* reverse transcriptase; *H* RNase H; *IN* integrase

Fig. 3 The ten currently approved HIV-1 protease inhibitors

the active site of the enzyme thereby preventing natural substrate processing and causing an arrest in the viral replication cycle.

2 Mechanism of Drug Resistance

Resistance to PIs was first described in 1993 when HIV-1 showing 30-fold decreased susceptibility to the PI RO31-8959 (saquinavir) was selected after in vitro passage in the presence of the inhibitor (35). The decreased susceptibility to saquinavir was genetically mapped to mutations in the protease gene of resistant viruses: G48V and L90M (36, 37). The in vivo development of resistance to saquinavir was soon after confirmed in patients receiving saquinavir monotherapy and genotyping revealed the presence of the same mutations in HIV-1 protease as those observed in vitro (7, 38). It became apparent therefore that, contrary to early expectations, the class of HIV-1 inhibitors targeting the protease enzyme was not immune to the development of resistance. Resistance to all currently marketed PIs has since been described (39) and Table 1 shows the mutations associated with decreased susceptibility to these inhibitors. Figure 4 shows the location of

Table 1 Mutations associated with in vivo and/or in vitro decreased susceptibility to the currently approved HIV-1 PIs[a]

Protease inhibitor	Primary mutations	Secondary mutations
Atazanavir	I50L, I84V, N88S	L10I/F/V/C, G16E, K20R/M/I/T, L24I, V32I, L33I/F/V, E34Q, M36I/L/V, M46I/L, G48V, F53L/Y, I54I/V/M/T, D60E, I62V, I64L/M/V, A71V/I/T/A, V82A/T/F/I, I85V, L90M, I93L/M
Amprenavir/fosamprenavir	I50V, I84V	L10F/I/R/V, V32I, M46I/L, I54L/M/V, G73S, V82A/F/T/S, L90M
Darunavir	I50V, I54L/M, L76V, I84V	V11I, V32I, L33F, I47V, G73S, L89V
Indinavir	M46I/L, V82A/F/T, I84V	L10I/R/V, K20M/R, L24I, V32I, M36I, I54V, A71V/T, G73S/A, V77I, L90M
Lopinavir	V32I, I47V/A, V82A/F/T/S	L10F/I/R/V, K20M/R, L24I, L33F, M46I/L, I50V, F53L, I54V/L/A/M/T/S, L63P, A71V/T, G73S, I84V
Nelfinavir	D30N, M46I/L, L90M	L10F/I, M36I, A71V/T, V77I, V82A/F/T/S, I84V, N88D/S
Ritonavir	V82A/F/T/S, I84V	L10F/I/R/V, K20M/R, V32I, L33F, M36I, M46I/L, I54V/L, A71V/T, V77I, L90M
Saquinavir	G48V, L90M	L10I/R/V, L24I, I54V/L, I62V, A71V/T, G73S, V77I, V82A/F/T/S, I84V
Tipranavir	L33F, V82L/T, I84V	L10V, I13V, K20M/R, E35G, M36I, K43T, I47V, I54A/M/V, Q58E, H69K, T74P, N83D, L90M

[a]Data adapted from Ref. (39)

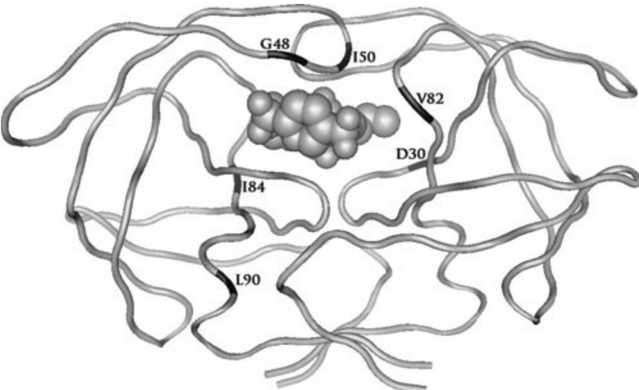

Fig. 4 Location of the most common primary resistance mutations in the three-dimensional structure of the HIV-1 protease

the most common primary resistance mutations in the three dimensional structure of HIV-1 protease.

In contrast to reverse transcriptase inhibitors where a single mutation in the target can confer >1,000-fold decreased susceptibility (40–42), high level resistance to PIs usually requires the presence of more than one primary mutation indicating that the genetic barrier to resistance to some PIs is somewhat higher than for the majority of reverse transcriptase inhibitors. For most PIs, single mutations generally confer from 2- to 10-fold resistance whereas high level resistance requires as many as ten mutations in the enzyme (7, 8, 43–45).

It is surprising to observe that the HIV-1 protease, given its small size and essential function towards multiple substrates, can withstand such considerable sequence variation. Compilation of data collected from viral isolates of treatment-naïve or PI-experienced patients indicate that 45 of the protease's 99 amino acids can show treatment-associated mutations (46). Of these however, only about 27 mutations are generally accepted as being directly involved in the development of resistance to PIs (39, 47, 48). This nonetheless highlights the great potential HIV-1 has to change its sequence to adapt to local environments such as inhibitor-induced selective pressure.

Mutations in the HIV-1 genome appear as the result of several mechanisms including the great propensity of the reverse transcriptase enzyme to make errors during reverse transcription and frequent recombination events that occur when multiple viral genomes are present in an infected target cell (49). These genetic irregularities occur at every replication cycle and given the high replication rate of HIV-1 (10^9 copies generated per day), a large spectrum of viral quasispecies is generated in an untreated infected individual in very little time (50). It is estimated that every possible point mutation in the HIV-1 genome occurs between 10^4 and 10^5 times per day in untreated patients (51). Most mutants have biological handicaps compared to the wild type virus and are rapidly outgrown by fitter viruses whose existence is imprinted in target cell genomes during the integration step of the viral replication cycle. When the environment becomes unfavorable to wild type virus replication, for example in the presence of drug-induced selective pressure, mutant viruses showing reduced susceptibility to this inhibitor can outgrow efficiently. The selection of mutant viruses is therefore extremely difficult to prevent if all viral replication is not completely suppressed.

Sustained viral suppression is challenged by several factors in treated patients including the pharmacologic properties of the antiretroviral agents (bioavailability, protein binding, and tissue penetration), toxicity and the lack of adherence of patients to therapy (51). The relationship between adherence and the development of HIV-1 resistance has been the focus of many studies over the recent years and the understanding of the correlation between these two parameters has rapidly evolved. Initially, the development of

resistance was thought to be directly dependent on the level of adherence of patients to therapy as patients having poor adherence showed rapid emergence of resistance. Recent studies however suggest that rather than being strictly linear, the relationship between adherence and resistance is more complex, adopting a bell-shaped configuration where the development of resistance not only depends on the level of adherence but also on the potency of the therapy (52–54). According to these studies, two clinical scenarios correlate with minimal occurrence of HIV-1 resistance: cases of very poor levels of adherence (no drug pressure) and cases of very high levels of adherence to treatment (viral suppression). The risk of developing resistance, peaks in intermediate conditions (ranging from 70 to 89% adherence) when viral replication occurs in a zone of high selective drug pressure (52–54). Conclusions from these studies are that very high levels of adherence are required to avoid the development of resistance and contrastingly, there is an increased risk of developing resistance when patients showing very poor compliance increase their adherence to treatment. However, these studies also demonstrated that adherence is not the only contributing factor to the development of resistance, as resistance was also observed in patients who were 100% adherent, but that insufficiently potent treatment also contributes (55).

2.1 Protease Mutations

PI resistance mutations have been characterized as "primary/major" or "secondary/minor" depending on their effect on inhibitor activity (39). Primary mutations usually have a direct impact on inhibitor activity and by themselves cause a significant change in the resistance phenotype of viruses. These are most often mutations of residues located in the active site of HIV-1 protease which are directly involved in inhibitor binding, and are mutations that are rarely seen as genetic polymorphisms in untreated isolates (46). Secondary mutations on the other hand do not, on their own, have a major impact on the susceptibility to the inhibitors but rather increase resistance in the presence of primary mutations either by influencing the affinity of the protease for the inhibitor or by compensating for the detrimental effects of primary mutations on enzyme function and/or viral replication.

Table 1 summarizes the primary and secondary mutations associated with resistance to each PI (39). The relative effect on susceptibility when different combinations of these mutations are present is complex and often results in difficulty in identifying the appropriate PI for treatment experienced patients. In the following section, examples of resistance to specific PIs will be described to illustrate many of the features associated with the development of PI resistance.

Resistance to certain PIs can develop either through the selection of unique resistance mutations or through alternate, sometimes non-exclusive mutational pathways. For example, the major pathway leading to the development of resistance and reduced clinical efficacy of nelfinavir in the absence of pre-existing PI mutations involves the selection of mutation D30N, a mutation unique to this inhibitor (10). In concordance, isolates from PI-naïve patients showing post-exposure reduced susceptibility to nelfinavir almost invariably harbor the D30N mutation. Other examples of mutations selected by a specific PIs include atazanavir (I50L) (56), amprenavir (I50V) (57), lopinavir (I47A) (58), saquinavir (G48V) (36, 37), and tipranavir (V82L) (59).

For several PIs however, several different mutational "pathways" to the development of resistance have been identified. The factors involved in the selection of specific resistance pathways include prevailing drug levels, codon usage, pre-existing resistance and/or natural polymorphisms and the replicative capacity of the resistant virus relative to the resistance conferred. A non-exhaustive description of these pathways follows.

The development of resistance to saquinavir, is associated with primary mutations located either in the protease active site (G48V) or at the dimer interface (L90M) of the enzyme (Fig. 4) (38). Both these mutations alone contribute to some level of resistance to saquinavir but studies have shown that L90M is the predominant mutational pathway observed in vivo while G48V is less frequently observed (60). Interestingly the G48V mutation was more frequently observed when higher drug dosages of saquinavir were used (60).

Nelfinavir, on the other hand, often selects the D30N mutation in treatment-naive patients, this mutation is rarely observed in treatment-experienced patients (61). It appears that the presence of other PI-associated mutations is sufficient to ensure that the D30N mutation is no longer the preferred route to resistance to nelfinavir. The mechanism behind the effect of the pre-existing mutations is unclear but the presence most likely renders incorporation of the D30N mutation unnecessary due to alternative routes to high level nelfinavir resistance without the replicative cost associated with the incorporation of the D30N mutation.

Divergent resistance pathways and the association with prevailing drug levels (Cmin) was demonstrated for unboosted amprenavir (57). Virological failure despite higher amprenavir levels selected for the I50V mutation whereas lower amprenavir concentrations were associated with the selection of the I54L mutation. Interestingly the I50V mutation is associated with greater resistance but at a higher cost to replicative capacity, indicating that higher drug levels may force the virus to select more extreme resistance solutions to overcome the drug pressure.

Another important, but often unappreciated aspect of PI resistance is that different mutations at identical positions

can have profoundly different effects on the resistance phenotype. For example, both atazanavir and amprenavir select for mutations at position I50 but this mutation involves a leucine upon treatment with atazanavir whereas a valine is observed following amprenavir/fosamprenavir therapy (13, 15). Studies in vitro have demonstrated that viruses showing reduced susceptibility to atazanavir due to the I50L mutation remain sensitive to amprenavir and I50V-containing viruses remain sensitive to atazanavir showing the non-overlapping effects of these two mutations (15). Similar observations have been made for other PIs and amino acids I54M/L/T/A/V and V82A/S/L/T/F.

For more recently approved PIs, phenotypic association with mutational patterns is so complex that mutation scores have been developed to assist in determining when a patient is likely to benefit from the drug. A mutation score was first developed for lopinavir with studies determining that between 4 and 8 mutations among those shown in Table 1 are required to confer diminished response to lopinavir (11, 43, 62). Recently, results from Phase II and III studies identified mutations at positions 82, 54, and 46 in viral isolates from patients showing incomplete virological response or viral rebound subsequent to initial response to lopinavir/ritonavir treatment in PI-experienced patients (63).

Mutation pathways leading to reduced susceptibility to the potent inhibitor tipranavir have also required detailed investigation. In vitro resistance to tipranavir is not dominated by a few signature mutations; instead, sequential accumulation of up to ten mutations in the protease gene is necessary to lead to substantial loss in potency (64). Recently, a mutation score based on clinical trial data was developed which consisted of a unique string of 16 protease positions and 21 mutations (59). While a number of these mutations correlate with positions identified in vitro to be linked with reduced susceptibility to tipranavir (positions 10, 13, 33, 36, 54, 82, and 84), several positions in the score have not been associated with resistance to other PIs, demonstrating a novel resistance profile for tipranavir. The importance of codon usage is also illustrated during the development of resistance to tipranavir. In the presence of the resistance mutation V82A, failure of tipranavir is often associated with the development of the V82T mutation whereas V82L is selected in viruses initially harboring a valine at this position. In both cases (A $\rightarrow$ T and V $\rightarrow$ L), a single nucleotide change is required to develop resistance at position 82 whereas a A $\rightarrow$ L or V $\rightarrow$ T mutation requires a double nucleotide change (65).

The most recently approved PI, darunavir, is chemically related to amprenavir and owes its improved antiviral potency, at least in part, to the addition of a bis-tetrahydrofuran moiety (66). Analysis of clinical trial data to identify mutations linked to decreased susceptibility to darunavir revealed that the presence of V32I, I47V, or I54M at baseline is associated with a lower virologic response (67). More recently, the presence of three or more mutations from V11I, V32I, L33F, I47V, I50V, I54L or M, G73S, L76V, I84V, or L89V was associated with decreased virologic response (68). Analysis of patients receiving darunavir who responded and then lost their antiviral response showed development of mutations V32I, L33F, I47V, or I54L (68).

Finally, in some cases, there is a certain advantage to the great mutability of the protease. Mutations selected by one PI can indeed confer increased susceptibility to another PI. The development of the N88S mutation following nelfinavir treatment leads to increased susceptibility to amprenavir (69). Similarly the I50V mutation selected by amprenavir leads to increased susceptibility and increased virological response to tipranavir-based PI therapy (70, 71). Virological failure of tipranavir, when the I50V mutation was present at baseline, resulted in the loss of this mutation from the circulating virus (71).

2.2 Structural Effects of Mutations

A substantial amount of work has been carried out to understand at the structural and biochemical levels how mutations in HIV-1 protease confer resistance to inhibitors. In its most simple terms, resistance is defined by the loss of affinity of an inhibitor for its target. It has long ago been determined that protease active site mutations increase inhibitor Ki, making the concentrations of drug required for full enzyme inhibition much higher than those required for wild type enzyme inhibition (72–77). In vivo, this is reflected by the inability of the effective drug plasma concentrations to inhibit viral replication and therefore viral rebound is observed. Crystal structure studies of inhibitor/protease complexes have demonstrated that although many protease mutations are chemically conservative they can distort the geometry of the active site so as to reduce the number of interactions between the inhibitor and the protein (van der Waals interactions, hydrogen bonds) (78–82). This can occur through several mechanisms including the formation of an extended active site cavity or by changes in the dynamics of flap opening and closing (24, 77, 81). Other mutations cause steric hindrance in the active site of HIV-1 protease due to the presence of larger side chains in the wild type residues (V82F) of mutant versus or affect protease dimer stability if the mutation is located at the dimer interface (L90M) (24, 83, 84). Thermodynamic studies further show that mutations that affect inhibitor binding act primarily through a reduction in binding enthalpy although a loss in binding entropy is also observed (24). Since inhibitors do not bind as well in the mutant active site compared to the wild type

active site in that they cannot bury as much area from the solvent as they did when binding to the wild type enzyme (23). This reduction in dissolving negatively affects the whole dynamic of protein–inhibitor interactions thereby contributing to the resistance phenotype. A recent study with tipranavir has revealed a unique thermodynamic response to mutations among PIs; tipranavir compensates entropic losses by actual enthalpic gains (or by sustaining minimum losses in enthalpy) when confronted to mutant proteases (32). Measurement of association/dissociation rates by surface plasmon resonance have also been used to explain the impact of mutations on PI affinity and the high genetic barrier to darunavir resistance (85).

Recent studies propose a novel approach to the understanding of how resistance mutations are selected in HIV-1 protease. In these studies, it was demonstrated that all natural substrates (cleavage site sequences) adopt a similar conformation upon binding to the wild type protease and fill a common space within the active site of the enzyme termed "substrate envelop" (86). The space occupied by the inhibitor upon binding mostly falls within this "envelop" space but some protruding regions are observed. It is mutations in these specific protruding sites that are detrimental to inhibitor binding since they primarily affect inhibitor binding without drastically compromising substrate binding. Mutations at positions I50V, I84V and V82A, three PI-associated resistance mutations are examples of such protruding contact site mutations (86, 87). According to this concept, the design of inhibitors that do not protrude beyond the substrate envelop space, should significantly impede on the development of resistance.

2.3 Effect of Protease Mutations on Enzyme Activity and Viral Replication

Protease mutations have been described not only to decrease inhibitor binding but also to affect enzyme catalytic activity mostly due to an elevation in the K_M of substrates (72–74, 76, 88). This is reflected in the virus by an altered pattern of polyprotein processing (89–91). Mutations outside the active site can partially compensate for this loss of substrate affinity (76, 77) as well as in some cases directly contribute to the decrease in inhibitor affinity (80, 92).

The phenotypic consequence of impaired protease activity is a reduced capacity of the virus to replicate (reduced replication capacity). Many PI resistant viruses have indeed been shown to have reduced replication capacity when assayed in culture (12, 45, 75, 93–101). Some secondary mutations have been reported to increase replication of mutant viruses but not always to wild type virus levels (75, 96, 100, 101). Since the inherent ability of HIV-1 to replicate partly determines the level of viremia in vivo (102), and therefore directly impacts on virus survival and propagation, it is not surprising that the virus has evolved to select mutations outside of the protease gene locus to compensate for the detrimental effects of PI resistance.

2.4 Cleavage Site Mutations in PI-Resistant HIV-1

A few years after resistance to PIs was first described, mutations in two gag precursor cleavage sites (NC/SP2 and SP2/P6) were reported in viruses selected in vitro to be highly resistant to PIs (89). These mutations (also referred to as A431V and L449F, respectively) seemed directly related to the presence of mutations in HIV-1 protease as they were not observed in viruses containing a wild type protease. Cleavage site mutations were shown to improve polyprotein processing in protease mutant viruses which was proposed to result from improved catalytic efficiency of HIV-1 protease towards these substrates (89). The biological outcome of the presence of cleavage site mutations was to improve replication capacity rather than to contribute to inhibitor resistance, although increased resistance to some PIs was observed in the presence of cleavage site mutations (12, 103). Cleavage site mutations were also identified in vivo and have now been identified in isolates exposed to most marketed PIs (12, 13, 15, 93, 104–106).

Cleavage site mutations are the first example of a substrate "naturally" adapting to an enzyme modification and their discovery expanded the already great potential of HIV-1 to develop resistance to PIs. It also demonstrated the significant selective pressure HIV-1 must undergo to maintain a high level of replication since the delicate balance between viral replication and immune response dictates the clinical outcome of the disease. Over the years, mutations in all nine *gag* and *gag-pol* cleavage sites have been described but mutations in NC/SP2 and SP2/P6 occur at significantly higher frequency than in any other site (105). The subtle interplay between the mutated enzyme and its mutated cleavage sites may make reversion of highly drug resistant HIV-1 protease enzymes more difficult than reversion of HIV-1 reverse transcriptase (96).

2.5 Impact of HIV-1 Subtypes and HIV-2 on PI Susceptibility

Because considerable variation in sequence between HIV-1 isolates is observed, HIV-1 strains are divided into distinct groups (M, N, and O) according to their sequence homology (107). Group M, the main group, can be further divided into

sub-types designated A, B, C, D, F, G, H, J, K, and a variety of circulating recombinant forms (CRF01_AE, CRF02_AG, etc.). Historically, the majority of infections in North America and Western Europe have been caused by subtype B and concordantly all currently marketed PIs have been developed to target this subtype. The discussion so far has also focused primarily on the development of resistance and mutations observed in HIV-1 subtype B. However it is now reported that more than 40% of new infections in some regions of Europe are caused by subtypes other than B (107, 108). Given the variation in sequence between sub-types as well as between HIV-1 and HIV-2, it is clinically important to determine if the mutations observed as polymorphisms in non-B subtypes can confer altered susceptibility to PIs, a phenomenon already described for NNRTIs. Some HIV-1 subtypes are indeed "naturally resistant" to NNRTIs due to polymorphisms in the reverse transcriptase, notably at position Y181 (107, 109).

Analysis of HIV-1 protease sequence of non-B subtypes indicated that no polymorphism in amino acid positions characterized as primary mutations are observed in any subtypes but mutations at secondary sites are commonly seen (110, 111). Differences in baseline susceptibilities to PIs are therefore not generally observed within HIV-1 subtypes (110–112).

In HIV-2, polymorphisms are observed in primary residues of protease 32 and 82 and they are observed in several secondary positions as well. There are also reports of reduced activity of the PIs amprenavir, atazanavir, nelfinavir, and tipranavir towards HIV-2 (111, 113). However resistance in these cases is generally not as extensive as that observed with NNRTIs although the question has been raised as to whether certain PIs should or should not be recommended in HIV-2 patients initiating HAART.

In general, despite significant sequence diversity in the protease of HIV-1 subtypes and HIV-2 strains, there is still little evidence to support the presence of natural occurrence of resistance to PIs in these strains. However, due to the polymorphisms observed at secondary sites, the development of resistance to PIs in non-B subtypes does not always involve the same mutational pathways as those observed in subtype B. For example, whereas nelfinavir selects for the mutation D30N in subtype B, a high proportion of resistant isolates were found to harbor mutations L90M and L63P in subtype C (114).

2.6 PI Boosting

More recently there has been a move away from using PIs without co-administration of low, sub-therapeutic doses (100–200 mg) of ritonavir. At these low, sub-therapeutic doses, ritonavir acts as a pharmacological enhancer of other co-dosed HIV-1 PIs, increasing their concentrations through the ability of ritonavir to inhibit Pgp and CYP3A4 enzymes (reviewed in (115, 116)). For most PIs, co-administration with low-dose ritonavir substantially "boosts" the PI exposure resulting in improved antiviral efficacy. Certain PIs (lopinavir, darunavir, and tipranavir) are only approved for clinical use when co-dosed with ritonavir.

The increased PI exposure has had profound consequences on the development of PI resistance especially in previously PI-naïve individuals. Previously PI-naïve patients experiencing virological rebound whilst receiving a Kaletra-based regimen (lopinavir/ritonovir) did not develop PI-associated viral mutations unlike the comparator nelfinavir (117) The difference between boosted and unboosted PI regimens was demonstrated in two subsequent fosamprenavir studies (118, 119). Patients experiencing virological rebound on a boosted fosamprenavir regimen did not select PI-associated mutations in contrast to those patient receiving unboosted fosamprenavir (118, 119). The absence of PI-associated mutations with other boosted PIs has been reported more recently (120).

Despite extensive use of boosted PI regimens in previously PI-naïve patients, the development of protease resistance has only been reported in exceptional cases (58, 121–123) and has usually been associated with prolonged periods of ongoing viral replication and/or the failure to fully adhere to the HAART regimen. Although the precise mechanism behind the absence of resistance to mutations is not established it is likely that the elevated drug levels require a significant number of mutations to escape the prevailing drug concentrations (a so-called high genetic barrier). By boosting the PI levels, more patients are now receiving drug concentrations well above a therapeutic threshold in which individual resistance to mutations could escape. It has also been proposed that the boosting effect increases the "forgiveness" of PI-based regimens and that even following a missed dose, a boosted PI regimen spends less time at drug level concentrations where resistance selection is likely (124). The implications for the future are that there will be lower incidences of PI resistance in patients initiating regimens with boosted PIs. However, in patients where PI-mutations are selected, the same mutations usually emerge whether or not the PIs are boosted, although the relative frequency of mutations may differ (39).

3 Cross-Resistance

Since several protease mutations are involved in the development of resistance to more than one inhibitor, it is not surprising to observe that resistance to one PI commonly confers some degree of cross-resistance to other inhibitors (44, 61, 125–132). The development of cross-resistance has clinical importance since it can significantly compromise the

virological response to salvage PI-based therapies upon treatment failure (133–137).

In a study of over 6,000 HIV-1 isolates tested for susceptibility to four PIs, it was found that 59–80% of isolates with a tenfold decrease in susceptibility to one PI had a tenfold decrease in susceptibility to at least one other PI (61). In general, there is a correlation between the number of resistance mutations in the HIV-1 protease and the levels of resistance and cross-resistance to multiple PIs. Combinations of ≥2 mutations at positions D30, G48, I50, V82, I84, and L90 generally contribute to the development of resistance to multiple PIs but there is also a significant contribution by the presence of the mutation V32I and non-active site mutations at residues 10, 46, and 54 (39, 138). One recent study revealed that when analyzing the protease genotype from a clinic-based population of 4,183 patients, several patterns of PI mutations conferred >2.5-fold resistance to seven of the currently marketed PIs. The most common mutations in these patterns were in this order: L90M, V82A, and I84V (139). Other studies have confirmed that L90M is the most prevalent PI-mutation in HIV-1 clinical isolates (61, 140).

To decrease the possibility of development of cross-resistance, a strategic use of PIs with distinct signature mutations must be considered. In this regard, neflinavir was an interesting option since prior use of this inhibitor, which selects for the uncommon mutation D30N (10) generally did not affect susceptibility to other PIs and patients failing nelfinavir could be successfully rescued by treatment with other PIs (141). This feature initially made nelfinavir the favored PI given in combination therapy. Nelfinavir however was usually unsuccessful in treatment of PI-experienced patients because most of the mutations that confer resistance to other PIs confer cross-resistance to nelfinavir (61, 128, 142). The advent of ritonavir boosting of potent PI inhibitors, has contributed positively to this situation. Thus, PIs like lopinavir, tipranavir, and darunavir (as well as some PIs in development) show remarkable activity towards PI-experienced isolates and constitute good alternatives for salvage therapies (43, 143, 144). The potent antiviral activity of lopinavir in combination with its high genetic barrier to resistance has made this PI one of the currently preferred PI in HAART regimens (5).

Virological rebound on a tipranavir-based regimen is most commonly associated with the development of V82L or V82T, and generally has minimal impact on the susceptibility to other PIs including darunavir (145). Interestingly, the I50V mutation, generally linked to resistance and/or decreased virological response to amprenavir, lopinavir, and darunavir, is associated with increased susceptibility to tipranavir (71). Consistent with this is the observation that I50V is de-selected following virological failure of a tipranavir-containing regimen.

Although mutations correlated with reduced clinical response to darunavir are similar to those known to confer reduced susceptibility to amprenavir, the predicted incidence of clinically meaningful cross-resistance is low; thus, high levels of resistance to, and prior use of amprenavir seem to have only a minimal effect on the virologic response to darunavir (112, 146, 147), although a recent report suggests that mutations I50V and V32I + I47V have a negative impact on darunavir efficacy (148).

4 Primary or Transmitted PI Resistance

HIV-1 showing resistance to PIs can be transmitted from one infected individual to another, including from infected mother to child (149). Transmission occurs mostly through patients who are untreated or who experience incomplete viral suppression following therapy as studies have shown that transmission rarely occurs when RNA viral loads are lower than 1,500 RNA copies/mL (150). With the advent of antiviral therapy and the development of resistance, transmission of resistant viruses has increased over the recent years (140). It is estimated that there are currently between 10 and 25% of new infections in the United States and Europe that involve resistant viruses, with 1–19% specifically involving PI-resistant viruses (149). This evolving pattern of new infections can significantly affect the virological response of patients upon initiation of antiviral therapy (151) and has therefore warranted the recommendation to consider genotypic/phenotypic resistance testing in acute/recently infected patients seeking medical attention and before initiating therapy in treatment-naïve patients (5, 138). In a study of 2,244 isolates from PI naïve patients, it was determined that even in the absence of any PI treatment, the median number of protease mutations/polymorphisms (mainly secondary mutations) in HIV-1 isolates from these patients was four (46). Other studies have detected PI-resistance mutations in untreated patients several years after infection, suggesting not only that PI-resistance mutations are transmitted but that they also persist in the absence of drug pressure (152–155). Since reversion to wild type protease genotype is not readily achieved in vivo in the absence of drugs, it seems unlikely that full susceptibility to PIs will re-emerge in these patients and careful screening and management of treatment-naïve, newly infected patients is required.

5 Other PIs in Development

The important role played by HIV-1 PIs in today's anti-HIV armamentarium justifies the ongoing efforts to discover improved members of this drug class with broad and potent antiviral activity against the increasing diversity of PI-resistant

PL-100 AG 1776

AG-001859 GS-8374

Fig. 5 HIV-1 protease inhibitors in clinical development

viruses. Several HIV-1 PIs are currently at different stages of pre-clinical and clinical development (Fig. 5).

PL-100, an L-lysine-containing compound, is a novel HIV-1 PI with potent antiviral activity ($EC_{50,wt}$ ~ 10 nM), a favorable cross-resistance profile, and a high genetic barrier to resistance (156). A study comprising 63 HIV-1 resistant strains and aimed at comparing the resistance profile of PL-100 against available PIs showed that PL-100 had significantly better antiviral activity; 76% of the viruses had a fold-change <10 with PL-100 compared to a range of 27–54% with other PIs. Only 3% of the viruses had a fold-change >50 with PL-100 (157). Under in vitro selective pressure with PL-100, a unique pattern of mutations was observed; T80I appeared first at week 8 followed by three simultaneously emerging mutations (K45R, M46I, P81S) at week 25. T80I and P81S are novel active site mutations and P81S severely impairs viral replication (unless it is accompanied by K45R, M46I, and T80I). Single, double, or triple viral mutants have a minimal fold-change reduction in activity (<2.5) against PL-100 while the quadruple mutant is only 11-fold less susceptible. No cross-resistance to other available PIs was detected (158). Phosphorylation of the hydroxyl moiety of PL-100 leads to the pro-drug PPL-100 which displays substantially improved pharmacokinetic properties (>1,000-fold more soluble and threefold greater oral bioavailability than PL-100). This pro-drug is currently in Phase I human clinical trials and is reported to have the potential to be a once daily, unboosted PI (159).

Another PI in development is AG-001859. This peptidomimetic inhibitor, which contains the unnatural amino acid allophenylnorstatine, maintains a median potency within the range observed for wild type virus against a panel of PI-resistant clinical isolates (containing a mean number of 5 PI-resistant substitutions) (160). There was no correlation between the level of antiviral activity of AG-001859 and the number of PI-resistant substitutions present. More recently, selective pressure experiments with AG-001859 indicated slow emergence of resistant variants, with a gradual increase in resistance observed as protease and *gag* cleavage sites mutations accumulated. Phenotypic analysis of virus containing the I84V (passage 33), V82I/I84V (passage 40), and M46L/V82I/I84V (passage 46) demonstrated 1-, 3-, and 12-fold reduced susceptibility to AG-001859 respectively (161). AG-001859 is reported as having entered Phase II clinical trials.

A few other PIs are at different stages of pre-clinical development. AG 1776 (previously known as KNI-764 and JE-2147) is a peptidomimetic PI (also containing allophenylnorstatine) that maintains potent antiviral activity (less than twofold change compared to wild type HIV-1) against several isolates resistant to most other available PIs. In vitro selective pressure experiments suggest a slow emergence of resistance to AG 1776. Two of the mutations conferring reduced susceptibility (I84V and I47V) map within the protease active site region result in negative hydrophobic and van der Waals interactions that disrupt binding of AG 1776 (162). Finally, GS-8374 is a diethylphosphonate-containing analog of TMC-126 (itself a close analog of darunavir) with a potency comparable to darunavir and atazanavir (163). It maintains a mean EC_{50} fold-change of 6.2 against a panel of

24 clinical isolates displaying an average of ten mutations in the protease. Interestingly, crystallographic analysis showed that the phosphonate moiety is highly exposed to solvent when bound to protease, making no obvious interactions with the active site or surface residues. This effect is driven by favorable entropy changes upon binding to mutant enzymes, allowing effective molecular adaptation to their larger cavity volumes (164).

6 Conclusion

The introduction of PIs in HIV-1 combination therapies has made a tremendous impact on disease mortality over the past 10 years but it is clear that the efficacy of this class of drugs is also challenged by the development of resistance. As our understanding of resistance evolves, better insight towards the requirements of next-generation inhibitors becomes available. Potent activity against wild type as well as against a broad range of mutant HIV-1 viruses will inevitably be required to reduce the risk of resistance. Recent breakthroughs in the field of PIs such as those described above as well as those in the fields of reverse transcriptase and other antiviral targets are already beginning to produce novel molecules with promising antiviral and biopharmaceutical properties (165–168).

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Chapter 35
Resistance to Enfuvirtide and Other HIV Entry Inhibitors

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1 Introduction

At the turn of the twenty-first century, only three classes of antiretrovirals were available for the treatment of HIV: nucleoside reverse transcriptase inhibitors (nRTIs), non-nucleoside RTIs, and protease inhibitors (PIs). Although combinations of these agents often provided potent suppression of HIV-1 RNA, and had dramatically improved clinical outcomes for many patients (1), the limitations of highly active antiretroviral therapy based on the available compounds had becoming increasingly apparent and problematic. Those limitations included adverse effects associated with treatment (2, 3), significant drug–drug interactions (4), and the selection of drug-resistant viruses with extensive intraclass cross-resistance (5). Consequently, there was a clear need for new classes of antiretroviral agents with both improved safety and tolerability profiles, and which act on alternative targets and thereby circumvent the problems associated with intraclass cross-resistance. The first such drug to be introduced in the new millennium was the HIV-1 fusion inhibitor enfuvirtide (formerly known as T-20), which became available in 2003. Enfuvirtide, when used in combination with previously available antiretrovirals, provided significant virological and immunological benefits to patients with few remaining treatment options (6, 7). As of early 2007, the entry inhibitors maraviroc and vicriviroc, which target CCR5 binding, were under review for regulatory approval or were in late-stage clinical trials (8–10). Although not discussed in this chapter, compounds targeting the viral integrase had also shown promising results in Phase 2 clinical studies and were in the late stages of clinical development (11).

The process of HIV binding and entry into target cells offers many potential opportunities for intervention and is an attractive target for antiretroviral development, as drugs targeting the entry process would not be expected to show cross-resistance to earlier classes of antiretrovirals. Three major advances in the understanding of the HIV binding and entry process have facilitated the identification of promising new drug targets: first, the identification of the chemokine coreceptors CCR5 and CXCR4, which interact with HIV gp120 after it has bound to CD4 on the cell surface (12–14); second, the description of the tertiary structure of gp120 (15–17) and the gp41–gp120 complex on the viral membrane (15, 18–20); and, third, an understanding of the molecular interactions and conformational changes within gp41 that lead to fusion of the viral and cell membranes (19, 21–25). Several HIV entry inhibitors, targeting many of the steps of the entry process, have progressed to various stages of clinical development. Counted among these are agents that interfere with CD4 binding either by targeting the viral gp120, such as the CD4 mimic PRO 542 (26) and the small molecule inhibitors BMS 806 (27, 28) and BMS 043 (29), or by targeting the cellular CD4 receptor, like the antibody TNX-355 (26, 30). Antagonists of the interaction of gp120 with the coreceptors CXCR4 and CCR5 have also entered clinical trials. The CXCR4 binding inhibitors AMD3100 (31, 32) and AMD11070 (33, 34) act by targeting the cellular CXCR4 coreceptor, while the CCR5 binding inhibitors SCH-C and SCH-D (vicriviroc) (35), UK-427,857 (maraviroc) (36), and GW873140 (aplaviroc) (37) target the cellular CCR5 chemokine coreceptor. Two peptide inhibitors of HIV-mediated fusion, enfuvirtide and T-1249, have been studied in clinical trials (24, 38–40). These compounds act by interfering with conformational changes in the viral gp41 transmembrane glycoprotein necessary for membrane fusion, which is downstream from and common to virus entry proceeding through the CXCR4 or CCR5 pathways. This chapter focuses on enfuvirtide, which is now marketed worldwide for use in combination with a background of PIs and RTIs in treatment-experienced HIV-infected individuals, and maraviroc and vicriviroc, two CCR5 binding inhibitors currently in the late stages of clinical development.

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2 Mechanism of Action

HIV entry into CD4+ target cells is a multi-step process mediated by the virus envelope glycoprotein. Prior to particle maturation, the envelope precursor, gp160, is cleaved into noncovalently associated gp120 and gp41 subunits, with gp120 on the virus surface and gp41 anchoring the subunits in the viral membrane; these are further organized into trimeric envelope complexes (41). The gp120 subunit is primarily involved in receptor and coreceptor binding (and thus cell targeting) and in shielding the virus from attack by the humoral immune system, while gp41 facilitates merging of the viral and target cell membranes to allow delivery of the viral core (Fig. 1).

2.1 HIV Interaction with Cellular Receptors

The process of HIV entry begins with the binding of gp120 to a CD4 molecule on the surface of a target cell. Binding to CD4 induces a conformational change that exposes an otherwise cryptic binding site on the gp120 molecule for one of the two major HIV coreceptors, CCR5 and CXCR4. Viruses obtained shortly after HIV infection are overwhelmingly CCR5-dependent (42, 43); in contrast, CXCR4-using strains emerge later in the course of disease progression in approximately half of patients in untreated populations (44–47), and were associated with reduced CD4+ cell counts in heavily treatment-experienced patients (48, 49). The appearance of strains able to use CXCR4 in addition to CCR5 was strongly associated with disease progression in natural history cohorts; however, the prognostic significance of detecting CXCR4-using strains emerging during antiretroviral therapy remains unclear (50, 51). The specificity of viral coreceptor binding is determined primarily by residues in the variable loop 3 (V3) region, with additional contributions from the V1/V2 region (52–55). These variable loops often contain insertions and/or deletions, as well as polymorphic residues within a virus population, making their study by population sequencing problematic.

CCR5 is a seven-pass transmembrane receptor present on macrophages and on many activated and memory CD4+ lymphocytes. The regions of the CCR5 coreceptor with which the viral gp120 typically interacts include the N-terminus and one or more additional extracellular domains,

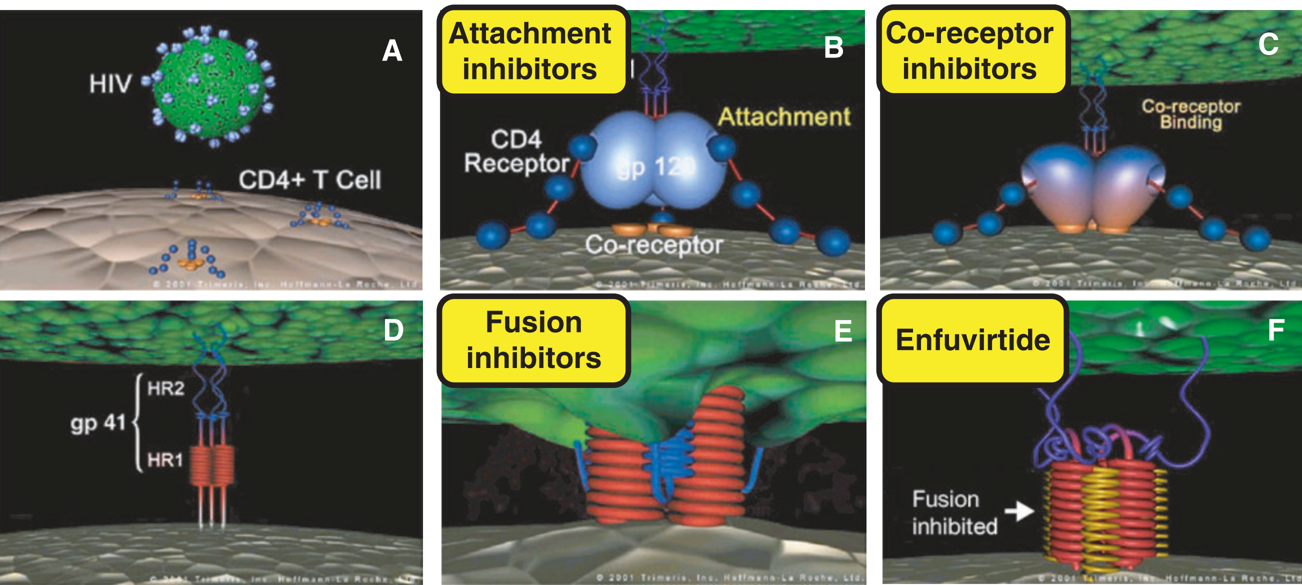

Fig. 1 The HIV entry process. HIV envelope-mediated entry begins with interaction between the viral envelope gp120, shown as *trimeric spikes* outside of the virus, and the cellular CD4 receptor (**a**, **b**). That interaction results in conformational changes that expose a binding site for the CCR5 or CXCR4 coreceptor (**c**). Gp120 binding to the coreceptor results in further changes that expose gp41 and allow insertion of the N-terminal fusion peptide of gp41 into the target cell membrane (**d**). Subsequently, formation of a six-helix bundle by the HR1 and HR2 domains of gp41 brings the viral and target cell membranes into close proximity, facilitating membrane fusion (**e**); HR2 binding to HR1 is inhibited by the fusion inhibitor enfuvirtide (**f**) (*See Color Plates*)

but appear to vary in a strain-dependent manner (56, 57). Natural history studies as well as in vitro selection experiments on cells expressing CCR5 with an N-terminal deletion have demonstrated that HIV can adapt to use alternate regions of the coreceptor for entry, suggesting that substantial plasticity may exist in the manner of viral interaction with CCR5 (58, 59).

2.2 The HIV Fusion Process

HIV fusion is mediated primarily by the gp41 envelope subunit, which is comprised, from the N-terminus to the C-terminus, of an ectodomain, a transmembrane, and an endodomain region. The ectodomain (protruding outward from the viral membrane) consists of four key regions (Fig. 2) (18, 60). A hydrophobic fusion peptide sequence is located at the N-terminus. Adjacent to the fusion peptide is the first of two leucine zipper-like 4–3 repeat regions, known as heptad repeat 1 (HR1, N terminal), and distal to this region is heptad repeat 2 (HR2, C terminal) (61). Between these two HR regions is a hinge region where two cysteine residues are able to form a disulphide-bonded loop.

The proposed model for the native form of the gp120–gp41 trimer predicts that each gp41 molecule is held in a high-energy conformation with the fusion peptide folded back toward the viral membrane (19). Following binding to either coreceptor, gp120 dissociates from gp41, and the hydrophobic fusion peptide region of gp41 is propelled toward, and inserts into, the target cell membrane (19). This process has been compared with the "spring-loaded" mechanism proposed for the action of influenza HA2 protein (62, 63). Opening up the structure of gp41 in this way reveals the HR1 regions of gp41 as a trimeric coiled-coil structure. The HR2 regions then fold over to associate with the hydrophobic grooves of the HR1 trimer, forming a "hair pin" structure containing a thermodynamically stable six-helix bundle (Fig. 1) (25, 64). It is thought that formation of the six-helix bundle brings the viral and cellular membranes into close proximity and leads to the creation of a fusion pore permissive to viral entry into the cell (19, 21, 22). Prior to the formation of the six-helix bundle, a "pre-hair pin" intermediate stage may exist for several minutes, thus exposing the N-terminal region of gp41 and providing an accessible target for the interaction of fusion inhibitors like enfuvirtide and other peptides that target the HR1 region (21, 65).

2.3 Inhibition of CCR5 Binding

Maraviroc and vicriviroc are small molecules that act through binding to the host CCR5 receptor and preventing its use as a coreceptor for HIV-1 entry. They appear to act allosterically by binding to a transmembrane pocket formed by several domains of the CCR5 molecule, and thereby altering the conformation of extracellular regions of CCR5 used by the virus for binding. Maraviroc and vicriviroc have both shown activity against a broad range of CCR5-tropic HIV viruses in vitro and in vivo. In a study of baseline susceptibility to maraviroc in vitro, a panel of 200 clinical isolate envelopes was tested in the PhenoSense™ Entry assay; the geometric mean IC_{50} to maraviroc was 13.7 nM (95% CI, 12.3–15.1 nM) with

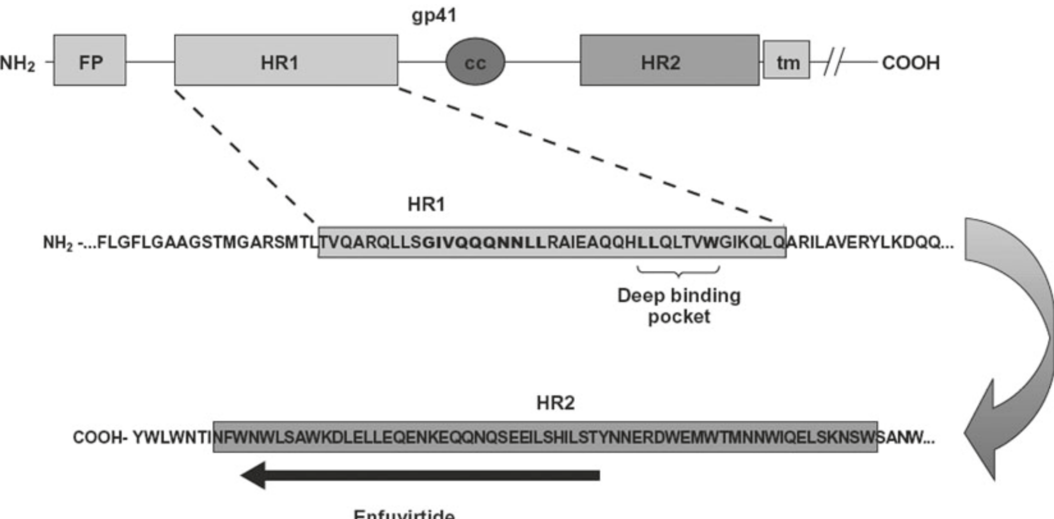

Fig. 2 Linear schematic of the HIV gp41 amino acid sequence. The amino acid sequences of the HR1 and HR2 regions are shown in detail. The deep binding pocket within HR1 is indicated and amino acids involved in the interaction between HR1 and HR2 are shown in *bold*. Amino acids thought to be involved in resistance to enfuvirtide are also shown in bold

a range for the geometric mean ± 2 standard deviations of 1.7-fold (36). Maraviroc was also active in a PBMC coculture system against a panel of 43 primary CCR5-using isolates with a geometric mean IC_{90} of 2.0 nM (95% CI, 1.8–2.4 nM) (66). Twenty-four-week data were recently reported for two Phase 2b/3 studies of maraviroc in treatment-experienced patients with exclusively CCR5-using HIV strains, as determined by phenotypic tropism testing. In this patient population (total $N > 800$ patients), the use of maraviroc with an optimized background regimen resulted in additional viral load reductions of −0.8 to −1.0 $\log_{10}$, relative to background therapy alone; patients receiving maraviroc also experienced significantly greater CD4+ cell count increases than the control group ($P < 0.001$) (8, 9). For vicriviroc, in vitro inhibition of a panel of 30 diverse CCR5-dependent HIV isolates was achieved with IC_{50}s ranging between 0.04 and 2.3 nM, and IC_{90}s between 0.45 and 18 nM (67). A Phase 2 study of vicriviroc compared to efavirenz in combination therapy in treatment-naive patients was halted due to vicriviroc inferiority; however, data have been reported for a second study, A5211, in treatment-experienced patients. Approximately 60 patients received vicriviroc at once-daily doses of 10 or 15 mg and, at 16 weeks, had experienced mean RNA reductions of 1.5–2.0 $\log_{10}$ copies/mL versus an approximately 0.5 log reduction for patients in the comparator arm (10). Although five malignancies of various types were reported among the patients receiving vicriviroc, two were also noted among patients receiving placebo, and no causal role for vicriviroc in their emergence (or, in some cases, re-emergence) has been described to date.

One of the original rationales for developing antiretrovirals targeting CCR5 binding came from studies showing that a deletion in the CCR5 coreceptor gene (CC5Δ32) conferred substantial protection from HIV-1 infection, and that individuals homozygous for this mutation appeared to be largely immunologically normal (68, 69). However, recent studies have demonstrated a critical role for CCR5 in the control of the West Nile virus infection, and have shown that individuals who are homozygous for the CCR5Δ32 mutation are at significantly increased risk of infection with the West Nile virus and of a fatal outcome if infected (70, 71). These findings could have implications for the safety of anti-HIV agents blocking CCR5, and indicate that long-term experience in clinical trials and clinical practice will be needed to fully assess the safety profile of these CCR5-blocking agents.

2.4 Inhibition of the HIV Fusion Process

Enfuvirtide is a 36-amino-acid peptide based on a portion of the gp41 HR2 region (gp160 amino acids 643–678) of the laboratory strain HIV-1_{LAI} (Fig. 2) (72). Enfuvirtide is thought to inhibit the fusion process by binding to HR1 and blocking its interaction with HR2 by competitively binding to the hydrophobic grooves of the HR1 trimer, thus preventing the formation of the “hair pin” structure and the subsequent membrane fusion (Fig. 3) (25, 40, 72–74). The in vitro antiviral activity of enfuvirtide was first demonstrated by Wild et al. (24, 40, 72), who investigated the ability of various synthetic peptides to block syncytium formation between cells expressing HIV envelope and uninfected CD4+ target cells. Enfuvirtide was able to inhibit fusion mediated by the envelope of various strains of HIV-1 with IC_{50} values in the nanomolar range. The activity of enfuvirtide against HIV-2 envelope-mediated cell fusion was about 1,000-fold lower. These results were confirmed by several other investigators, using various combinations of cell types and reporting IC_{50} values within a similar range (75–77). Enfuvirtide concentrations approximately 10–100 times higher than those required to inhibit cell–cell fusion are generally required to block infection with cell-free virus. For example, for the laboratory isolate HIV-1_{LAI}, Wild et al. report enfuvirtide IC_{50} values of 90 ng/mL (20 nM) in CEM cells and 1,100 ng/mL (240 nM) in peripheral blood mononuclear cells (72). In vitro studies with clinical isolates recovered from patients participating in Phase 2 trials of enfuvirtide demonstrated that enfuvirtide exhibited a range of IC_{50}s against primary isolates, with a geometric mean IC_{50} of 20 ng/mL (4 nM) in a cMAGI-based assay (78).

In the pivotal Phase 3 clinical studies TORO 1 and TORO 2, highly treatment-experienced patients received enfuvirtide in combination with an antiretroviral background regimen optimized with the aid of resistance testing and previous treatment history. At baseline, in vitro sensitivity of the study patients’ viruses to inhibition by enfuvirtide was assessed using a recombinant reporter virus assay (PhenoSense Entry assay, Monogram Biosciences). The baseline range of susceptibilities to enfuvirtide extended over a wide range of IC_{50} values, from close to 10 ng/mL to over 7 µg/mL, with a geometric mean IC_{50} of 260 ng/mL (79). At 24 weeks, patients experienced additional virological suppression of 0.8–0.9 $\log_{10}$ copies/mL, relative to control patients; at 48 weeks, patients randomized to receive enfuvirtide maintained a least-squares mean reduction of 1.5 $\log_{10}$ copies/mL from baseline in plasma viral load (6, 7, 80). Significantly, patients harboring viruses across the range of baseline susceptibility to inhibition by enfuvirtide responded with comparable decreases in plasma virus levels (81).

3 Mechanism of Drug Resistance

3.1 Resistance to CCR5 Binding Inhibitors

Three potential mechanisms have been suggested through which CCR5-using HIV strains could develop resistance to CCR5 binding inhibitors: coreceptor switching to use

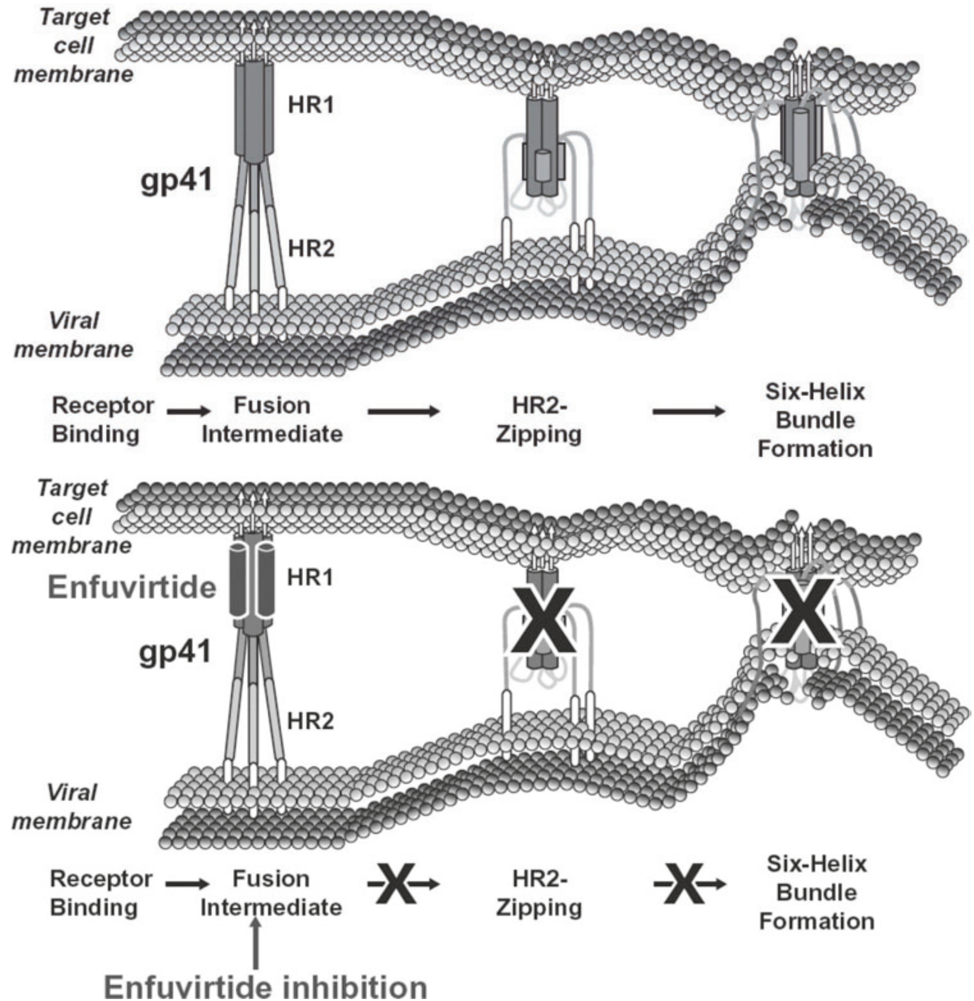

Fig. 3 Proposed mode of action of enfuvirtide. Enfuvirtide binds to the conserved hydrophobic groove of the HR1 region, preventing the association of HR1 and HR2 and the resultant formation of the folded fusion active six-helix bundle

CXCR4; increased affinity for unbound CCR5; or selection of variants capable of using CCR5-inhibitor complexes for entry (82, 83). While each of these has been observed to some degree, the main mechanism for resistance reported to date has been the emergence of the strains capable of using inhibitor-coreceptor complexes (66, 82, 84, 85). However, it should be noted that the results described for CCR5 inhibitors to date have largely been from in vitro selection experiments rather than from viruses developing resistance in the presence of the inhibitors in vivo.

The issue of coreceptor switching as a potential mechanism of resistance has received much attention, due to the strong association demonstrated between CXCR4-using viruses and disease progression in natural history cohorts (47, 86–89). However, the emergence of CXCR4-using strains may be a rare event due, in part, to a substantial genetic barrier to emergence of strains without major defects in viral fitness; this notion is supported by the low incidence of CXCR4 use emerging during in vitro selection experiments with a number of CCR5 binding inhibitors (82, 83, 90, 91). In addition, CXCR4-using strains appear to be under significant negative selective pressure from the immune system, which may further reduce the likelihood of their early emergence in many patients (92, 93). Nonetheless, strains capable of using CXCR4 have been detected in approximately 50% of patients with low CD4+ cell counts in both treated and untreated populations (47–49, 51) and have emerged in a small number of patients receiving CCR5 binding inhibitors in clinical studies (84), and therefore the question of the impact of their emergence in the context of CCR5 inhibition remains highly germane (50). Results touching on this point were reported from a Phase 2 study of maraviroc versus placebo (in combination with an optimized background regimen) in patients with dual/mixed virus populations at baseline (94). In that study, approximately 20% of patients receiving maraviroc (compared to 4% in the placebo arm) developed exclusively CXCR4-using strains during treatment; those patients, nonetheless, experienced average CD4+ cell increases of approximately 40 cells/mm^3. It appears that further studies will be needed to understand the clinical impact of the emergence of CXCR4-using strains under conditions different from those permissive for their emergence in untreated patients.

A second potential mechanism of resistance to CCR5 binding inhibitors lies in the emergence of strains with higher

affinity for the unbound coreceptor, which might allow fusion at lower concentrations of free CCR5. Theoretical support for this mechanism was provided by Reeves et al. in a report demonstrating that increased coreceptor affinity as well as increased coreceptor expression levels resulted in faster fusion kinetics and reduced susceptibility to a range of entry inhibitors, including both enfuvirtide and a small molecule CCR5 binding inhibitor. Studies of viruses developing resistance to other CCR5 binding inhibitors have demonstrated that modest levels of resistance (approximately threefold) could develop through this mechanism (83), and variations in coreceptor affinity have also been proposed as an explanation for some of the differences in sensitivity to CCR5 antagonists observed among naive viruses (82).

Experiments using in vitro selection to obtain viruses resistant to CCR5 binding inhibitors have been performed for several compounds and have consistently selected for viruses that appear to be capable of using CCR5-inhibitor complexes (Fig. 4). In general, resistant viruses have been more easily obtained for clinical isolates than for laboratory adapted strains, as would be expected, given the diverse envelope quasispecies typical of primary isolates (82, 90). For maraviroc and vicriviroc, strains obtained following serial passage of the CC1/85 strain in the presence of increasing concentrations of the drug remained dependent on CCR5 for entry, but had became highly resistant (>1,000-fold) to the agent used for selection (66, 90). In the case of maraviroc, high-level resistance was conferred by the presence of two mutations in the V3 loop; using an analogous starting virus, resistance to vicriviroc developed without mutations were in the V3 loop (83, 95). Given that the vicriviroc-resistant strains retained a largely CCR5-dependent phenotype, it was considered likely that other changes in the gp120 glycoprotein were responsible for the observed resistance phenotype (90). In most other previously reported examples of in vitro selection for resistance with CCR5 binding inhibitors, mutations in the V3 loop have played a primary role in the resistance phenotype (Table 1) (66, 82, 83, 90, 95, 96).

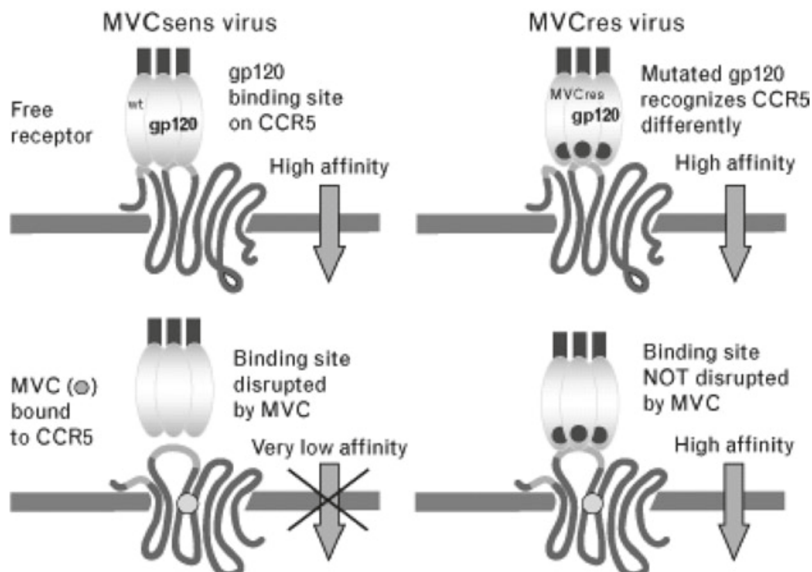

Fig. 4 Allosteric mechanism of action and resistance to CCR5 binding antagonists. CCR5-using HIV-1 binds to the extracellular domains of the CCR5 receptor with high affinity. Small molecule CCR5 binding inhibitors such as maraviroc (MVC) bind to a pocket formed by multiple CCR5 transmembrane strands and alter the conformation of exposed epitopes used for HIV binding. Resistant viruses could harbor mutations allowing the virus to bind to native and inhibitor-bound CCR5 with comparable efficiencies, or may, in some cases, recognize a binding domain that is not disrupted by maraviroc. (Adapted from Westby (82))

Table 1 Summary of selection experiments in vitro for resistance to small molecule CCR5 binding inhibitors

Study	Compound	Input virus	Alterations in V3 loop sequence	Regions potentially impacting resistance	Postselection coreceptor use
Trkola et al. (2002), Kuhmann et al. (2004)	AD101	CC1/85	H308P, K305R, A316V, G321E	Residues 271–386, gp41	R5
Marzosan et al. (2006)	Vicriviroc	CC1/85	none	V1, V2, C2, C3; gp41 FP, pre-HR2	R5 PBMC; R5/X4 U87
		CC101.6	(H308P baseline)	V4, gp41 FP, and pre-HR1	R5
Westby et al. (2007)	Maraviroc	CC1/85	A316T, I323V	V2, V3, C3, V4, and gp41	R5
		RU570	QAI deletion	V1, C4, V3, V4, and C5	R5
Baba et al. (2006)	TAK-652	KK	T306K, Q309E	V2, C2, V3, V4, C4, gp41	R5/X4 mix

FP fusion peptide

3.2 Resistance to Enfuvirtide

As enfuvirtide is thought to act by binding to gp41 in HR1 and preventing the interaction between HR1 and HR2, it might be expected that mutations leading to resistance would arise in the enfuvirtide binding target in HR1. This notion was validated by early experiments reported by Rimsky et al. (97), who passaged HIV-1_{IIIB} in the presence of increasing concentrations of enfuvirtide in vitro. One highly resistant subclone was examined that exhibited an approximately 100-fold decrease in sensitivity to enfuvirtide, and that carried amino acid substitutions in a highly conserved region of the enfuvirtide binding target region at positions 36 (glycine to serine) and 38 (valine to methionine). Clones with only one of these substitutions were less resistant than the double mutant, exhibiting five- to tenfold changes in sensitivity to inhibition by enfuvirtide.

The findings of Rimsky and colleagues were validated and extended based on studies of viral resistance to enfuvirtide observed during Phase 2 clinical trials. In those studies, mutations were common within a gp41 region encompassing residues 36–45 (78, 98, 99). Site-directed mutagenesis experiments investigating the region between amino acid position 29 and position 45, and in vitro binding studies with recombinant HR1 fusion proteins containing these same mutations showed that mutations at codons 36, 39, 40, and 43 altered the binding affinity of enfuvirtide to the HR1 fusion proteins in a manner that was proportional to the effect of the mutations on enfuvirtide susceptibility of mutant viruses (Table 2). The reductions ranged from no effect for the Q39H substitution to reductions in enfuvirtide binding of more than 40-fold and decreases in susceptibility of more than 20-fold resulting from the substitutions Q40H and N43D, supporting the notion that reduced binding affinity for enfuvirtide is possibly the major mechanism by which mutations in HR1 confer resistance to enfuvirtide (98).

The primary role of mutations in gp41 residues 36–45 was confirmed by population sequencing of samples from 279 patients on enfuvirtide who were experiencing protocol-defined virological failure in the TORO 1 and TORO 2 Phase 3 clinical trials (81). In that analysis, the most frequently observed mutations (occurring either alone or as mixtures with the wild-type residue in samples from ≥15% of patients) were G36D, V38A, and N43D; the mutations V38M, Q40H, N42T, N43K, and L45M were also each detected in samples from more than 5% of patients (81). Overall, 92.7% of the virological failure samples tested harbored mutations in gp41 residues 36–45; furthermore, of those demonstrating at least fourfold decreases from baseline in susceptibility to enfuvirtide, 98.8% carried mutations in gp41 residues 36–45.

Studies of these substitutions by site-directed mutagenesis experiments, either alone or in combination, has helped further elucidate their impact on enfuvirtide susceptibility (98). Using an NL4-3 background, more than tenfold reductions in enfuvirtide susceptibility (compared with the parental wild type) resulted from the single amino acid substitutions V38A, Q40H, and N43D, and for all the double amino acid substitutions tested (Table 3) (98). While mutations at codon 42 alone had only a modest impact on enfuvirtide susceptibility, the greatest impact (>100-fold reductions in enfuvirtide susceptibility) was observed for combinations of mutations at codons 38 and 42 (Table 3). High levels of resistance have also been observed for primary isolates with two amino acid substitutions between gp41 amino acids 36 and 45 that were recovered from patients during enfuvirtide

Table 2 Effects of amino acid substitutions at positions 36 to 43 of HIV-1 gp41 on enfuvirtide binding to the first heptad repeat, and on the antiviral activity of enfuvirtide

Amino acid substitution	Binding (B_{50} μg/mL)	Fold change compared with NL4-3G	Antiviral activity (EC_{50} μg/mL)	Fold change compared with NL4-3G
NL4-3G[a]	0.10		0.009	
G36D	0.70	7.0	0.091	10.1
G36S	0.95	9.5	0.063	7.0
Q39H	0.25	2.5	0.011	1.2
Q40H	4.33	43.3	0.256	28.4
N43D	4.96	49.6	0.210	23.3

[a]NL4-3 altered to match the consensus sequence at amino acid position 36 (aspartic acid replaced by glycine)

Table 3 Enfuvirtide susceptibility of HIV-1 mutants carrying single and double substitutions in gp41 amino acids 36–45

Substitution(s)[a]	Enfuvirtide EC_{50} (μg/mL)	Enfuvirtide fold change[b]
NL4-3G[c]	0.012	
G36D	0.091	8
G36S	0.088	7
V38A	0.188	16
Q40H	0.256	21
N42T	0.045	4
N42E	0.015	1
N42S	0.006	1
N43D	0.210	18
N43S	0.067	6
N43K	0.063	5
L44M	0.021	2
L45M	0.017	1
G36S + L44M	0.181	15
N42T + N43K	0.388	32
N42T + N43S	0.727	61
V38A + N42D	1.685	140
V38A + N42T	1.782	149
V38E + N42S	6.156	513

[a]Relative to a consensus wild-type sequence of GIVQQQNNLL (NL4-3G)
[b]Relative to NL4-3G
[c]NL4-3 altered to match the consensus sequence at amino acid position 36 (aspartic acid replaced by glycine)

Table 4 Fold-change from baseline in susceptibility to enfuvirtide observed for common mutations during Phase 2 or Phase 3 clinical trials

Substitution(s)[a]	N[b]	Range of change from baseline	Fold range
G36D (cMAGI)	3	4–450	113
G36D	8	15–344	23
G36V	5	15–124	8
V38A	35	10–215	22
V38E	4	132–6,217	47
V38M	10	7–68	10
Q40H	3	19–97	5
Q40H + L45M	3	65–161	2
N42T	3	9–185	21
N43D	25	5–401	80

[a]Substitutions in the on-treatment virus isolate relative to a consensus wild-type sequence of GIVQQQNNLL
[b]Data displayed for $n = 3$ or more
[c]This virus also harbored the N42S polymorphism

therapy (78). These findings underscore the primary role of this locus in development of resistance to enfuvirtide.

As discussed above, baseline susceptibility to enfuvirtide varied widely between virus strains from different patients. Similarly, individual mutations conferred a wide range of loss of enfuvirtide susceptibility in both primary isolates and pseudotyped virions. For example, in primary isolates, the G36D substitution resulted in fold changes in EC_{50} relative to pretreatment virus of 450, 17, and 4 in three different patients' virus pairs, while the range of decreased susceptibilities observed in strains from eight patients harboring G36D mutations in the Phase 3 studies was 15- to 344-fold (Table 4) (78, 81). Together, these data suggest that the capacity of any specific genotypic change in HR1 to confer alterations in phenotypic sensitivity to enfuvirtide is strongly influenced by the rather variable genetic context of the viral envelope in which it emerges. In addition, at least two mutations in the HR2 domain, N126K and E138A, have been reported, which may act as secondary or compensatory changes in viruses with resistance mutations in HR1 (81, 100, 101). Intriguingly, another baseline polymorphism, E137K, has been identified as being permissive for the emergence of the N43D mutation, possibly due to electrostatic interference between the side chains of the wild-type HR2 residue and glutamic acid at position 43 in the six-helix bundle (102).

3.3 The Role of Regions Outside gp41 HR1 in Determining Fusion Inhibitor Susceptibility

In spite of the conserved nature of the enfuvirtide primary resistance locus of HR1 amino acids 36–45 in fusion inhibitor-naive viruses, phenotypic studies show that clinical isolates from fusion inhibitor-naive patients display a broad range of susceptibilities to inhibition by enfuvirtide (78, 81). This clearly implies that regions other than the primary resistance locus impact susceptibility to enfuvirtide.

Several studies have attempted to identify functional or genetic correlates of the variation in HIV-1 susceptibility to fusion inhibitors (103–108). Reeves et al. demonstrated that susceptibility to enfuvirtide could be modulated through alterations in coreceptor binding affinity. These differences are possibly due to changes in the rate of fusion following initial receptor binding, which would alter the length of time during which the enfuvirtide binding target is exposed in the pre-hairpin intermediate state and thus vulnerable to interference (107). This notion highlights the possibility that differences in viral susceptibility to enfuvirtide could occur based on coreceptor-specific differences in fusion kinetics. Several studies have examined this question; however, results to date have been contradictory. The studies of Reeves and colleagues, for example, followed work by Derdeyn et al., which examined 14 fusion inhibitor-naive primary isolates and reported reduced sensitivity to enfuvirtide for CCR5 tropic isolates (mean IC_{50} for R5 isolates was 0.8 log higher than mean IC_{50} for X4 isolates) (108). Using NL4-3 chimeras, these investigators suggested that enfuvirtide sensitivity was modulated by the V3 loop, a major determinant of coreceptor specificity. A subsequent study by this group examined a larger set of 55 primary isolates, and found a difference in enfuvirtide susceptibility between CCR5-tropic and CXCR4-tropic strains of 0.3 $\log_{10}$ ($P < 0.001$, Wilcoxon rank sum test) (106). However, in a study from Greenberg et al. analyzing enfuvirtide susceptibility in 111 virus isolates, no differences in susceptibility were seen between CXCR4, CCR5, or dual-tropic viruses from separate patients, or between serially obtained isolates from patients who underwent a phenotypic switch from CCR5 to CXCR4 isolates during the course of their disease (109). Finally, Melby et al. examined susceptibility and coreceptor-use data from 724 baseline samples obtained in the enfuvirtide Phase 3 studies (48). In that work, dual tropic strains exhibited significantly lower IC_{50}s to enfuvirtide than either exclusively CXCR4 or CCR5 tropic strains; however, no difference in virological or immunological response to enfuvirtide-based therapy was observed between patients with CCR5 versus CXCR4 or dual/mixed isolates. Intriguingly, a significant bias was observed toward changes from dual/mixed to CCR5-dependent virus populations in patients whose regimens contained enfuvirtide when compared to similar patients receiving the background regimen alone (48). The disparate results from the various studies highlight the difficulties involved in comparing results obtained using different virus isolates and across different assay systems (110, 111).

A different approach to examine the impact of coreceptor tropism on susceptibility to enfuvirtide was used by Stanfield-

Oakley and colleagues, who constructed reciprocal envelope gp120–gp41 chimeras between R5 and X4 tropic isolates of widely differing sensitivities to enfuvirtide (104). Their findings indicated that the major determinants of sensitivity to enfuvirtide resided in gp41. Although these results did not rule out a role for gp120, they did strongly suggest that if gp120 had an influence on enfuvirtide susceptibility, it was relatively minor compared to that of gp41. Additional work from Heil et al. and Stanfield-Oakley et al. (103, 104) to further map susceptibility determinants within gp41 found that neither the HR1 region nor the HR2 region of gp41 was sufficient to account for enfuvirtide sensitivity; rather, both regions appeared to contribute. Thus, changes in single amino acids in HR1 (at position 45) and HR2 (at position 135) were sufficient to significantly modulate enfuvirtide susceptibility (104). These residues are in close proximity in the six-helix bundle complex (20), thus suggesting that contacts and affinities between the viral envelope HR1 region and the HR2 region may be major determinants of enfuvirtide sensitivity in fusion inhibitor naive viruses. This conclusion is supported by reports of an association between the primary N43D mutation in HR1 and the secondary S138A mutation in HR2 (100), and the association between the emergence of N43D and the presence of a baseline polymorphism at position 137 (E137K) in HR2 (102).

3.4 The Impact of Fusion Inhibitor Resistance Mutations on Viral "Fitness"

Data addressing the impact of resistance to CCR5 binding inhibitors on viral replicative capacity are relatively limited. One study of resistant isolates selected through in vitro passage for resistance to maraviroc found that clones from one strain replicated poorly compared to wild type, while those from another strain replicated comparably to wild type (66). Given the highly variable nature of the envelope gene, this result is not surprising, and studies of replicative capacity in strains selected in vivo during treatment with a CCR5 binding inhibitor will be needed to address this question in a more clinically relevant context.

The HR1 binding target of enfuvirtide is highly conserved and is thought to play an essential role in viral entry (78, 81, 112, 113); mutations that emerge in this region during treatment with enfuvirtide might thus be predicted to have a negative impact on the replicative capacity or "fitness" of the virus. This hypothesis was confirmed by Lu et al., who performed growth competition assays with either NL4-3 clones carrying various HR1 mutations or with recombinant viruses expressing envelopes derived from clinical isolates (114). Comparable results were obtained for site-directed mutant viruses and for envelopes derived from clinical samples; in the absence of the drug, the wild-type virus was able to replicate with faster kinetics than viruses bearing mutations, and a relative order of GIV > DIV > DTV > DIM > SIM was determined. In the presence of enfuvirtide, the relative order of fitness was reversed. Further studies by these workers established a highly significant inverse correlation between the decreases in enfuvirtide IC_{50} conferred by the mutations and increases in virus replication kinetics. In addition, in vivo fitness was evaluated by Marconi and colleagues, who examined the decay of the V38A mutation during enfuvirtide interruption in 3 patients. That study found a fitness defect of 25–50% for V38A, relative to wild-type (115).

In addition to an apparent impact on viral replicative capacity, specific enfuvirtide resistance mutations have also been associated with variations in CD4+ cell responses in patients continuing enfuvirtide-based therapy after virological rebound. In a report by Aquaro et al. on a cohort of 54 patients who added enfuvirtide to a failing antiretroviral regimen, patients developing the most common resistance genotype, V38A/E, experienced significant CD4+ cell gains through 36 weeks of treatment, while those developing the Q40H mutation experienced a loss of CD4+ cells (116). Those results were confirmed and extended in an analysis of 134 patients in the enfuvirtide Phase 3 TORO studies. Patients were classified based on enfuvirtide resistance genotype at the time of meeting protocol-defined virological failure criteria, and were examined for changes in CD4+ cell counts from the time of virological failure onward. After an additional 48 weeks of enfuvirtide-based therapy, the group of patients with V38 mutations had experienced a significant further increase in CD4+ cell counts, those with N43D or other genotypes had largely maintained their CD4+ cell gains, and the group of patients with the Q40H mutation had experienced a CD4+ cell decline back to baseline levels (124). These findings may help to explain earlier reports of continued CD4+ cell benefits after virological rebound in small observational cohorts (117, 118).

3.5 Interactions Between Entry Inhibitors

The potential for cross-resistance between fusion inhibitors and coreceptor binding inhibitors is expected to be limited because the former bind to gp41, while the latter bind to the cellular chemokine coreceptor. This notion was supported by a study examining the activity of small molecule CCR5 binding inhibitors against enfuvirtide-resistant viruses, and another study examining enfuvirtide activity against maraviroc-resistant viruses (66, 119, 120). In both cases, viruses resistant to an entry inhibitor targeting one stage of viral entry remained fully sensitive to the other; thus, their use in sequence or in combination may well be possible and

advantageous. Furthermore, because maraviroc and vicriviroc are active only against the subset of viruses that enter cells through the CCR5 receptor, additional advantages may be gained by combining them with an agent, such as enfuvirtide, that is fully active against CXCR4-using strains. However, it should also be noted that increased affinity for CCR5 has been suggested to confer some degree of reduced susceptibility to both enfuvirtide and to CCR5 binding inhibitors, a notion supported by a reported correlation between reduced susceptibility to enfuvirtide and TAK779 in patients experiencing disease progression in the absence of CXCR4-using strains (121). It would therefore be of interest to evaluate the degree of covariation in susceptibility to enfuvirtide for strains with varying susceptibility to other small molecule CCR5 binding inhibitors.

Within the class of CCR5 binding inhibitors, it might be anticipated that cross-resistance would be observed for agents that bind to similar sites on CCR5, such as the transmembrane pocket. However, if resistance to these agents occurs via a noncompetitive mechanism, such as viral adaptation to use a coreceptor–inhibitor complex, then cross-resistance would depend not upon the site of binding, but rather upon the degree of similarity between the CCR5 conformations conferred by binding of the various inhibitors. Consistent with this idea, results reported to date indicate that cross-resistance between chemically unrelated compounds is usually minimal, even among small molecule CCR5 inhibitors that compete with one another for binding to CCR5 (66, 82). These data suggest that sequential use of at least some CCR5 antagonists may be possible even after development of resistance to one member of the class. However, it should be noted that in vitro selection for resistance to vicriviroc resulted in strains that were also resistant to the related compounds SCH-C and AD101, although not to enfuvirtide (90). Furthermore, in the same experiments, resistance was selected more rapidly in strains that started with partial resistance following selection with a different but related compound. If initial low-level resistance is indeed a result of increased coreceptor affinity, as previously suggested, a similar effect could be expected across the CCR5 inhibitor class, and could thus potentially lower the genetic barrier to development of resistance to second-line compounds within the class.

Studies have already demonstrated significant in vitro synergy between enfuvirtide and aplaviroc, PRO 542 (76), and SCH-C (122). This is consistent with work suggesting that agents that inhibit the initial steps toward entry, especially interactions with CCR5 or CXCR4, may increase the window of opportunity for peptide inhibitors such as enfuvirtide to interfere with gp41-mediated fusion (107). It may thus prove to be more beneficial to use these compounds in combination, rather than in sequence. In addition, strong synergy has been reported between the CCR5 monoclonal antibody PRO140 and maraviroc, vicriviroc and TAK779 (123).

4 Conclusion

The fusion inhibitor enfuvirtide has been a valuable addition to the armamentarium of anti-HIV therapies, particularly for patients harboring viruses resistant to other drugs. Enfuvirtide has been shown to act by binding to the HR1 region of HIV gp41 and preventing the formation of the six-helix bundle that draws the viral and cellular membranes together during the fusion process. In vitro and in vivo studies clearly implicate substitutions within HR1 at gp41 amino acids 36–45 in the development of resistance to enfuvirtide. In addition, secondary mutations have been observed at a limited number of positions in HR2 in viruses developing resistance through mutations in HR1. Viruses harboring enfuvirtide resistance mutations have been shown to have reduced viral replicative capacity in the absence of the drug, both in vivo and in vitro, and preliminary results have shown that specific mutations may also be associated with continued CD4+ cell increases, even in patients experiencing virological rebound. Maraviroc and vicriviroc are small molecule CCR5 binding inhibitors, presently in the late stages of clinical development. Maraviroc and vicriviroc have been shown to bind to a transmembrane pocket on CCR5 and alter the presentation of extracellular CCR5 domains to HIV-1, thereby inhibiting CCR5-using viruses at an early stage of the viral entry process; they do not, however, block entry via the CXCR4 coreceptor. Although low-level resistance may be acquired through the development of increased coreceptor binding affinity, the primary mechanism of resistance appears to be acquisition of mutations in gp120, usually including residues within the V3 loop, that allow the virus to use CCR5-inhibitor complexes for entry. Consistent with their allosteric mechanism of action, resistance to these compounds is characterized by reduced maximal levels of inhibition rather than a right-shifted inhibition curve, and extensive cross-resistance is usually observed only for chemically related compounds. The lack of cross-resistance between enfuvirtide and small molecule CCR5 binding inhibitors, their reported in vitro synergy, and the high degree of suppression of CXCR4-using strains reported during enfuvirtide-based therapy support the importance of exploring the concomitant use of enfuvirtide and CCR5 binding inhibitors in combination antiretroviral therapies.

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Chapter 36
Resistance to Inhibitors of Human Immunodeficiency Virus Type I Integration

Daria J. Hazuda

1 The Role of Integrase in HIV-1 Replication

The genome of the human immunodeficiency virus type 1 (HIV-1), as well as all other retroviruses, encodes three proteins with enzymatic activities that are essential for viral replication: reverse transcriptase (RT), protease (Pr), and integrase (In). The development of multiple, orally bioavailable drugs that target the viral reverse transcriptase and protease revolutionized the treatment of HIV-1 infection and AIDS by providing antiretroviral regimens which combine multiple agents to enhance the overall efficacy of therapy and forestall the development of drug resistance, as reviewed in (56). However, as a consequence of the persistent nature of HIV-1 infection and the complexities of adherence, the selection and spread of viral variants resistant to one or more drug classes have steadily increased (36, 39, 62). Effective treatment of multidrug-resistant infections in both treatment-naive and treatment-experienced patients requires the development of new therapies to manage HIV-1 as a chronic disease.

Integrase inhibitors are among the emerging opportunities for novel antiretroviral agents to treat drug-resistant HIV-1 infections (9, 55, 63). Integrase catalyzes the integration of the newly reverse-transcribed HIV-1 DNA into the host cell's genomic DNA, as reviewed in (1). Integration is a characteristic and essential feature of retroviral replication, required for both the stable maintenance of the viral genome and the expression of viral genes. Each of the sequence-specific events in integration, *assembly* with the viral DNA, *3′ endonucleolytic processing*, and *strand transfer* are carried out by the virally encoded enzyme integrase (16). In the context of HIV-1 replication, these events proceed in a successive manner as follows (Fig. 1a). Integrase first *assembles* as a stable complex [termed preintegration complex (PIC)] at specific sequences within the long terminal repeat (LTR) regions at each end of the viral DNA. Integrase then *processes* the terminal 3′ dinucleotide from the DNA, leaving a recessed 3′-OH at both termini. In the subsequent *strand transfer* reaction, integrase nicks the host cell or target DNA on each strand, and covalently links the 5′ phosphate of the target DNA to the recessed 3′-OH of the viral DNA. In the final step of the process, the integrated intermediate is repaired by cellular enzymes to create an intact double-stranded product.

While integrase is entirely sufficient to catalyze each of these three specific steps required for integration in vitro, in the infected cell various host cofactors are not only essential to mediate the final repair but are also believed to facilitate and enhance the overall efficiency of integration during retroviral infection (2, 10, 37, 52). In the case of HIV-1, several putative host integration cofactors have been identified, including integrase interacting protein (INI1), lens epithelium-derived growth factor (LEDGF), and barrier to autointegration factor (BAF) (3–6, 32, 35, 41, 46). These cellular factors interact either with integrase and/or the viral DNA, and may act to prevent autointegration, traffic or target the PIC to the cellular DNA, and/or tether the PIC to the nuclear membrane. The observation that integrase interacting factors may be required for anchoring or trafficking PICs during HIV-1 infection suggests that integrase may participate in several processes required for integration.

In addition to this complex role in integration, integrase has been shown to influence other steps in the HIV-1 infection cycle, including reverse transcription and virus assembly. Many of these effects on HIV-1 replication have been discriminated on the basis of mutagenesis and/or complementation studies (20, 40, 43, 44, 61). As integrase is required both directly and indirectly for integration, as well as for reverse transcription and viral assembly, and may interact with several host factors, there may be opportunities for multiple inhibitory mechanisms directed against this single protein target. However, this functional complexity also presents interesting and exciting challenges for understanding the potential implications of integrase inhibitor resistance and effects on viral replication capacity. As will be discussed, specific mutations within integrase can differentially affect one or more activities in the HIV-1 infection process (15, 34, 42, 43, 61),

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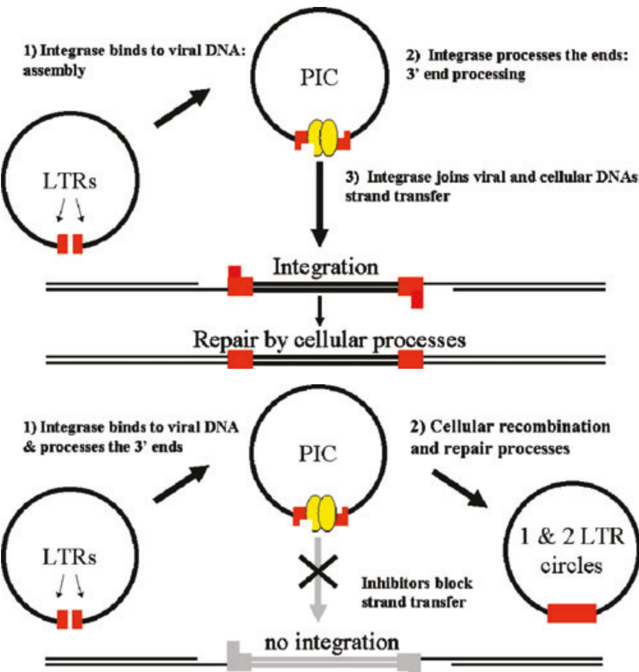


Fig. 1 (**a**) Schematic representation of the multistaged process of integration. (**b**) Strand transfer inhibitors shift the metabolic fate of the HIV-1 DNA during infection (*See Color Plates*)

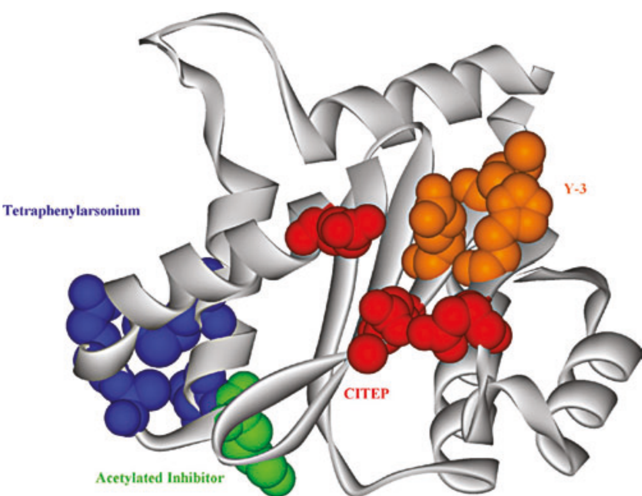


Fig. 2 Four inhibitor binding sites have been mapped in the HIV-1 integrase catalytic core domain. In the first inhibitor binding site, the Y-3 binding residues identified by co-crystallization of the inhibitor with avian sarcoma virus integrase catalytic domain (45) are shown in *orange* (I60, Q62, and H114, which corresponds to K119 in ASV integrase). The second inhibitor binding site was identified using an acetylated inhibitor that specifically modified K173 (shown in *green*) (58). The third site binds tetraphenylarsonium; residues that interact with this compound are shown in *blue* (50). The catalytic triad (D64, D116, E152) and metal are highlighted in *red*. These residues and the metal are required for high-affinity binding of the diketo acids (23) and related strand transfer inhibitors (*See Color Plates*)

and resistant viruses selected with prototypic integrase inhibitors have shown a variety of phenotypes that are consistent with the pleiotropic role of integrase in HIV-1 replication.

Although a large number of structurally and mechanistically distinct integrase inhibitors have been described, to date only compounds that selectively inhibit the strand transfer activity of integrase have been validated as bona fide inhibitors of integration in vitro (19, 25, 29) and demonstrated antiviral activity in vivo (30). Given the promising clinical results for the most advanced integrase inhibitors in development, raltegravir and elvitegravir, both of which belong to the specific class of inhibitors known as integrase strand transfer inhibitors or InSTIs (38, 51), this monograph will primarily focus on what is known from studies of integrase inhibitors with this mechanism of action. Only two such InSTIs have thus far progressed into advanced clinical development; however, a variety of structurally diverse compounds with this mode of action have been described. A complex spectrum of overlapping and differential cross-resistance to these agents has emerged, and is only beginning to be appreciated in detail (27).

2 Integrase Inhibitor Mechanism of Action

HIV-1 integrase is a 288 amino acid residue protein composed of three independently folded domains (13, 14). Although the structure of a full-length integrase has yet to be determined, structures of the subdomains have been elucidated for the HIV-1 enzyme and the homologous proteins from the simian immunodeficiency virus (SIV) and avian sarcoma virus (ASV), by either NMR or X-ray crystallography, as reviewed in (7). The dimeric structures observed for each of the subdomains are consistent with studies that have shown integrase functions as a multimer, both in vitro and in cells. The N-terminal and C-terminal domains (amino acid residues 1–50 and 213–288 in HIV-1 integrase) contain the "HH–CC" zinc binding and DNA binding regions, respectively, whereas the catalytic core domain (amino acids 51–212 in HIV-1 integrase) includes the active site residues, aspartates 64 and 116 and glutamate 152, that coordinate the divalent metal ion cofactor (or cofactors) required for phosphodiester bond cleavage/formation (22). This "DDE" motif is conserved among all retroviral integrases and is analogous to the metal binding architecture found at the active sites of many magnesium-dependent phosphotransferases (12, 47).

Cocrystallography studies using the integrase catalytic core domain have elucidated four independent binding sites for inhibitors (45, 50, 53, 58) (Fig. 2), and at least two distinct inhibitory mechanisms have also been demonstrated in vitro. From the perspective of drug development, the least advanced mechanism is one in which compounds interfere with the interaction between integrase and DNA and prevent assembly of the stable strand transfer complex (25, 29).

Assembly inhibitors such as Y3, or catechols such as dihydroxyphenyltriphenylarsonium, may bind at the DNA binding site and/or at the intra- or inter-subunit interfaces, blocking the interactions required for assembly on the DNA. Therefore, in biochemical assays assembly inhibitors block all integrase biochemical functionalities, including 3′ end processing, strand transfer, and disintegration. The second mechanism of action is the one exemplified by the clinical compounds, raltegravir and elvitegravir. In this mechanism, compounds selectively interfere with strand transfer, the last catalytic step in the reaction, with minimal effects on either assembly or 3′ end processing (17). *Strand transfer inhibitors* bind at the enzyme active site and exhibit high affinity for the enzyme/DNA complex. Among the potential mechanisms for integrase inhibitors, only strand transfer inhibitors have been validated in vivo, both in SHIV-infected rhesus macaques (30) and in HIV-1-infected patients (11, 24, 38, 48, 51). (For a historical perspective on inhibitors of HIV-1 integrase, the reader is referred to the following general review (31).)

The 4-aryl-2,4-diketobutanoic acids (or diketo acids) L-731988 and L-708906 (Fig. 3) represent the archetypical inhibitors of strand transfer (28). These compounds were the first integrase inhibitors demonstrated to have activity against HIV-1 in cell culture, directly as a consequence of their effect on integration. The validation of integrase as the molecular target of action of these compounds was established by selecting resistant variants, identifying mutations in integrase, and demonstrating an association between these mutations and the resistant phenotype (19, 25, 29), as well as by elucidating the inhibitor mechanism of action in biochemical assays and correlating this activity with the effect of the inhibitor on HIV-1 replication. In biochemical assays and in HIV-1 infected cells, the diketo acids were shown to inhibit integration, but to have little or no effect on integrase-mediated processing of the viral DNA (25, 29). In the context of HIV-1 infection, blocking integration allows the viral DNA to be metabolized by cellular enzymes, resulting in nonfunctional circular byproducts and an irreversible block of the viral replication cycle (Fig. 1b). The accumulation of 1 and 2 LTR circular DNA byproducts is a defining feature of the effect of integrase strand transfer inhibitors, but was first noted in HIV-1 viruses with integrase mutations that are defective in integration (59, 61).

Studies designed to understand the biochemical and molecular basis of strand transfer inhibitors have identified distinct properties that characterize compounds with this mechanism of action. First, high-affinity binding of these inhibitors is restricted to integrase in a specific complex with the HIV-1 LTR sequence DNAs (17). The inhibitor bound complex is not competent to bind the cellular or target DNA substrate, and the net result is a selective inhibition of strand transfer. Strand transfer inhibitors are inactive in disintegration assays, as the substrates used in those assays are designed to mimic integration intermediates wherein the donor and target DNAs are covalently linked (8). Second, a variety of evidence suggests there is a direct interaction between the critical diketo carboxylate (or isosteric) pharmacophore in the inhibitor and the divalent metal ion (or ions) in the integrase active site (23). Binding of strand transfer inhibitors to the integrase/DNA complex is dependent on divalent metal, and the specific chemical structure of the pharmacophore has been shown to influence the overall activity of the inhibitor in biochemical assays, depending on whether magnesium or manganese is used in the reaction. In addition, although certain nonacid pharmacophore replacements exhibit only a modest loss in binding affinity, they are completely inactive in enzymatic transfer assays. Therefore, the overall affinity and specificity of these molecules is largely determined by the pendant substituents (R groups), but the sequestration of the active site metal cofactors by the pharmacophore is critical for inhibition (Fig. 4).

Based on these observations, and on information derived from X-ray crystallography studies of integrase and other metal-dependent phosphotransferase enzymes, a model for the interaction between the diketo acid moiety and two metals at the integrase active site has been proposed (23) (Fig. 4). The bond lengths and angles for the diketo acid pharmacophore represented in this model were based on the crystal structure of the diketone inhibitor 5-CITEP (1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone) (21) and are entirely consistent with a distance of 3.62 observed

L-731,988

L-708,906

IC_{50} = 0.01 uM Strand Transfer Inhibition IC_{50} = 0.04 uM

IC_{95} = 9.6 uM Antiviral Activity (HIV-1 IIIB) IC_{95} = 17.4 uM

Fig. 3 Structures of the two original diketo acid inhibitors of strand transfer: L-708906 and L-731988 (25, 29)

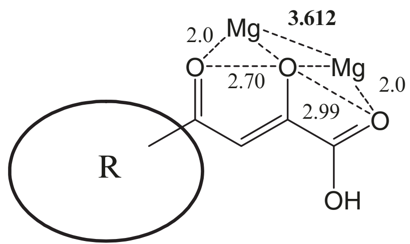


Fig. 4 Model of the interaction between the diketo carboxylate pharmacophore and two metals ions in the active site of integrase (23)

between the two active site metals in the crystal structure of ASV integrase. This model is also consistent with the known structural requirements for the pharmacophore and the proposed mechanism of action for these inhibitors, as well as with the observation that mutations engendering resistance map near the active site "DDE" residues that coordinate the metal cofactors (27).

3 In Vivo Activity of Integrase Strand Transfer Inhibitors

Subsequent to the identification of the diketone-based strand transfer inhibitors L-708906 and L-731988, numerous structurally diverse analogs that elaborate novel, isosteric replacements for the essential diketo acid pharmacophore were developed (Fig. 5a, b). Many of these analogs were designed to obviate pharmacologic liabilities such as protein binding and reactivity inherent to the diketone moiety. Although these novel inhibitors display a remarkable range of chemical diversity, in each case the critical elements of the metal-binding pharmacophore encompassed in the original diketo acid series are preserved. Notable among these compounds is a series of 8-hydroxy-(1,6)-naphthyridine carboxamides exemplified by the two inhibitors L-870810 and L-870812 (27, 30) (Fig. 6a), which provided proof of concept for the efficacy of integrase strand transfer inhibitors as antiretro viral agents in vivo.

Like the diketo acids, the naphthyridine carboxamides L-870810 and L-870812 are selective inhibitors of integrase strand transfer in vitro and in HIV-1 infected cells. In competition binding experiments, the naphthyridine carboxamides displace radiolabeled diketobutanoic acid L-731988 from the integrase/donor complex, indicating that these analogs bind to the assembled DNA complex within the same or overlapping regions of the active site. L-870810 and L-870812 are specific for HIV-1 integrase and the highly homologous enzyme from SIV, and are also effective inhibitors of HIV and SIV viral replication in vitro (30). While the robust antiviral L-870812 in rhesus macaques infected with the virus SHIV 89.6P provided the first proof of concept for integrase inhibitors in vivo, in the context of incomplete suppression extended treatment with L-870812 monotherapy selected for viruses with resistance. Interestingly, the appearance of the resistant variants containing an N155H mutation in integrase was not associated with a full rebound in plasma viremia, and there was no decline in CD4 cell levels despite ongoing viral replication. HIV-1 engineered with the N155H mutation in integrase was shown to exhibit decreased replication capacity in vitro, suggesting that decreased fitness of these resistant mutants could be associated with reduced pathogenicity.

The rhesus studies performed with L-870812 provided the first demonstration of efficacy for integrase strand transfer inhibitors in vivo. The related naphthyridine analog L-870810 was shown to have potent antiretroviral activity in HIV-1-infected patients, providing the first clinical proof of concept for the class (38). Although L-870810 was subsequently placed on hold due to preclinical toxicity, additional integrase strand transfer inhibitors have since advanced into clinical study. The two novel integrase strand transfer inhibitors to reach advanced clinical development, raltegravir (MK-0518) and elvitegravir (GS-9137, JTK303) (11, 24, 48, 51) are distinct from the naphthyridines, and each is from a different chemical series (Fig. 6b). Both compounds have demonstrated robust antiviral responses in ten-day monotherapy studies, and have proven effective in phase 2 studies conducted in patients with triple-class resistant HIV-1 infections. Based on phase 3 data, raltegravir was licensed for the treatment of HIV-1 infection in patients with drug-resistant virus in late 2007, in both the US and the EU.

4 Mechanism of Integrase Strand Transfer Inhibitor Resistance and Cross-Resistance: Genetics and Structural Analysis

Serial passage of HIV-1 in cell culture in the presence of inhibitors from either the diketone or naphthyridine carboxamide series selects for viral variants that contain multiple mutations within the integrase coding region. For a variety of different strand transfer inhibitors, the selection of resistance has been shown to require multiple passages of HIV-1 in cell culture, possibly as a result of both the sequential accumulation of mutations in integrase, as well as the reduced fitness of these mutants (18, 19, 27) (Shimura et al. 2007). Limited data from the clinical experience with raltegravir and elvitegravir corroborate these in vitro observations. Multiple mutations appear to be associated with high-level resistance, and are acquired sequentially over time.

In site-directed mutagenesis studies, only a subset of the observed mutations appear to reduce the susceptibility of the virus to the integrase inhibitor used for selection when introduced as a single change in the virus; however, the overall magnitude of resistance to the inhibitor can be significantly enhanced when these "primary mutations" are combined with specifically co-selected but otherwise silent "secondary mutations" (27). Thus, secondary mutations result in high-level InSTI resistance. While unique mutations have been observed with different integrase strand transfer inhibitors, the affected residues are all localized within the integrase active site (Fig. 7). Many of the mutations that have a primary effect on resistance (e.g., residues 155, 153, and 121) are proximal to the amino acid residues involved in

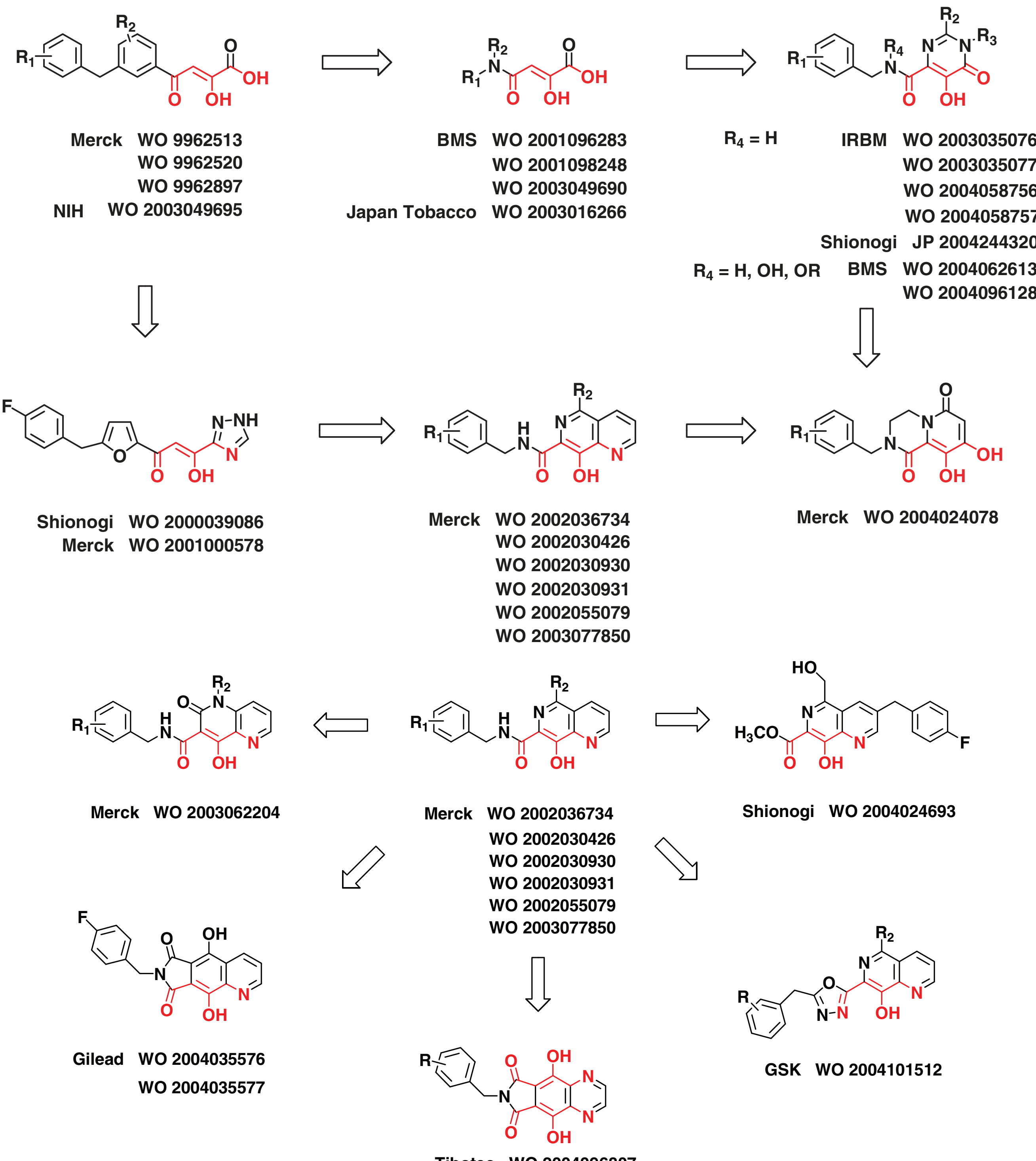


Fig. 5 Evolution of structurally diverse strand transfer inhibitors from the original diketo acids; the preservation of the metal binding pharmacophore in each chemical series is shown in *red*. Relevant references to the patent literature are as indicated (*See Color Plates*)

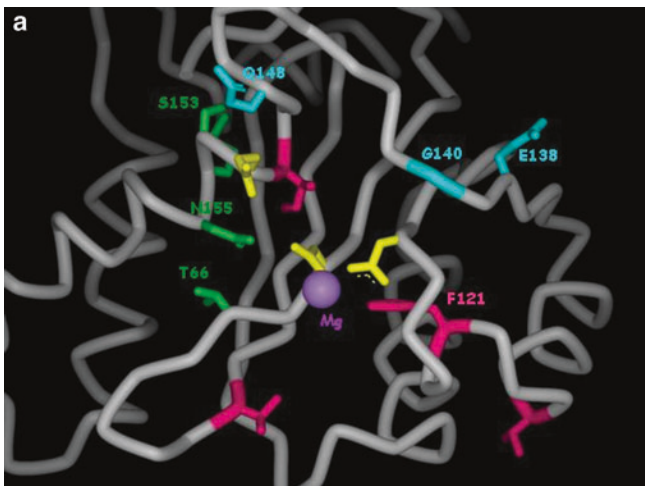
Fig. 6 (**a**) The two napthyridine carboxamides L-870812 and L-870810 provided proof of concept for the activity in integrase strand transfer inhibitors as a new class of antiretro viral agents in SIV-infected rhesus macaques and in HIV-1-infected patients. (**b**) Chemical structures of the two integrase inhibitors, raltegravir and elvitegravir, which have reached advanced clinical development status. These compounds are distinct from the naphthyridines and belong to structurally different chemical series

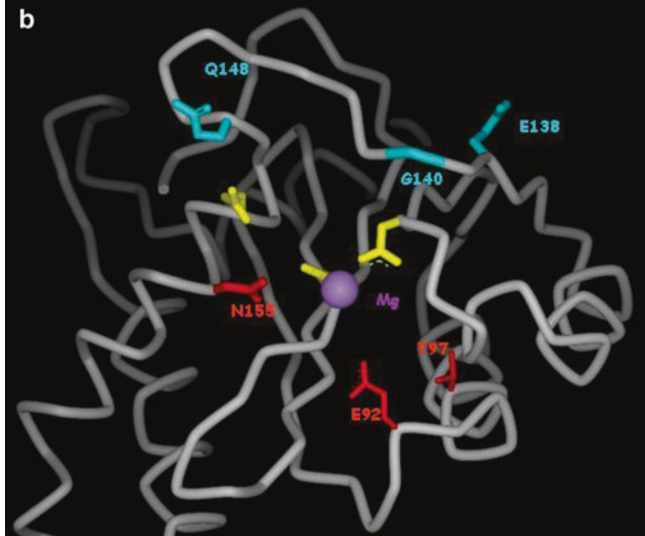
coordinating the metal cofactors. The proximity of the resistance mutations to the "DDE" motif is consistent with the metal sequestration mechanism of action proposed for these inhibitors. Importantly, mutations that engender resistance to integrase strand transfer inhibitors do not affect susceptibility to other antiretroviral agents, including PIs, NNRTIs, RTIs, and the various classes of entry inhibitors.

Although similar mutation patterns have been observed with integrase strand transfer inhibitors from different chemical series, structurally diverse analogs can exhibit discordant resistance profiles and select for unique mutations in integrase (Fig. 7a) (27). For example, the mutations F121Y/T125K and N155H identified with the napthyridine carboxamides L-870810 and L-870812, in vitro and in SIV-infected rhesus, respectively, engender resistance to these agents but have no effect on viral susceptibility to the diketo acid inhibitors. Conversely, the mutations T66I, S153Y, and M154I selected with different diketo acid inhibitors reduce susceptibility to the diketo acids without affecting the activity of either of the naphthyridine carboxamides. Interestingly, different mutations at the same residue can also have distinct effects on inhibitor susceptibility: for example, the N155H mutation observed in infected rhesus macaques treated with l-870812 results in reduced susceptibility to the naphthyridine carboxamides and not the diketo acids. In contrast, viruses with the N155S mutation exhibit resistance to both chemical series.

The limited cross-resistance observed between the naphthyidine carboxamides and the diketo acids is consistent with selection of distinct resistance mutations by prototypical inhibitors in each of the two chemical series. Although each of the mutated residues identified with these inhibitors is seen to surround the metal binding site (D64, D116, and E152, in yellow in Fig. 7a), those residues that are exclusively associated with resistance to either the naphthyridine carboxamide or diketo acid cluster on distinct sides of the active site. The discrete clustering of the mutations for each inhibitor suggests unique interactions that extend beyond the metal binding center, in opposite directions. The naphthyridine carboxamide pharmacophore was originally designed to orient the pendant groups in this series with the hydrophobic substituents of the diketo acids (64). However, given the pseudo-symmetrical nature of the naphthyridine carboxamide structure, the critical metal-binding elements in each pharmacophore can also be overlaid in a "reverse" orientation. When docked in the integrase active site such that the pharmacophore engages two metals, aligning the two inhibitors in the reverse orientation enables their respective pendant hydrophobic substituents to establish unique interactions with the enzyme (27). The inverted binding mode of the two molecules is consistent with the divergent resistance profiles of these inhibitors, and also explains distinct aspects of the structure activity relationships observed in each chemical class.

The results of resistance analyses such as these have suggested the potential for (at least) two ligand-binding surfaces for strand transfer inhibitors in the integrase active site (27). Compounds designed with substituents extended in both directions from the pharmacophore can exhibit enhanced affinity relative to analogs, with either substitution alone supporting this hypothesis. Molecular dynamics simulation studies have also suggested an extended ligand-binding surface based on the potential for multiple binding modes of the flexible diketone 5-CITEP (57). It has been proposed that the specific orientation that is adopted by the more constrained naphthyridine carboxamides is in part a result of the requirement to accommodate the increased rigidity and bulkiness of this pharmacophore. Differences in the flexibility of





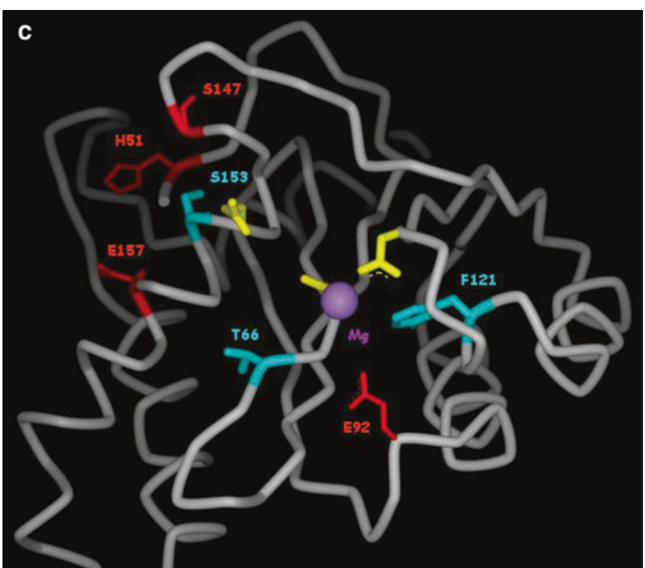


Fig. 7 Integrase strand transfer inhibitor resistance mutations map within the integrase active site. The three-dimensional structure of the integrase active site is shown as a *grey* α-carbon pipe. The active site residues (D64, D116, and E152) highlighted in *yellow* are believed to coordinate two divalent metals, although only one is shown (as a *purple ball*) in this representation. (**a**) Residues associated with the development of resistance to representative diketo acids, naphthyridine, and pyrimidine (raltegravir) inhibitors observed in vitro are shown in *green*, *magenta*, and *blue*, respectively (27). (**b**) Residues associated with resistance to raltegravir reported from the phase 2 clinical studies (26). (**c**) Residues associated with resistance to elvitegravir reported from the phase 2 clinical studies (49) (*See Color Plates*)

I. Elvetigravir Phase 2*	
E92Q	H51Y, S147G, E157G
T66I	F121Y, S153Y, R263K
Q148R/H/K	**E138K, G140C/S**
II. Raltegravir Phase 2**	
N155H	L74M, **E92Q**, T97A, Y143H, V151I, G163K/R
Q148R/H/K	L74M. E138K/A, G140S
III. Raltegravir Phase 3**	
N155H	L74M, **E92Q**, T97A, V151I, G163R
Q148R/H/K	E138K, G140S
Y143R/C	L74M/I, **E92Q**, T97A, G163R, I203M, S230R

Note: International Resistance Workshop
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the structures of the pharmacophore may also account for the disparity in resistance between the naphthyridine carboxamides and diketo acids with respect to mutations at residue 155. N155 points directly into the integrase catalytic center, and hydrogen bonds with the metal binding residue D64. The N155S mutation selected with the diketo acids disrupts this hydrogen bond, and may therefore alter the metal binding architecture of the active site. This would explain the observation that N155S confers resistance to both chemical classes. In contrast, the N155H mutation preserves the hydrogen bond with D64, and results in a more limited perturbation at the catalytic center. However, the rigid structure of the naphthyridine carboxamide pharmacophore may be less able to accommodate this more subtle change than the flexible diketo acid, resulting in the more restricted cross-resistance observed with this mutation.

The suggestion that resistance mutations can influence metal liganding at the active site, and/or occur at amino acid residues located in opposing directions distal to the catalytic center, suggests that resistance to InSTIs may be mediated either through influencing metal binding by the pharmacophore (e.g., N155) or by affecting interactions between the pendant groups in the inhibitor and residues within the enzyme active site. While the latter suggests a potential for limited cross-resistance, the former (i.e., influences on the metal binding architecture) suggests the possibility of broader class resistance with some mutations. Understanding the extent to which specific mutations engender cross-resistance among InSTIs is still quite limited, especially for those mutations that have thus far been observed with raltegravir and elvitegravir in clinical studies to date (Fig. 7b, c). However, for these two compounds, many of the same mutations have been observed in vitro and in vivo (e.g., E92, Q148, and N155) (26, 49) (Shimura 2007), and this extensive overlap therefore suggests the potential for significant cross-resistance. Despite these disappointing results, efforts to address resistance from these first-generation agents have already demonstrated that it is possible to find InSTIs that retain potent activity against resistant variants commonly observed with raltegravir and elvitegravir (60). The observation that one can identify potential second-generation InSTIs with activity against viruses with resistance to first-generation compounds in the class offers the hope that the InSTIs class will be a long-lived component of the antiretroviral armamentarium.

5 Consequences of Resistance

The complex role of integrase in integration, as well as in reverse transcription and viral assembly, is manifest in the different phenotypes exhibited by various integrase mutants with respect to their effect on the HIV-1 infection cycle (15, 61). Integrase mutations that specifically affect integration without influencing reverse transcription or viral assembly are referred to as class I mutants. These mutants are characterized by having normal levels of HIV-1 DNA synthesis early in infection, and increased levels of unintegrated circular byproducts at later stages of infection. In contrast, viruses with class II integrase mutations exhibit defects in either reverse transcription or viral assembly, with or without concomitant affects on integration. Class II replication defects have been observed with a variety of integrase mutations, including some highly conserved residues within the integrase active site. As class II mutations can affect both early and late stages of the viral infection cycle, these mutations can have effects on HIV-1 replication that are not manifest in single cycle assays. Therefore, relative to other classes of antiretroviral agents such as reverse transcriptase and protease inhibitors, the complexities engendered by the pleiotropic role of integrase in HIV-1 replication may present unique challenges for characterizing the effects of different integrase resistance mutations on viral replication in standard phenotypic susceptibility assays based on the single-cycle infection format (54). However, these complexities could also present some additional hurdles to the virus with respect to the development of integrase inhibitor resistance. Due to the multifaceted phenotype of class II mutants, unique compensatory mutations may be required to enhance fitness under drug pressure. As will be discussed, both class I and class II strand transfer inhibitor resistant mutants have been identified.

Each of the strand transfer inhibitor primary resistance mutations identified thus far occurs at conserved residues within the integrase active site: 121, 153, 154, and 155. It is perhaps not surprising, therefore, that HIV-1 variants with these mutations display reduced replication capacity, with an associated defect in integration. These primary mutations all engender a 50% or greater loss in specific infectivity when evaluated in single-cycle replication assays, and the addition of secondary mutations frequently leads to a further reduction in overall replicative capacity. When studied, the purified enzymes containing these mutations have also been shown to display decreased activity consistent with the effect on integrase enzymatic function observed in cell culture (19, 25, 29). However, the degree of resistance measured in enzyme activity assays is generally less than that observed in viral replication assays. Although this discordance is not well understood, the overall magnitude of the effect measured in vitro can be influenced by the choice of metal cofactor used in the reaction, and may partly result from the need to perform integrase biochemical assays at superphysiological concentrations of the metal cofactor and DNA substrate.

While each of the aforementioned primary resistance mutations exhibits defects in integration, HIV-1 isolates

with either S153Y or N155S exhibit a class II phenotype with an additional defect on virus assembly, but not reverse transcription. Interestingly, viral variants with the N155S and N155H mutations are indistinguishable in single-cycle infection assays (with approximately 30% of the replication capacity of wild-type HIV-1). However, the N155S variant displays a much more profound phenotype in multiple-cycle replication assays consistent with the class II defect resulting from this mutation. These results, in conjunction with the discordant resistant profiles of the two N155 mutants, explain the observation that the less profound N155H class I mutation is preferentially selected over the N155S class II mutation with strand transfer inhibitors in the naphthyridine carboxamide class.

6 Alternative Agents

As structurally diverse integrase strand transfer inhibitors can both select for and be influenced by different mutations localized to distinct regions within the integrase active site, it should prove possible to developing novel inhibitors, which have a limited potential for cross-resistance, within this class. Structural information would ideally be used to guide the development and selection of strand transfer inhibitors that display an orthogonal resistance profile. However, in the crystal structures of the various integrase subdomains constructs, the active site is highly variable and certain structural elements in a region (or regions) predicted to be relevant to the interaction of some chemical classes of known strand transfer inhibitors is not precisely defined. Although structural information is available for the diketone strand transfer inhibitor 5-CITEP (21), many important features relevant to the mechanism of action of strand transfer inhibitors are not represented, limiting the overall utility of this model for drug development. Most notably, there is no DNA present, and neither the dicarbonyl oxygens nor the tetrazole nitrogens in the 5-CITEP molecule engage the catalytic metal (or metals) in integrase. Since high-affinity interaction of strand transfer inhibitors with integrase requires a specific DNA bound conformation (17) and obtaining a structure of this complex has not been achieved, the lack of sufficiently detailed structural information, as required to facilitate inhibitor design, continues to present a challenge for integrase inhibitor development. In the absence of such information, resistance profiling studies have suggested valuable insights into how these inhibitors interact with the integrase active site, and should prove useful for developing new strand transfer inhibitors with discordant resistant profiles. Integrase strand transfer inhibitors that display orthogonal resistance patterns could be used in combination or in the sequencing of HIV-1 therapy, to address the development of resistance that is certain to be inevitable as compounds in this promising new class of antiretroviral agents are introduced into clinical practice. In addition, the complex role of integrase in HIV-1 replication suggests the potential for additional opportunities to develop multiple agents that target this essential HIV-1 protein in a mechanistically distinct fashion.

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Chapter 37
The Hepatitis B Virus and Antiviral Drug Resistance: Causes, Patterns, and Mechanisms

Stephen Locarnini

1 Background and Introduction

The hepatitis B virus (HBV) is a DNA-containing virus (Fig. 1a, b) that belongs to the family *Hepadnaviridae*. Under normal circumstances, viral infection and subsequent replication within the hepatocyte does not directly result in cell death. The inability of the host's immune response to clear HBV from infected hepatocytes within the liver is the basis for disease. As most patients once chronically infected do not resolve their infection, the course and clinical outcome of chronic hepatitis B (CHB) infection is determined by the generation and selection of viral escape mutants. Several unsuccessful attempts by the host's immune response to clear wild-type and escape mutants of HBV from infected hepatocytes leads to a cycle of ongoing necroinflammation and viral replication, resulting in the liver damage recognized as CHB (1). The emergence of these 'immune escape' mutants as dominant populations during active HBV replication may have important consequences for the severity of a disease (2–7) such as hepatitis Be antigen (HBeAg)-negative CHB. Similarly, selection of HBV quasispecies with mutations in the viral reverse transcriptase (rt) during antiviral therapy can result in further progression of liver disease and, in some cases, significant clinical deterioration (8, 9).

The hepatitis B virus utilizes reverse transcription to copy its DNA, thereby generating mutant viral genomes at a much higher rate than other DNA viruses. Particular selection pressures, both endogenous (host immune clearance via innate and adaptive responses) and exogenous (vaccines and antivirals), readily select out these escape mutants. Not surprisingly then, the introduction of nucleoside/nucleotide analog therapy has resulted in the emergence of drug resistance to every approved agent, thereby limiting drug efficacy. Factors determining treatment failure include patient compliance, drug regimen potency, and the drug's inherent genetic barrier to resistance. The development of antiviral drug resistance depends on a number of parameters, such as the magnitude and rate of viral replication, the fidelity of the viral polymerase, the selective pressure of the drug, the amount of replication space in the liver, and the fitness of the resistant virus.

Thus, in the treatment of chronic hepatitis B, the development of drug resistance is not unexpected if viral replication continues in the setting of ongoing treatment, especially monotherapy. Prevention of resistance will require the adoption of strategies that effectively control virus replication.

This chapter will briefly review the major aspects of the molecular virology and replication of HBV, and summarize the major viral mutants of clinical significance that are associated with drug resistance. Also, the factors and mechanisms of drug resistance in hepatitis B will be discussed. Finally, strategies to prevent the emergence of drug resistance will be addressed.

2 Molecular Virology and Lifecycle

HBV is distantly related to the retroviruses and replicates its genome by the reverse transcription of an RNA intermediate, referred to as pregenomic RNA (pgRNA). The 3.2 kb double-stranded DNA HBV genome is organized into four overlapping and frame-shifted open-reading frames (ORFs) (Fig. 1b.) The longest of these encodes the viral polymerase (Pol ORF). The second ORF, referred to as the envelope ORF, encodes the viral surface proteins and is located within the Pol ORF but in a frame-shifted manner. Two smaller ORFs, which encode the precore/core proteins and the X protein, partially overlap the Pol ORF. The viral lifecycle of HBV is well characterized, and has been reviewed recently (9) (see Fig. 2).

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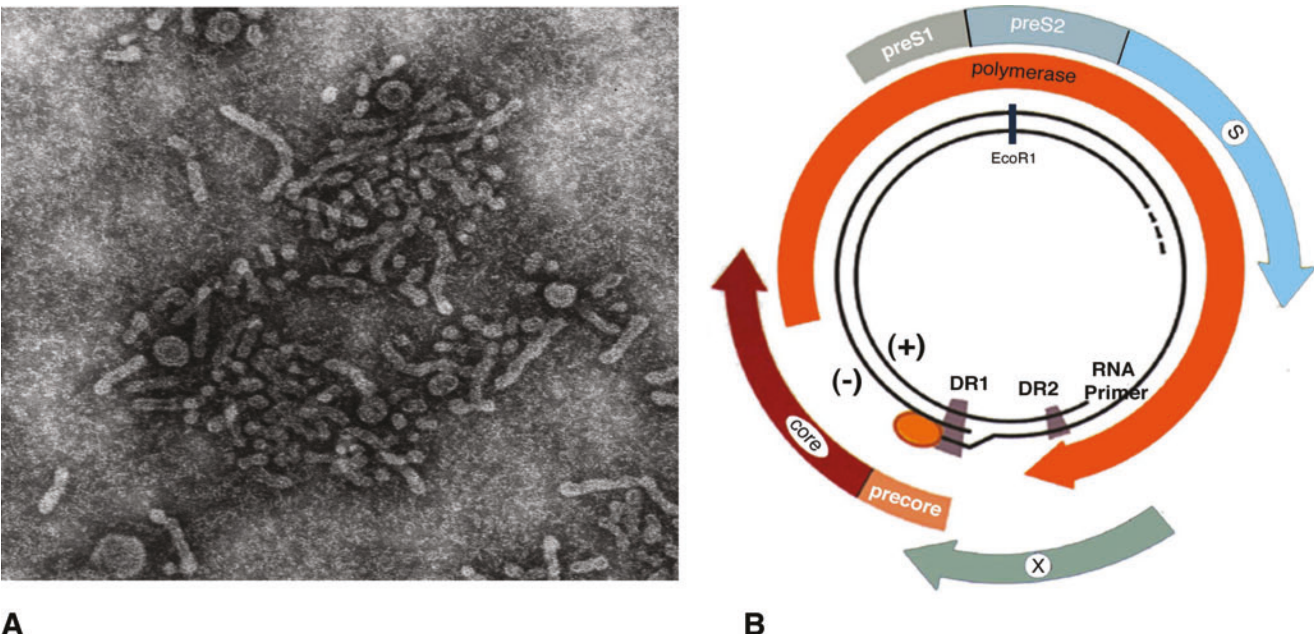

Fig. 1 (**a**) Electron micrograph of the HBV virions (42 nm). Filamentous structures and 22 nm small particles of HBsAg can be seen. (**b**) The genetic organization of the HBV genome; the four major open reading frames are shown (see text). The nicked and gapped molecules of DNA are held in a relaxed circular (RC) arrangement by the direct repeat 1 (DRI) and DR-2 regions

2.1 *Attachment, Penetration, and Uncoating*

The first stage of infection involves attachment to a susceptible hepatocyte and the penetration of HBV into the cell cytoplasm following the binding of the HBV envelope to its specific cellular receptor/coreceptor (10). The subsequent events of penetration and uncoating are not well defined, but it has been assumed by most investigators that a process of receptor-mediated endocytosis is responsible for delivery of the DNA-containing cores to inside the cell.

2.2 *Conversion of Genomic RC DNA into cccDNA and Transcription of the Viral Minichromosome*

Following viral penetration and envelope uncoating, the cytoplasmic viral nucleocapsids are transported to the nuclear membrane, where they uncoat (11). The genomic relaxed circular DNA (RC DNA) (12) is released into the nucleus and then converted into covalently closed circular DNA (cccDNA) using host cell enzymes, resulting in the formation of the viral minichromosome, the major template of HBV that is used for the transcription of all the viral mRNAs involved in viral protein production and replication (13, 14).

Using this transcriptional template, five major unspliced RNA species, two of 3.5 kb, and one each of 2.4, 2.1, and 0.7 kb, are generated. The transcripts can be classified into two classes: subgenomic and genomic (15). Both classes contain heterogeneous transcripts that are of positive orientation, are capped at the 5′ end, and are polyadenylated at the 3′ end. The synthesis of these transcripts is controlled by the enhancer II/basal core (BCP), large surface antigen (Pre-S1), major surface antigen (S), and enhancer I/X gene promoters (15).

The smaller, subgenomic transcripts, which measure 2.4, 2.1, and 0.7 kb, function exclusively as mRNAs for the translation of the viral envelope proteins (Pre-S1, Pre-S2, and S) and the accessory protein, X. The 2.4 and 2.1 kb mRNAs translate the large (Pre-S1), middle (Pre-S2), and small (S) envelope proteins. Both the Pre-S2 and S envelope proteins are translated from the 2.1 kb mRNAs. The Pre-S1 is translated from the 2.4 kb transcript and is required for the formation of the virions as well as the filamentous forms of the hepatitis B surface antigen (HBsAg). The S protein forms the small 22 nm spherical particles of HBsAg. The 0.7 kb mRNA translates the X protein, a modest transactivator of transcription that also appears to have a regulatory function in viral replication (16). The X protein is regarded as an accessory protein of HBV.

The greater than genomic transcripts measure 3.5 kb and are greater than one genome in length, and serve as the pgRNA and precore RNAs. The pgRNA encodes the viral nucleocapsid (core protein, HBcAg) and the HBV polymerase (Pol), and also acts as a template for reverse transcription. The

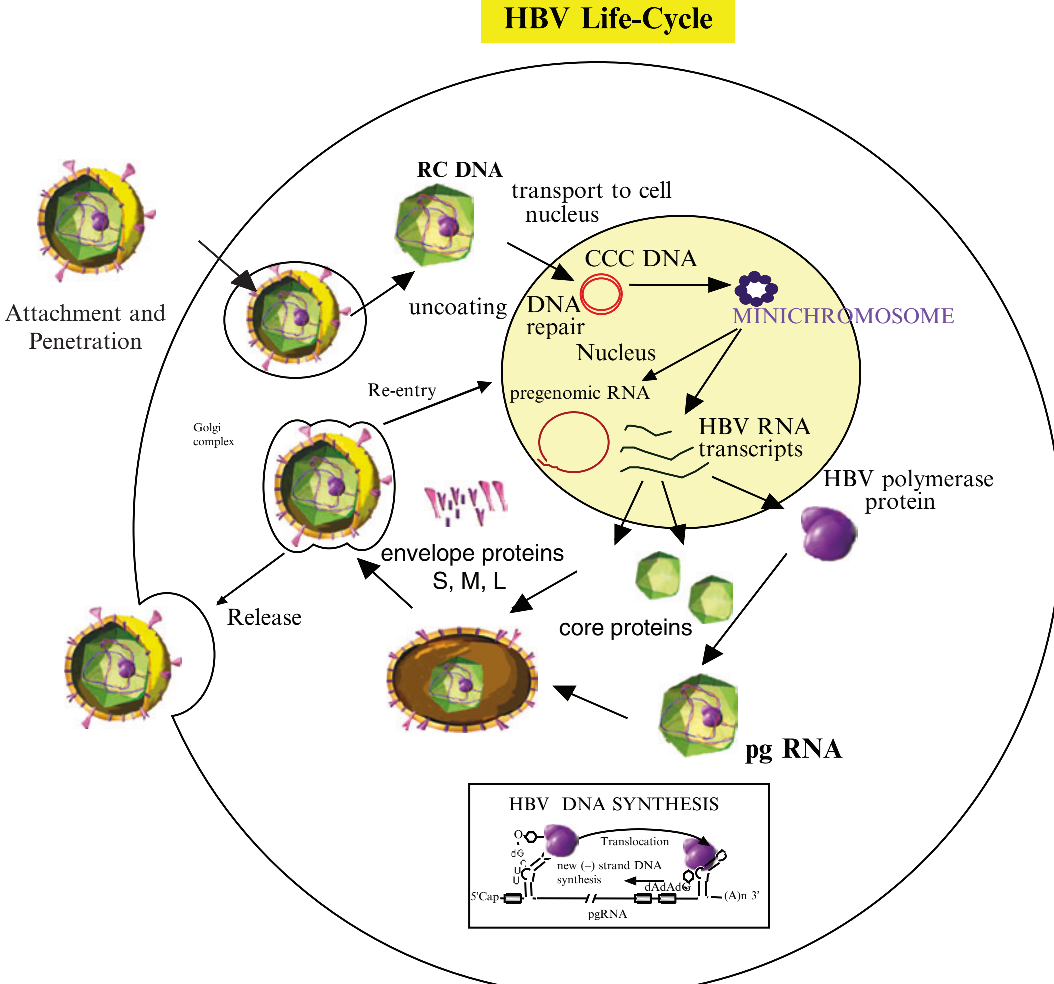

Fig. 2 HBV life cycle. The major processes involved in (HBV) genome replication: conversion of replication complex (RC) DNA, reverse transcription of cccDNA to produce pregenomic RNA (pgRNA), reverse transcription of pgRNA to make minus HBV DNA, and HBV DNA polymerase activity to make the RC DNA, completing the cycle

precore RNA is slightly longer than the pgRNA at the 5′ end and encodes the second accessory protein of the HBV, HBeAg.

2.3 Viral Reverse Transcription

The process of reverse transcription used by HBV to convert its pgRNA into double-stranded DNA (dsDNA) has been reviewed (17–19). Reverse transcription is initiated upon binding of the viral polymerase to the encapsidation signal (epsilon) on the pgRNA. This then signals the binding of core protein dimers to form nucleocapsids. A series of interactions, including the involvement of host chaperone proteins, results in the synthesis of minus-strand DNA-strand, followed by positive-strand synthesis and circularization of the genome (15). The viral envelope, the small particles, and the filamentous forms are synthesized and assembled at the endoplasmic reticulum (ER) membranes and then bud into its lumen. The HBcAg protein is synthesized in the cytosol and assembled independent (17) of the enveloped proteins (15, 17–20).

2.4 Assembly and Release

The assembly of nucleocapsids containing mature relaxed circular DNA occurs in the cytosol, and these nucleocapsids are selectively enveloped before exiting the cell (15). Minus-strand DNA synthesis appears to be coupled to phosphorylation of the nucleocapsid, which is required for envelopment to occur. Incomplete dsDNA/RNA genomes that have completed minus-strand DNA synthesis and at least started plus-strand synthesis can readily be found in the blood as secreted virions.

2.5 Replication and Diversity of HBV Genomes

The unique replication strategy of HBV provides it with at least two selective advantages. First, the HBV cccDNA minichromosome that acts as the major transcriptional template for the virus is very stable. Second, the error-prone HBV reverse transcriptase generates a high rate of mutations, resulting in a population of viral quasispecies. The high mutation rate of HBV rt has resulted in a substantial diversity in the nucleotide sequence of HBV. Currently, eight major genotypes, A through to H, have been identified based on nucleotide (nt) diversity of ≥8% at the whole genome level (21, 22). These genotypes typically have a distinct global geographic distribution, with A and D mainly found in Europe and North America, and B and C in Asia. This geographical clustering is now starting to merge, reflecting the substantial population migrations that have occurred from Asia over the last 50–100 years.

3 Antiviral Drug Resistance

Antiviral drug resistance reflects the reduced susceptibility of a virus to the inhibitory effect of a drug, and results from a process of adaptive mutations under the selection pressure of antiviral therapy. Approved and/or available medications for CHB include lamivudine (LMV), a synthetic deoxycytidine analog with an unnatural L-conformation, and related L-nucleosides, including emtricitabine (FTC), telbivudine (LdT), and clevudine (CLD). A second group of nucleos(t)ide analogs is the acyclic phosphonates, which include adefovir dipivoxil (ADV), a prodrug for the acyclic 2′-deoxyadenosine monophosphate (dAMP) analog adefovir, and the structurally similar tenofovir disoproxil fumarate (TDF), which is currently used to treat patients with the human immunodeficiency virus (HIV) infection. A third group of agents has recently been developed that contains a cyclopentane/cyclopentene sugar moiety and includes the most potent anti-HBV drug discovered to date, the deoxyguanosine analog entecavir (ETV) (23).

Two types of mutations have been identified that have been associated with treatment failure to these agents: primary resistance mutations (Fig. 4), which are directly responsible for the associated drug-resistance; and secondary or compensatory mutations, which probably occur in order to promote or enhance replication competence. Compensatory mutations emerge because the selection of genetic resistance is usually associated with some cost in replication fitness for the virus. Compensatory mutations are important, as they ‘fix’ the discriminatory primary drug-resistant mutations into the genetic archive of the HBV minichromosome, thus providing quasispecies memory (25).

With several different nucleos(t)ide analogs now approved for the treatment of CHB in many countries, it has become important to describe drug resistance in terms of clinical and laboratory relevance. For example, antiviral drug resistance can be described in terms such as high (>100-fold increase in EC_{50}), intermediate (10–99-fold increase in EC_{50}), or low-level (2–9-fold increase in EC_{50}), with respect to the fold

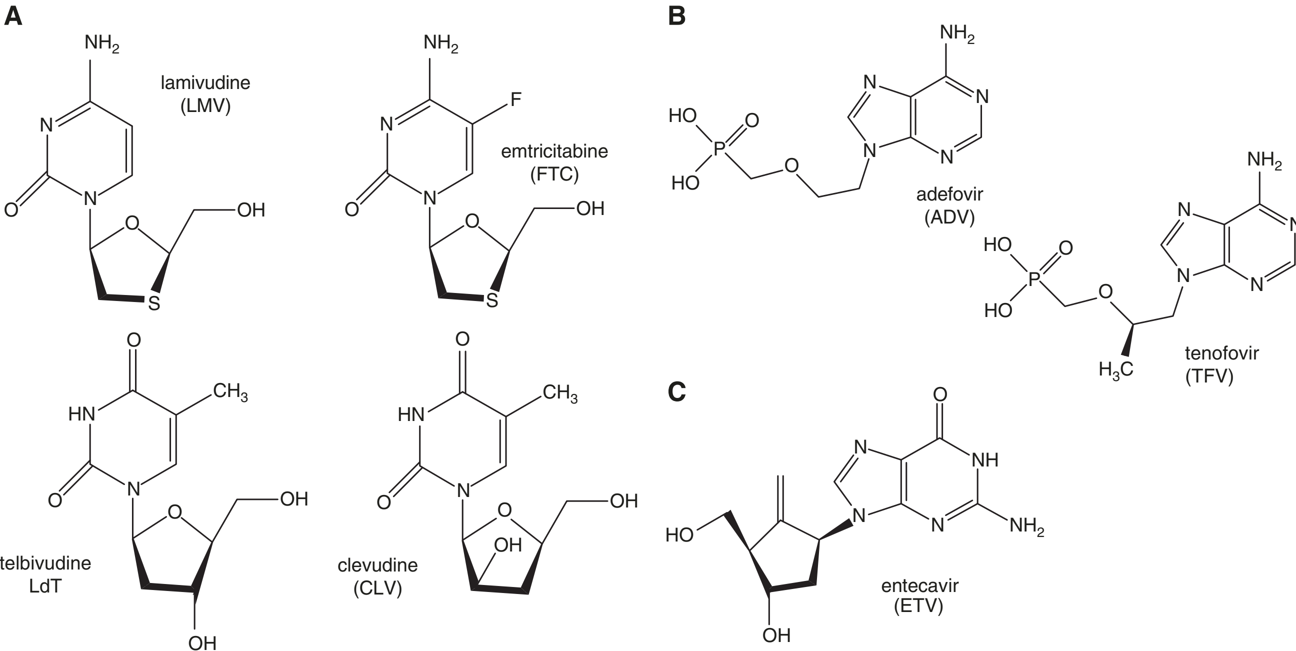

Fig. 3 Structures of nucleoside and nucleotide analogs. (a) L-nucleosides; (b) acyclic phosphonates and (c) entecavir

increase observed in EC_{50} (effective concentration 50%) from in vitro studies (Table 1).

3.1 Lamivudine Resistance Mutations (L-Nucleosides)

Antiviral resistance to LMV has been mapped to the YMDD locus in the catalytic or C domain of HBV Pol (24). The primary resistance mutations within the Pol gene that have been selected during LMV therapy are designated rtM204I/V/S (domain C) +/−rtL180M (domain B) (34). Other primary mutations include rtA181T/V (35). Compensatory mutations can be found in other domains of the HBV Pol, such as rtL80V/I (36), rtI169T (30), rtV173L (37), rtT184S/G, rtS202I, and rtQ215S (38), that enhance viral replication levels (Fig. 5).

Lamivudine resistance increases progressively during treatment, at rates of 14–32% annually, exceeding 70% after 48

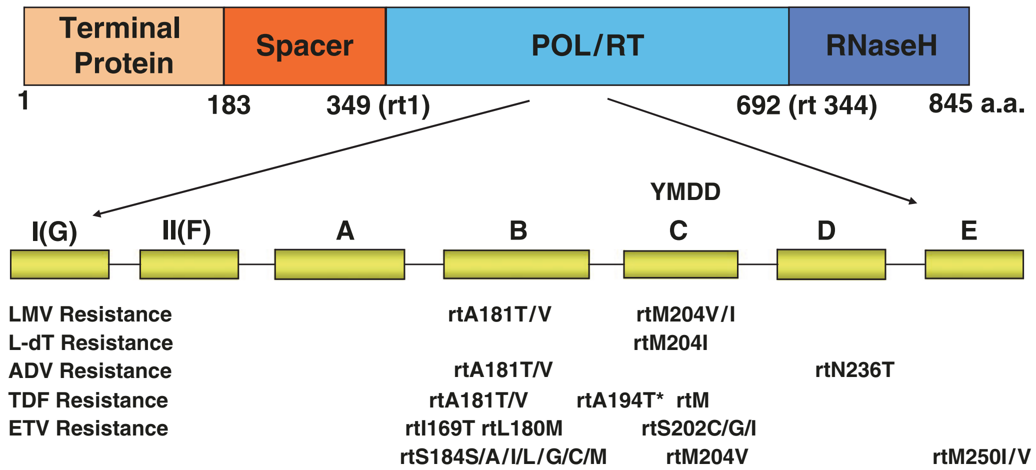

Fig. 4 The location of major drug-resistance mutations on the HBV polymerase. According to convention and for consistent identification of mutations conferring resistance to antiviral nucleos(t)ide analogs, amino acids are numbered from the beginning of the Pol/RT (rt1 to rt344) domain (24). Mutations associated with resistance to lamivudine (LMV), telbivudine (LdT), adefovir (ADV), tenofovir (TDF), and entecavir (ETV) are indicated

Table 1 Antiviral sensitivity profiles of drug-resistant HBV in vitro

HBV mutant[a]	Lamivudine[1,2,3]	Adefovir[2]	Clevudine[1,4]	Telbivudine[5]	Entecavir[5,6]	Tenofovir[3,7,8]
HBV	Fold resistance	Fold resistance	Fold resistance	Fold resistance	Fold resistance	
Wild-type	1.0	1.0	1.0	1.0	1.0	1.0
L180M	1.7	0.5	>120	12	1.0	1.0
M204I	>106	0.7	>120	236	30	3–5
L180M + M204V	>105	0.2	>120	133	30	1.0
A181T/V	2–6	1–5	NA	NA	NA	3–5
N236T	3–8	7–10	4.7	2.4	0.67	3–5
I69T/M250V[a]	>1,000	1	NA	>100	>1,000	NA
T184G/S202I[a]	>1,000	2	NA	>1,000	>1,000	NA
V214A/Q214S	10–20	7–10	NA	NA	NA	>10
A194[a]	>1,000	>10	NA	NA	NA	>10
L80V[a]	>1,000	5–10	NA	NA	NA	NA

[a](+ L180M + M204I/V); *NA* not available

[1]Chin et al. (26); [2]Delaney et al. (27); [3]Ono-Nita et al. (28); [4]Sozzi et al. (29); [5]Tenney et al. (30); [6]Brunelle et al. (31); [7]Sheldon et al. (32); [8]Delaney et al. (33)

2–9-fold → no or low level of resistance; 10–99-fold → medium level of resistance; >100-fold → high level of resistance

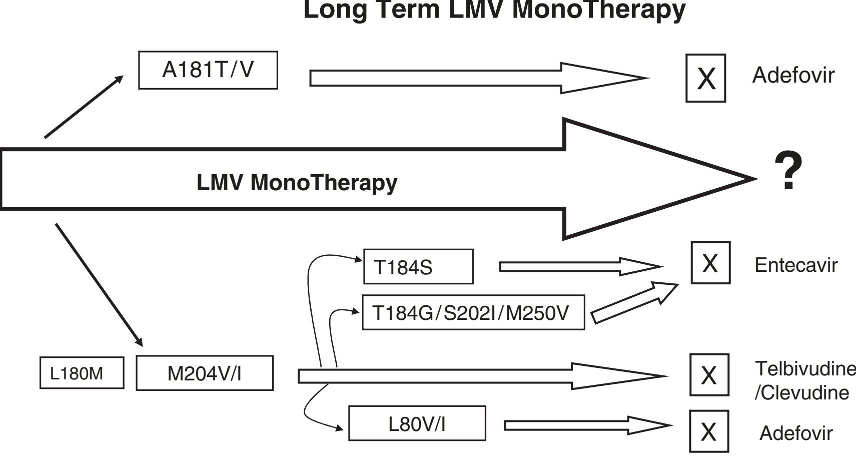

Fig. 5 Pathways of evolution for the HBV Pol during emergence of lamivudine (LMV) resistance in patients undergoing long-term LMV monotherapy. If the selection pressure of the drug is maintained once resistance has emerged, then further compensatory mutations can be found, some of which will compromise future rescue therapy options

Table 2 Annual prevalent resistance rates for lamivudine, adefovir, entecavir, emtricitabine, and telbivudine

	Resistance at year of therapy expressed as percentage of patients				
DRUG	1	2	3	4	5
Lamivudine[a]	23	46	55	71	80
Adefovir[b]	0	3	6	18	29
Entecavir[c] (naïve)	0.1	0.4	1.1	–	–
Entecavir[c] (LAM-resistant)	6	14	32	–	–
Emtricitabine[c]	9–16	19–37	–	–	–
Telbivudine[d]	4[d]	–	–	–	–

[a]Modified and updated from Lai et al. (39) and Leung et al. (40)
[b]From Locarnini et al. (41)
[c]From Perrillo et al. (42), Colonno et al. (43)
[d]In the LAM comparator arm, the percentage was only 8%, based on a complex case definition of antiviral drug resistance/treatment failure. One would thus expect a comparable relative level of 10–12% based on genotypic resistance compared with lamivudine (25% per annum)

months of treatment (39) (Table 2). Factors that increase the risk of development of resistance include high pretherapy serum HBV DNA and ALT levels, and the incomplete suppression of viral replication (39, 44). The main LMV resistance mutations rtM204V/I do not confer cross-resistance to ADV (Table 1), but the rtA181T/V does (38). The rtI169T, rtT184S/G, and rtS202I contribute to entecavir resistance (30) (Fig. 5). The rtM204V/I is cross-resistant with all other L-nucleoside analogs tested, such as emtricitabine (FTC), telbivudine (LdT) and clevudine (L-FMAU) (see Table 1 and Fig. 4)

Mutations that confer LMV resistance decrease in vitro sensitivity to LMV from at least 100- to >1,000-fold. The rtM204I substitution has been detected in isolation, but rtM204V and rtM204S are found only in association with other changes in the A or B domains (45). The five common patterns of resistance that can be identified are (1) rtM204I, (2) rtL180M + rtM204V, (3) rtL180M + rtM204I, (4) rtV173L + rtL180M + rtM204V, and (5) rtL80V/I±rtL180M + rtM204I. The dominance of particular patterns tends to be influenced by the HBV genotype (46). The molecular mechanism of LMV resistance is steric hindrance caused by the β-branched side group of the valine or isoleucine amino acids colliding with the oxathiolane ring of LMV with the deoxynucleotide triphosphate (dNTP)-binding site (47). This results in a greater than 100-fold increase in EC_{50} (Table 1).

3.2 *Adefovir Dipivoxil Resistance Mutations (Acyclic Phosphonates)*

Resistance to ADV was initially associated with mutations in the B (rtA181T) and D (N236T) domains of the enzyme (48) (Fig. 4). HBV resistance to ADV occurs less frequently than resistance to LMV, with a prevalence of around 2% after 2 years, 4% after 3 years, 18% after 4 years, and 29% after 5 years (49) (Table 2).

These ADV-associated mutations in HBV Pol result in only a modest (three- to eightfold) increase in the concentration of the drug required for 50% inhibition for viral replication in vitro (EC_{50}) (Table 1), and are partially cross-resistant with TDF, probably because the molecular mechanism of resistance is similar in both, with indirect perturbation of the triphosphate binding site between the A and D domains (47, 50). The rtN236T does not significantly affect sensitivity to LMV (48), but the rtA181T/V changes are partially cross-resistant to LMV (Table 1). Recently, another mutation (rtI233V) mapped to the reverse transcriptase domain has been identified, that confers resistance to ADV (51) (Fig. 6). In clinical studies, the rtI233V

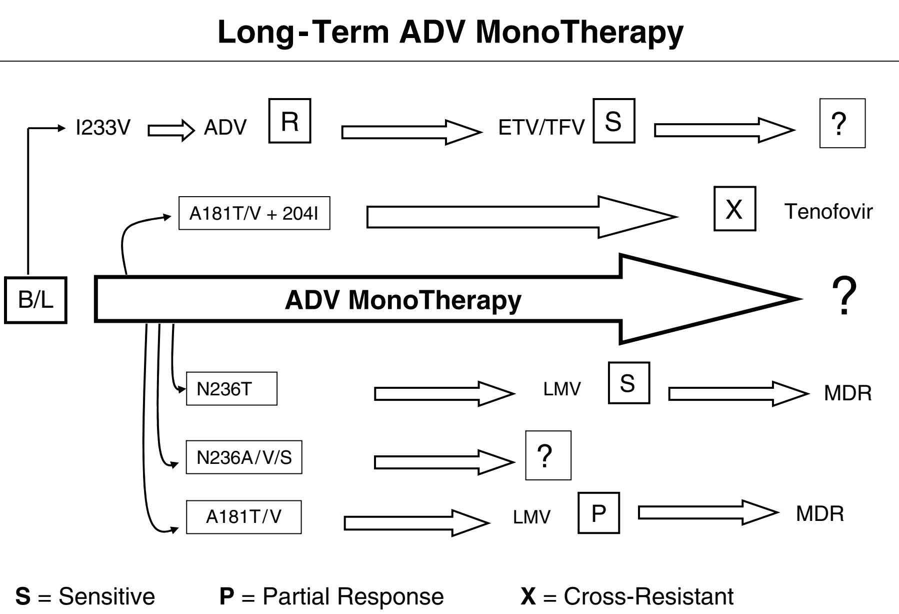

Fig. 6 Pathways of evolution for the HBV Pol during emergence of adefovir (ADV) resistance in patients undergoing long-term ADV monotherapy. B/L refers to baseline, pretherapy (51)

mutation appears to occur in approximately 2% of all patients with CHB (51, 52), and the final significance of this mutation will need independent confirmation as other groups have not found an association between the rtI233V and ADV resistance (53) (Locarnini, S. and Yuen, L.; unpublished observations).

3.3 *Entecavir Resistance Mutations (Cyclopenta(e)ne Sugar)*

Resistance to ETV has been observed in patients who are naïve to therapy (43) and are also LMV-resistant (30). Mutations in the viral polymerase associated with the emergence of ETV resistance were mapped to the B domain (rtI169T, rtL180M, and/or rtS184G), C domain (rtS202I and rtM204V), and E domain (rtM250V) of HBV Pol (Fig. 4). In the absence of LMV mutations, the rtM250V causes a ninefold increase in IC_{50}, whereas the rtT184G + rtS202I changes have only a modest effect (Table 1) (26, 28, 30, 54–56). The mechanism of ETV resistance for the rtT184G + rtS202I is an allosteric change with altered geometry of the nucleotide-binding pocket and DNA template binding of the polymerase near the YMDD site (56). The molecular mechanism of resistance for the rtM250V change is thought to be an alteration of the binding interaction between the DNA primer strand and DNA template strand with the incoming dNTP (56).

Recent clinical experience with ETV failure has indicated that at least three mutations, rtL180M⊕rtM204V, and either rtT184G/S or rtS202I are required in the HBV Pol for ETV resistance to develop (Fig. 7). This accounts for the low rate of resistance in treatment-naive patients after 1 year (0.1%), 2 years (0.4%), and 3 years (1.1%) of ETV monotherapy (Table 2). In contrast, in LMV-experienced patients, it should be noted that as well as rtL180M and rtM204V/I mutations, changes at codon 184 occur in 4.5% of patients and the frequency of ETV genotypic resistance changes in LMV-experienced patients is 6% (year 1), 14% (year 2), and 32% (year 3) (see Table 2). In this group, viral breakthrough as well as genotypic resistance occur in 1% (year 1), 10% (year 2), and 25% (year 3) (57) of patients.

3.4 *Multidrug Resistance*

Recently, multidrug-resistant HBV has been reported in patients who have received sequential treatment with NA monotherapies (30, 31, 58–60). The development of multidrug resistance will certainly have implications on the efficacy of rescue therapy, as in the case of multidrug-resistant human immunodeficiency virus (61, 62). Successive evolution of different patterns of resistance mutations have been reported during long-term LMV monotherapy (35, 63) (Fig. 5). The isolates of HBV with these initial mutations appear to be associated with decreased replication fitness compared with wild-type HBV; however, additional mutations that can restore replication fitness are frequently detected as treatment is continued (37, 64) (Fig. 5).

A recent study by Yim et al. (65) characterized multidrug-resistant HBV in more detail, in six patients receiving alternating monotherapies, typically LMV and ADV (see Figs. 5 and 6). Using conventional cloning techniques with subsequent

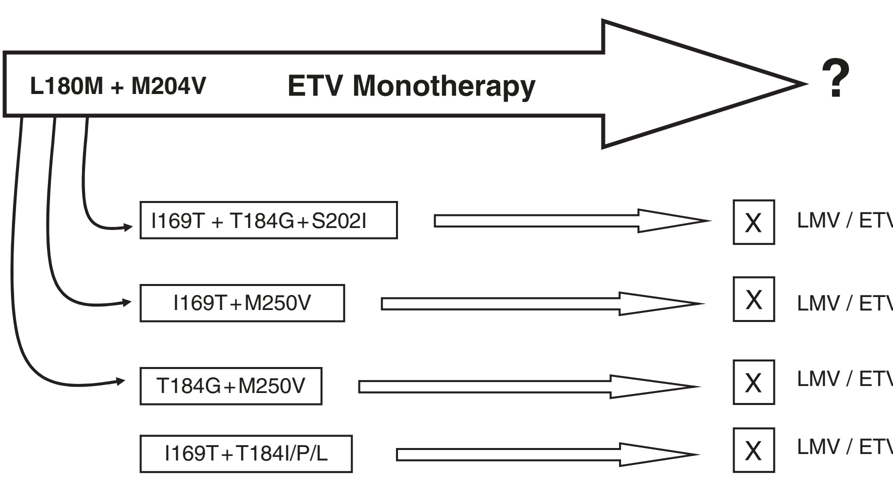

Fig. 7 Possible pathways for evolution of the HBV Pol during the emergence of entecavir (ETV) resistance

PCR sequencing, the majority of the clones sequenced (85%) had mutations to both therapies on the same genome. The remainder had LMV-resistant clones only. In three of the patients, analysis of successive samples revealed progressive evolution from the clones with LMV-resistant HBV mutations only, to mixtures of clones that had multidrug-resistant mutations. These studies strongly support the role for combination therapy in managing patients with CHB (66) (see below).

4 Why HBV Antiviral Drug-Resistant Mutants Are Selected

Antiviral drug resistance depends on at least five factors: (1) magnitude and rate of virus replication, (2) the fidelity of the viral polymerase, (3) selective pressure of the drug, (4) amount of replication space in the liver, and (5) replication fitness of the drug-resistant virus.

4.1 Magnitude and Rate of Virus Replication

The natural history of CHB is highly variable, but can be generally divided into four phases: immune tolerant (high replicative), immune elimination (intermediate replicative), non-replicative phase, and a “reactivation phase”, generally associated with HBeAg-negative CHB (49, 67). During the HBeAg-positive immune tolerant phase, there is a very high daily production of virions, approximately $10^{12–13}$.

Over the various phases of CHB, the HBV replication rate is considered to be approximately 10^{11} virions per day (68). This substantial daily production, coupled with the mutational frequency of the HBV Pol (see below), equates to at least 10^{10} point mutations produced per day in individuals who have a high level of replication. HBV genomes typically contain approximately 3,200 nucleotides, thus all possible single-base changes can be produced each day (69). HBV thus exists in an infected individual as populations of HBV quasispecies. However, the organization of the ORFs into a frame-shifted overlapping arrangement within the HBV genome does place some restriction on the final number of viable mutants that are actually generated. The stability of the predominate HBV within the quasispecies pool is maintained by particular selection pressures from the host’s innate and adaptive immune system, and by viability and replication competence of the virus.

4.2 Fidelity of the Viral Polymerase

The HBV mutation frequency has been estimated to be approximately $1.4–3.2 \times 10^{-5}$ nucleotide substitutions per site per year (70, 71). This rate is approximately tenfold higher than that for other DNA viruses, and more in keeping with the RNA viruses such as retroviruses. Unlike cellular polymerases, the HBV Pol is a reverse transcriptase that lacks proofreading function. As discussed above, the mutation rate of HBV is also influenced by the clinical phase of the patient, such as whether the patient is in the immune-tolerant phase (low error rate) or the immune-elimination phase (higher rate), HBeAg-negative CHB, and by clinical settings such as immunosuppression and transplantation (34). Thus, prior to antiviral therapy and because of the quasispecies pool, there is preexistence of variants carrying single and double mutations potentially associated with drug resistance (72).

4.3 Selective Pressure of the Drug

The probability of a mutation associated with drug resistance being selected out during therapy depends on the efficacy of that drug; the probability has been depicted graphically as a bell-shaped curve (73). Hence, a drug with low antiviral activity does not exert significant selection pressure on the virus, and the risk of drug resistance emerging is not high. Conversely, complete suppression of viral replication allows almost no opportunity for resistance to emerge because, as highlighted above, mutagenesis is replication-dependent (69). Because monotherapies exert varying degrees of antiviral activity directed at one single target site, they result in the highest probability of selecting for drug resistance. The ideal treatment regimen exerts antiviral activity targeted at different sites in the viral lifecycle, to significantly reduce the risk of selecting drug-resistant quasispecies. Resistance emerges when replication occurs in the presence of drug-selection pressure. The corollary of this is that "no replication" translates into "no resistance".

4.4 Amount of Replication Space in the Liver

Replication space for HBV has been described as the potential of the liver to accommodate new transcriptional templates or molecules of cccDNA (74, 75). This indicates that the eventual takeover by a mutant virus is dependent upon the loss of the original wild-type virus, and is governed by factors such as replication fitness as well as the turnover and proliferation of hepatocytes (74, 75). Hepatocyte turnover in the normal liver is slow, displaying a typical half-life of over 100 days (68). This can be reduced to less than 10 days in the setting of increased necroinflammatory activity or associated toxicity (68). In a fully infected liver, synthesis of new HBV cccDNA molecules can only occur if uninfected cells are generated by normal growth within the liver, hepatocyte proliferation and turnover, or loss of wild-type (dominant) cccDNA from existing infected hepatocytes (76, 77). The enrichment of one species over another suggests that the expanding virus has augmented its population through an expansion of cccDNA synthesis (76, 77). In other words, the expansion of a (drug-) resistant mutant in the infected liver can be possible only with the creation of new replication space (77).

4.5 Replication Fitness of the Drug-Resistant Virus

Replication fitness has been defined as the ability to produce offspring in the setting of natural selection (72). This is not a yield measurement of viral replication, and can be measured using in vitro coinfection competition assays. Unfortunately, this cannot be conveniently done with HBV because of the lack of a suitable cell culture system for viral infectivity.

Several clinical observations demonstrate the fitness of lamivudine-resistant HBV. Thibault et al. (78) were the first to document the transmissibility of LMV-resistant HBV from patient to patient (78). Several groups have described the persistence of LMV-resistant HBV as codominant quasispecies with wild-type HBV posttreatment for at least 3 months (79), or as a minor quasispecies with wild-type HBV posttreatment for almost 1 year (80).

4.6 Other Factors

Host factors effecting antiviral therapy includes previous drug experience, compliance, host genetic factors (e.g. inborn errors of metabolism), and the ability to efficiently convert the nucleos(t)ide analog to its active metabolite via several intracellular phosphorylations (intrahepatic salvage enzymes) (81, 82). In addition, there are sequestered sites/sanctuaries of viral replication that may not be accessible to the antiviral agent, and HBV replicative intermediate, as the cccDNA form is typically recalcitrant to conventional therapy (14, 83).

5 Strategies to Overcome Resistance

Currently, interferon, lamivudine, adefovir dipivoxil, and entecavir can all be considered as first-line therapy for individuals with noncirrhotic liver disease (84). In the context of rescue or salvage therapy, mutations that confer resistance to lamivudine confer cross-resistance to other L-nucleosides and reduce sensitivity to entecavir, but not to adefovir or tenofovir (Table 1 and Figs. 5–7). Generally, mutants that are resistant to adefovir and tenofovir remain sensitive to L-nucleosides and entecavir (Table 1). Multiple mutations are required for high-level resistance to entecavir (Table 1) (30, 57). The lower risk of resistance to adefovir dipivoxil and entecavir (Table 2) supports their use in liver transplantation patients and in patients with cirrhosis or decompensated liver disease, given that development of drug resistance is more likely to precipitate clinical deterioration in these individuals (69).

In the future, combination chemotherapy will be used to treat CHB, especially in patients with more advanced disease. Provided that appropriate drug combinations are used, this approach should yield well-recognized benefits, including reduction of the risk of drug resistance. This is because, even though the pre-existence or rapid evolution of viral mutants with the potential to resist individual drugs is almost guaranteed by high HBV loads and rapid turnover in vivo, the preexistence or evolution of multidrug-resistant mutants is much less likely. This is known as the combinatorial ledge (85). Drugs used in

combination should have different mechanisms of action, and should act additively or synergistically (66). Unfortunately, in the management of CHB, the nucleoside/nucleotide analogs have essentially similar mechanisms of action (82, 86). However, the selection of the individual drugs in the combination regimen can also be based on resistance mutation profile and cross-resistance potential (see Figs. 5, 6, and 7).

6 Public Health Implications of the Polymerase Envelope Genes Overlap

The polymerase gene overlaps the envelope gene completely and changes in the HBV Pol selected during antiviral resistance can cause concomitant changes to the overlapping reading frame of the envelope (see Fig. 1b). Thus, the major resistance mutations associated with LMV, L-dT, ADV, and ETV failure would also have the potential of altering the C-terminal region of HBsAg. For example, changes associated with LMV resistance, such as the rtM204V, result in a change at sI195M in the surface antigen, whilst the rtM204I change is associated with three possible changes, sW196S, sW196L, or a termination codon. To date, there has been only one published study that has examined the effect of the main LMV resistance mutations on the altered antigenicity of HBsAg (87). One of the common HBV quasispecies that is selected during LMV treatment is rtV173L + rtL180M + rtM204V, that result in change in the HBsAg at sE164D + sI195M. Approximately 20% of HIV-HBV co-infected individuals (88) and 10% of mono-infected individuals encode this “triple Pol mutant” (89). In binding assays, HBsAg expressing these LMV-resistant associated residues had reduced anti-HBs binding (87). This reduction was similar to the classical vaccine escape mutant, sG145R.

The ADV resistance mutation rtN236T does not affect the envelope gene, and overlaps with the stop codon at the end of the envelope gene. The mutation selected by ADV and/or LMV at rtA181T results in a stop codon mutation at sW172stop. The ADV-resistant mutation at rtA181V results in a change at sL173F. HBV with mutations that result in a stop codon in the envelope gene, such as those for LMV and ADV, would be present in association with a low percentage of wild-type to enable viral packaging.

The ETV-resistant associated changes at rtI169T, rtS184G, and rtS202I also affect HBsAg and result in changes at sF161L, sL/V176G, and sV194F. The rtM250V is located after the end of HBsAg. The sF161L is located within the region that was defined as the “a” determinant or major hydrophilic region (MHR), which includes amino acids 90–170 of the HBsAg (90). This region is a highly conformational epitope, characterized by multiple di-sulphide bonds formed from sets of cysteines at residues 107–138, 137–149 and 139–147 (90). Thus, distal substitutions such as sE164D significantly affect anti-HBs binding (87). The influence of other changes to HBsAg, such as sF161L, needs further investigation to determine the effect on the envelope structure and subsequent anti-HBs binding.

While evidence for the spread of transmission of antiviral-resistant HBV is limited, there has been a report of the transmission of LMV-resistant HBV to an HIV patient undergoing LMV as part of antiretroviral therapy (91).

While some mutations that confer resistance in the HBV polymerase can affect the immunogenicity of the envelope gene products, likewise it is possible that changes brought about by immune selection can influence antiviral drug sensitivity. To date, the common surface changes sG145R and sP120T, which are associated with vaccine escape and failure to respond hepatitis B immune globulin therapy can cause concomitant amino acid changes in the HBV polymerase protein. However, these changes have not been shown to alter antiviral drug susceptibility.

7 Conclusions

Antiviral drug resistance now poses a major problem in the management of patients with chronic hepatitis B. The probability that viral resistance will develop is directly proportional to the potency of the drug regimen and the diversity of quasispecies. Inhibition of HBV replication should be able to prevent the development of drug resistance, mainly because mutagenesis is replication-dependent. If viral replication can be suppressed for a sufficient length of time, viral load will theoretically decline to a point where the continued production of quasispecies with the potential for resisting new drug treatments is no longer possible. Whether this end point also translates to other benefits such as HBeAg seroconversion, sustained virological suppression with histological improvement, or even HBsAg seroconversion, is presently unknown. However, a reasonable clinical goal at present is the application of this concept via the optimization of combination therapies analogous to the highly active antiretroviral therapy regimens used for HIV infection (12, 66).

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Chapter 38
Mechanisms of Hepatitis C Virus Drug Resistance

Samir Ali and George Kukolj

1 HCV-Associated Diseases

Hepatitis C Virus (HCV) is a pathogen that poses a serious and a growing threat to human health with 170–200 million people infected globally. Approximately four million people (1.8%) in the US are HCV seropositive, and 2.7 million of these (70%) are chronically infected (1–4). The prevalence of HCV in Western Europe is similar to that in the US, and it is much higher in other countries such as Egypt, where the prevalence exceeds 20% (5). HCV transmission can often be linked to bloodborne routes, and following an acute infection the virus effectively evades immune recognition with an extremely high rate of viral persistence in the infected individuals. Most HCV-infected individuals succumb to chronic infection, which is normally associated with a significant risk of severe active hepatitis and cirrhosis of the liver (6–8). Persistent HCV infection may also directly modulate liver cell proliferation and viability and further increases the risk factor for development of hepatocellular carcinoma (HCC) (4). In addition, HCV infection is frequently associated with the development of autoimmune diseases, such as mixed cryoglobulinemia and glomerulonephritis (9). Without an effective antiviral treatment, HCV-related mortality is expected to triple in the next 10 years (8).

2 Hepatitis C Virus

HCV is a positive, single-stranded RNA virus classified in the *Hepacivirus* genus within the Flavivirus family (3). Its genome is composed of a large central open reading frame (ORF) of approximately 9,600 nucleotides encoding for a ~3,010-amino-acid polyprotein that is bracketed by two untranslated regions at the 5′ and 3′ ends (Fig. 1). This polyprotein precursor is post-translationally cleaved by host proteases to form structural proteins and by viral-encoded proteases to form the nonstructural proteins necessary for viral propagation. The structural proteins, encoded in the *N*-terminal region, include the core protein followed by two envelope glycosylated proteins E1 and E2, and the small transmembrane protein p7 whose function is somewhat obscure. The nonstructural domain encodes for six proteins; NS2, 3, 4A, 4B, 5A, and 5B. The NS3 protein has helicase and protease activities, whereas NS5B contains the RNA-dependent RNA polymerase activity. Both the NS3 and NS5B are essential for HCV replication (1) and are major targets in drug research.

3 Interferon Therapy

Current guidelines both in Europe and the US recommend using pegylated-interferon (IFN) α-2a or pegylated-IFN α-2b either alone or in combination with ribavirin in patients with chronic hepatitis C. IFNs are broad-spectrum natural host proteins harboring a variety of cellular actions, including induction of an antiviral state in their target cells, secretion of cytokines, recruitment of immune cells, and induction of cell differentiation. They were initially discovered as antiviral agents during studies on influenza virus interference. IFNs were shown to directly inhibit HCV replication on the basis of mathematical modeling of HCV kinetics *in vivo* (10, 11), and several *in vitro* studies reveal that IFNs inhibit replication of HCV RNA in cell culture systems (12–15). With respect to HCV, IFN apparently stimulates both innate and adaptive immune responses. After subcutaneous administration, IFNs bind specifically to high-affinity receptors at the surface of target cells. IFN receptor ligation triggers a cascade of intracellular reactions that activates numerous IFN-inducible genes. The

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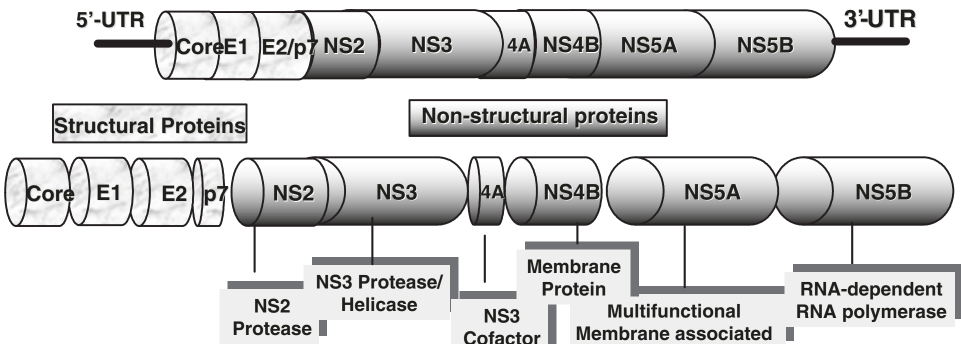

Fig. 1 Organization of the HCV genome. The genome plus strand encodes one long open reading frame that is flanked by 5′ and 3′ untranslated regions (UTRs). The structural proteins core, envelope E1 and E2, and p7 are processed by host cell proteases. The NS2 protein encodes an autoproteolytic cysteine protease. The NS3 serine protease catalyzes the cleavage of the remaining proteins from the nonstructural region; NS3 is also an RNA helicase. NS4A is a membrane-bound cofactor for NS3. NS4B is an intracellular membrane protein whose function is not well defined. NS5A is a membrane-associated protein with a number of purported functions. The NS5B encodes an RNA-dependent RNA polymerase

products of these genes mediate the cellular actions of IFNs (16). The antiviral effects of IFNs occur through two distinct but complementary phases. The first phase involves the induction of a general antiviral state in infected cells, resulting in direct inhibition of viral replication; this phase is characteristic of the innate immune response phase. The second phase encompasses immunomodulatory effects that enhance the host's specific antiviral immune responses and may accelerate the death of infected cells (17). This second phase mainly involves the adaptive immune response. Only a subset of the IFN-induced proteins and enzymatic pathways have been characterized (18) and include host factors such as the 2′–5′ oligoadenylate synthetase (2′–5′OAS) system, Mx proteins, and double-strand-RNA-dependent protein kinase (PKR). A combination of a number of IFN-activated cellular factors may ultimately contribute to directly inhibit HCV replication. The second-phase decline in HCV load during IFN-α-based therapy has been suggested to result from gradual clearance of infected cells by the host immune system, which may be accelerated by IFN-α (10, 11). Binding of IFN-α to its receptors at the surface of immune cells triggers complex and intricate effects. In particular, IFN-α induces class I major histocompatibility complex antigen expression; activates effector cells such as macrophages, natural killer cells, and cytotoxic T lymphocytes; and has complex interactions with the cytokine cascade (19, 20). It also stimulates the production of type 1 T helper (Th1) cells and reduces the production of Th2 (suppressor) cells (19, 20). However, the role of the immunomodulatory properties of IFN-α in the clearance of infected cells has not yet been convincingly demonstrated *in vivo*, and it is possible that infected cells are simply cleared by the normal immune response while IFN-α efficiently suppresses viral replication.

4 Advances in HCV Treatment

4.1 *Patterns of HCV Response*

Plasma HCV load values measured during and after IFN-based treatment categorize patients into different virological response groups. The primary objective of treatment is a "sustained virological response" (SVR), defined as the lack of detectable HCV RNA in serum 24 weeks after the end of therapy. Even with the best IFN-α treatment regimens (described below), a significant proportion of patients fail to achieve an SVR. These patients are said to be in "treatment failure". Treatment failure has been proposed to correspond to specific virological patterns. First, "non-responders" lack a significant decline in HCV RNA load by less than one log at any point during treatment. Second, "partial responders" experience a significant drop of greater than one log in HCV RNA through therapy; however, the viral genome can still be detected in patient's serum. The third class, "responders with breakthrough" initially become HCV RNA-negative but relapse at any time during treatment. The final class, "responder relapsers" appear to have maintained an SVR on the basis of negative detection of HCV RNA in serum during treatment but experience a relapse after treatment withdrawal, typically within 24 weeks (7, 21).

4.2 *Monotherapy with IFN*

Initial therapy for chronic hepatitis C was limited to IFN-α alone (22). This treatment regimen had limited outcome. HCV RNA becomes undetectable in 40–60% of patients during

treatment with IFN-α; however, most of these patients relapsed after the treatment was discontinued, and less than 20% of patients had a durable sustained virological response (21, 23, 24). In general, if HCV RNA is still detectable after 12 weeks of therapy with IFN-α, the patient is not expected to develop a virological response. Larger or more frequent dosing has resulted in higher SVR rates, but is often poorly tolerated (25).

4.3 Combination IFN and Ribavirin Treatment

Ribavirin, a synthetic broad-spectrum guanosine analog, has been found to have inhibitory actions *in vitro* against a wide range of DNA and RNA viruses (26). Among the proposed modes of action is the inhibition of inosine 5′ monophosphate dehydrogenase, resulting in direct inhibition of viral replication by interfering with RNA metabolism and in indirect effects on the host's immune response. It is unclear how ribavirin might modulate the immune response. There is evidence that it can shift the Th1/Th2 balance towards Th1 responses, leading to more efficient elimination of infected cells by specific immune effectors (27, 28) and modulation of immune response by alteration of the levels of Th1 and Th2 cells and direct cytoprotection that reduces hepatic inflammation. In addition, ribavirin is a potential RNA virus mutagen, increasing mutations in the RNA virus genome and driving viral quasispecies to "error catastrophe"; this results in a loss of fitness through a lethal acceleration of the accumulation of nucleotide mutations during replication (29, 30), ultimately reducing viral infectivity. Evidence for such a phenomenon in HCV infection is covered below in the section on NS5B polymerase nucleoside inhibitors.

A documented principal benefit of ribavirin is that it prevents relapse in IFN-α combination therapy (21, 31, 32). Ribavirin monotherapy has a moderate and transient inhibitory effect on HCV replication, observed in about 50% of patients (less than 0.5 log reduction at days two and three of administration) (33). When combined with standard IFN-α administration, ribavirin augments the initial response by increasing the proportion of patients who clear HCV RNA during therapy (21, 31, 34).

4.4 Pegylated-IFN

A significant breakthrough in the treatment of HCV infection was the development of pegylated forms of IFN-α. Polyethylene glycol (PEG) molecules are covalently attached to IFN-α (either large, branched pegylated IFN α-2a (40 kDa), or a small linear moiety for pegylated IFN α-2b (12 kDa)), resulting in higher sustained levels of IFN-α (35–38). The increased and sustained efficacy of pegylated IFN-α is due to longer serum half-lives attributable to decreased renal clearance. The optimized pharmacokinetic properties of the two pegylated IFNs reduced administration to a once-weekly dosing regimen that yields enhanced efficacy against HCV infection. When administered in monotherapy for 48 weeks, pegylated IFNs showed an improvement in efficacy relative to standard IFN; moreover, combination therapies with pegylated IFN and ribavirin further enhance SVR rates to 46–56% (34, 39).

5 Mechanisms of Resistance to Current Therapy

Only approximately 50% of chronically infected patients achieve a sustained virologic response to the combination pegylated IFN-α and ribavirin therapy. HCV strains appear to have the propensity to attenuate or evade host antiviral responses. The underlying mechanisms that regulate the responses and resistance to IFN-α-based therapy appear to be complex and are currently not well defined. The remainder of this section reviews some of the factors that are known or predicted to play a role in sustaining chronic infections with the premise that interplay between these factors is responsible for poor outcomes in terms of establishing an SVR to IFN-based therapies.

5.1 HCV Genotype

The particular HCV genotype remains the best predictor of SVR. Patients infected with HCV genotypes 1 and 4 are less likely to respond to IFN-α-based therapies than those infected with HCV genotypes 2 or 3. This trend has been consistently observed regardless of whether standard or pegylated IFN was used either alone or in combination with ribavirin (31, 32, 34, 36, 38, 39). Patients with HCV genotype 1 require a longer treatment regimen with the peg-IFN-α and ribavirin combination (48 weeks) to achieve SVR rates of only around 51% (40). The SVR rates among non-genotype 1 infected patients (primarily genotypes 2 and 3) are about 73–78% and can be attained with 24 weeks of combination therapy.

5.2 HCV Quasispecies

HCV persists in patients as a quasispecies, defined as a complex mixture of coexisting viral subpopulations harboring

genetically unique but closely related variants (reviewed in (41)). This quasispecies distribution confers a significant survival advantage. The simultaneous presence of multiple variant genomes and the high rate of generation of new variants allow the rapid selection of mutants with survival characteristics that suit new environmental conditions. Quasispecies arise from the high replication error rate of the HCV RNA-dependent RNA polymerase, as the enzyme lacks "proofreading" activity. This results in the accumulation of mutations that can confer survival benefits. The emergence of a specific variant is controlled primarily by selection pressures exerted by the environment in which the virus replicates, particularly the host immune responses, which may fluctuate both spontaneously and under the influence of exogenous factors such as IFN-α treatment (41). Generally, no clear pattern of viral mutations has been identified in patients failing to achieve SVR, despite extensive sequence analysis of HCV genes during and after IFN-α therapy (42–44). Perhaps one exception is the quasispecies changes found after IFN-α treatment failure in a 27-amino-acid stretch located at the *N*-terminus of the E2 envelope glycoprotein known as the hypervariable region 1 (HVR1). HVR1 differs considerably among genotypes and also among strains within a given genotype, and varies substantially during the course of acute and chronic infections, both spontaneously and under the influence of external factors. Significant HVR1 changes are observed during and after IFN-α treatment (42, 43, 45–47). These changes are probably driven by treatment-enhanced humoral responses (43). Breakthrough during therapy or post-treatment relapse is generally characterized by a peak of replication that corresponds to reinfection of the liver by the treatment-selected quasispecies.

5.3 Evasion of Immune Responses

Qualitative and quantitative defects in both CD4 + and CD8 + immune clearance of infected cells appear to be important determinants of viral persistence. Early and sustained CD4 + and CD8 + T-cell responses are crucial for controlling HCV infection (48, 49). This is evident from the observed dramatic decrease in the magnitude of T-cell responses in chronic versus acute HCV infections. The factors that control CD4 + and CD8 + T-cell response may also influence the outcome of IFN-α therapy, and the principal determinants (viral and/or host) are only partially known and understood.

5.4 Viral Proteins

A number of different HCV proteins have been associated with IFN-α resistance mechanisms *in vitro*. The envelope E2 protein, the nonstructural NS3/4A protein, and the NS5A proteins have been proposed as viral antagonists of cellular antiviral activities.

A 12-amino-acid sequence within the carboxyl-terminal part of the genotype 1 E2 protein, termed the PKR/eIF-2a phosphorylation homology domain (PePHD), is analogous to the phosphorylation sites of RNA-dependent protein kinase (PKR) and the eukaryotic translation initiation factor eIF-2α. Binding of HCV subtype 1a/b-derived PePHD to the PKR abolished its kinase activity and blocked its inhibitory effect on protein synthesis *in vitro*, while these effects were not detectable for PePHD sequences based on HCV genotype 2 and 3 isolates (50). The interaction of the PePHD with PKR may, in part, account for the relatively enhanced resistance of HCV-1 isolates to INF-α-based antiviral therapy (50, 51).

A similar PKR antagonistic function has been ascribed to the carboxyl-terminal part of the NS5A protein. A 66-amino-acid NS5A sequence in HCV genotype 1 isolates is proposed to interact with PKR and result in the inhibition of protein synthesis *in vitro* (52). Specific mutations in the NS5A protein within a region known as the interferon sensitivity determining region (ISDR) has been loosely linked to antagonism of innate cellular antiviral responses (52). Furthermore, the functions of the NS5A protein are incompletely defined, but a wide variety of mutations in genotype 1 NS5A adapt HCV genomic RNA to enhance replication in cell culture.

The strongest evidence to have emerged for the role of a virally encoded protein in modulating the infected cell's innate antiviral response is with the HCV NS3/4A protease. An intracellular antiviral response can be initiated by host proteins such as RIG-I or MDA-5 that sense replicating viral RNA and instigate a signaling cascade to activate NF-κB and IRF-3, and induce type I interferon gene expression. The HCV NS3/4A serine protease cleaves an adaptor protein, MAVS (also known as VISA, IPS-1 and Cardif), downstream of RIG-I/MDA-5 to prevent the activation of IRF-3 and block interferon gene expression (53).

5.5 Negative Regulators of Jak/Stat Signaling Pathway

Although the NS3/4A protease suppresses intracellular induction of an antiviral response, precisely how the HCV can resist the action to ectopically administered interferon is unclear. The action of IFN-α is mediated by the Jak/Stat signaling pathway. IFN-α binding to the extracellular portion of its transmembrane receptor activates tyrosine kinases (Jak1, Tyk2) that recruit and phosphorylate the signal transducers and activators of transcription (Stat). A tetrameric complex of activated Stat1/2 and p48/IRF-9 translocates to the nucleus and forms interferon-stimulated gene factor 3 (ISGF3). ISGF3

ultimately increases the transcription of a broad group of interferon effector genes.

The expression of the HCV proteins has been shown to interfere with the IFN-α-induced intracellular signaling in various animal models as well as human disease samples chronically infected with HCV (54–56). The structural HCV proteins are presumed to be engaged in the inhibition of the Jak/Stat signaling pathway, but further investigation is required to decipher the details of these interactions.

5.6 *Host Negative Regulators of IFN Signaling*

Under normal condition, host cells express various negative regulators of INF-driven Jak/Stat-mediated signaling and gene activation. Despite the absence of direct evidence linking deregulation of these cellular proteins in HCV resistance to IFN-α therapy, it is conceivable that HCV may interfere with the levels of these proteins shifting the cell state toward resistance. Among the cellular proteins that downregulate Jak/Stat-mediated signals are the family of suppressors of cytokine signaling (SOCS), protein inhibitor of activated Stat (PIAS), Stat-induced Stat inhibitors (SSI), and cytokine-inducible SH2 protein (CIS) (57–61).

There are eight known members of the SOCS proteins (57). Multiple host cytokines, including IFN-α, induce SOCS-1, which interacts with the kinase domain of all four members of the Jak family of kinases to suppress signaling and activation of Stat1. SOCS-1 is also known to inhibit the antiviral and antiproliferative activities of IFNs. Members of the family of cellular proteins, PIAS-1 and PIAS-3, are also negative regulators of Stat signaling. PIAS-1 protein directly associates with Stat1 in response to treatment with IFNs and blocks the DNA-binding activity and therefore the Stat1-mediated gene activation (59). Negative regulation of Jak/Stat signal transduction is also achieved by dephosphorylation mediated by the SHP-1 protein tyrosine phosphatase. SHP-1 suppresses the signal transduction process of a variety of cytokines, including IFNs (62), by directly interacting with Jak kinases and catalyzing their dephosphorylation.

Evidence that some HCV gene products may serve as agonists in the suppression of cytokine signaling is seen with the HCV core protein. Ectopic expression of the HCV core induces SOCS-3 expression and inhibits IFN-α-induced nuclear translocation of Stat1 (63). These observations were acquired with the core from genotype 1b virus, and it will be interesting to determine the extent of IFN-α signal suppression by the genotype 2 and 3 core proteins.

A combination of viral and host factors likely determine the response to current treatments that predominantly rely on augmenting the interferon-mediated antiviral defense systems. HCV has apparently evolved a number of effective strategies to resist these responses, and the next generation of therapies will undoubtedly attempt to overcome these limitations by specifically targeting virus-encoded functions.

6 Resistance: *In Vitro* Models

6.1 *HCV Replicons*

The inability to robustly culture the HCV virus *in vitro* has been a major impediment to studying the HCV life cycle and establishing authentic antiviral assays. The first major breakthrough in overcoming this limitation to basic HCV research was the development of a robust tissue culture system based on subgenomic replicons (64). This system fulfilled a long-standing need to evaluate the cell culture efficacy of antiviral compounds that target HCV nonstructural proteins as well as provide an *in vitro* genetic screen for virology and resistance studies. The first generation of HCV subgenomic replicons were designed in a bicistronic fashion by replacing the structural region of the genome with the gene encoding for neomycin phosphotransferase (NPT) and the internal ribosomal entry site (IRES) from encephelomyocarditis virus (EMCV) such that the 5′ HCV IRES in the first cistron directs the translation of NPT and the second cistron directs translation of the HCV nonstructural proteins from the EMCV IRES (Fig. 2). Introduction of this RNA into the human hepatoma Huh-7 cell line and selection with neomycin permitted the isolation of rare clones that harbored high levels of

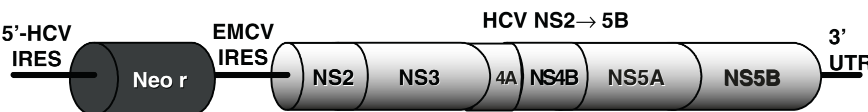

Fig. 2 Schematic representation of a bicistronic subgenomic HCV RNA replicon. The 5′ and 3′ untranslated regions (UTRs) mimic the HCV viral RNA. The first cistron is translated by the HCV IRES and encodes the neomycin phosphotransferase gene. The second cistron is translated by the EMCV IRES and encodes the entire HCV nonstructural region. Replicon RNA is transfected into cultured cells and selected for high level of RNA replication with neomycin. Antivirals that target HCV nonstructural protein function effectively reduce RNA replication. Resistance to HCV antivirals is selected in cell culture by dual selection with the specific inhibitor and neomycin

subgenomic RNA whose replication was catalyzed by the cascade of functions encoded by the HCV nonstructural region. These replicon clones confer resistance to neomycin, and their low frequency of formation reflected the selection of tissue-culture-adaptive mutations in the HCV nonstructural region (65). The authenticity of cell-culture-adapted replicons is somewhat disputable, as specific adaptive mutations can severely attenuate viral growth in chimpanzees and there is an apparent discordance with replication of HCV genotype 1 RNA in tissue culture and replication in animal models (66). Nonetheless, a genotype 2a viral RNA clone was recently isolated from a patient with fulminant hepatitis (67), which replicates efficiently in the replicon model without the need for adaptive mutations and produces infectious particles both in tissue culture and animal models (68–70). The HCV replicons have thus served as an invaluable model to progressively improve our study of the HCV life cycle in *ex vivo* models. With the discovery of specific inhibitors that target the well-characterized HCV enzymatic functions, the bicistronic genetic system of the subgenomic replicons has permitted the selection of replicons in the presence of neomycin and specific inhibitors of the HCV nonstructural proteins. Such a dual selection system facilitated the isolation and identification of antiviral-resistant mutants in HCV targets because they are genetically linked to the neomycin resistance cistron (Fig. 2). The remaining part of this chapter summarizes the emerging work that has been initiated with novel NS3 protease and NS5B polymerase inhibitors and the discovery of various mutations that confer resistance to these diverse compounds. As some of these HCV antivirals progress through clinical development, it will be interesting to see which of these drugs actually select for resistant viruses in infected patients and whether the mutations identified *in vitro* were predictive of the mutations isolated *in vivo*. From an early drug discovery perspective, the HCV replicon resistance studies have certainly provided more insight into the mechanisms of action for compounds that specifically target essential HCV functions.

6.2 NS3 Protease Inhibitors

NS3 protease is a chymotrypsin-like serine protease encoded by the amino terminal 180 amino acids of NS3 that catalyzes four cleavages of the polyprotein to produce mature NS3, NS4A, NS4B, NS5A, and NS5B proteins (1). Enzymatic studies have shown that amino terminal products derived from cleavage of peptide substrates based on the NS5A/5B and NS4A/4B cleavage sites are competitive inhibitors of the enzyme (71, 72). These peptides have served as a useful starting point in medicinal chemistry efforts to rationally design NS3 protease inhibitors (73) that led to the discovery of potent anti-HCV compounds (74, 75). BILN 2061, selected from a series of macrocyclic inhibitors, is a competitive inhibitor of the NS3 proteases of HCV genotypes 1a and 1b with low nanomolar K_i values, blocks NS3-mediated polyprotein processing, and inhibits HCV RNA replication in replicon-containing cells with an EC_{50} value of 3 nM. In a clinical proof-of-concept study with patients infected with HCV genotype 1, BILN 2061 was highly effective at reducing virus load in a 2-day treatment (74, 76). These early results clearly demonstrated the great potential of selective and potent anti-HCV agents. Other inhibitors of the NS3 protease have also entered clinical development (Fig. 3); VX-950 (telaprevir) and SCH-503034 (boceprevir) are

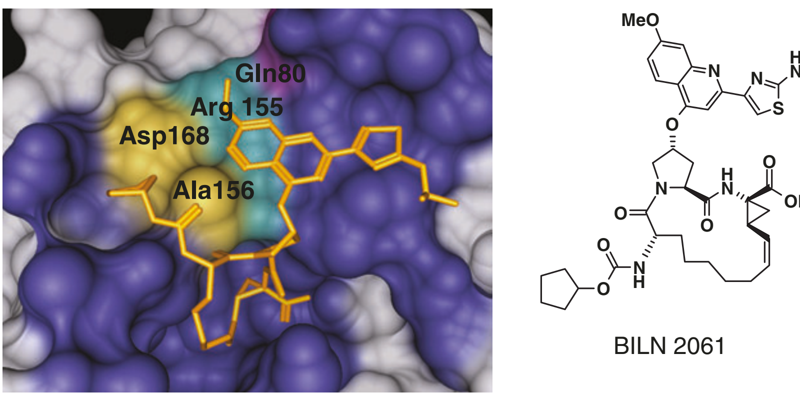

Fig. 3 NS3 protease inhibitors and a model of the macrocyclic BILN 2061 in the protease active site. Residues located within 5 Å of the inhibitor are shaded in color. Residues responsible for resistance to BILN 2061 and related protease inhibitors are identified: Gln80, Arg155, Ala156 and Asp168 (*See Color Plates*)

also peptidomimetics based on *N*-terminal cleavage products but, unlike BILN 2061, are reactive ketoamide inhibitors of the serine protease (77). A number of replicon resistance studies have been reported for BILN2061, VX-950, and SCH-503034 (78–81).

Long-term culturing of replicon cells in the presence of both the selective agent neomycin and BILN 2061 identified resistant mutants that predominantly encoded NS3 protease amino acid substitutions at either A156 or D168. Changes at either of these two positions were also selected with VX-950, SCH-503034, and related peptidomimetic inhibitors (78, 80–83). The effect of these major mutants on replicon fitness, as determined in a direct measure of transient replication, revealed that most mutants reduce (1–10% of the wild-type replicon) RNA levels. The D168A mutant is severely compromised with no transient replication detectable above background, although rare stable cell lines with this mutant replicon are selected at a very low frequency, and presumably reflect the need for additional compensatory mutations that restore replication (82).

Though the A156 and D168 mutations affected transient RNA replication to varying extents, none of these mutations, including D168A, has a remarkable effect on the catalytic efficiency of purified NS3/NS4A protein. Changes at these positions decreased the potency of BILN 2061 up to 500-fold in both enzymatic and cell-based assays. A model of BILN 2061 bound to the active site of the NS3 protease domain highlights the proximity of R155, A156, and D168 residues to the bound inhibitor (Fig. 3). D168 is proposed to form a salt bridge with R155, and changes in D168 indirectly affect BILN 2061 affinity by reorienting the R155 side chain. The inhibitor P2 quinoline substituent is affected by this reorientation, and inhibitors such as VX-950 with smaller P2 segments can apparently tolerate D168 substitutions (78). D168 substitutions to glycine or alanine decrease BILN 2061 potencies 17- and 96-fold, respectively (81). The size of the amino acid side chain, in addition to the change in charge at position 168, affects BILN 2061 affinity. Aspartate168 is not conserved among HCV genotypes and is substituted with a glutamine in genotype 3a. Notably, the affinity of the genotype 3a (relative to the 1b) NS3 protease for BILN 2061 is approximately two orders of magnitude lower, a characteristic that can be mainly attributed to the D168Q polymorphism (84). These results apparently account for the reduced efficacy of BILN 2061 in short-term Phase Ib clinical trials involving patients infected with non genotype-1 HCV (85).

The effect of an R155Q substitution on BILN 2061 inhibition in cell-based replicon assays (~50-fold decrease in potency) was not as large as those conferred by select A156 or D168 changes (80, 81). A Q80R-resistant mutant displayed a small shift (threefold reduction) in BILN 2061 potency; this particular mutant was only selected in the presence of low BILN 2061 concentrations and demonstrates the broad capability of selecting HCV replicons that encode both major and minor determinants of resistance (81).

The NS3 protease A156T or V substitutions significantly diminish (>100-fold) both BILN 2061 and VX-950 affinity, presumably by a steric clash with the inhibitors. However, an A156S substitution that was selected with either VX-950 or SCH-503034 presents a smaller side chain than T or V, and does not affect BILN 2061 potency (78). In contrast, the A156S substitution is proposed to disrupt the VX-950 P4 cylcohexyl hydrophobic interaction with the protease subsite (79). The varying effect of different A156 mutants demonstrates that related yet distinct inhibitors do not necessarily share the same resistance profile. The clinical relevance of the A156 position to viral resistance has emerged from a 14-day Phase Ib trial with VX-950 wherein all patients initially had a substantial (>3 log) decline in viral load during treatment (86). A subset of patients on treatment also demonstrated either a plateau or an apparent rebound in viral load, and sequencing of the NS3 protease domains during this selection identified a series of mutations that were not found in samples from the placebo controls. A highly sensitive clonal sequencing method was used to identify the VX-950-resistant variants. Single amino acid NS3 substitutions A156S/T/V, R155K/T, as well as V36A/M and T54A, were selected during VX-950 monotherapy; rare double mutants were also identified that combined substitutions at position 36 with changes at residue 155 or 156 (87). In the absence of VX-950 selective pressure, following the short treatment, most resistant variants were replaced by wild-type virus, which presumably reflects reduced fitness of the mutant viruses (87).

The potency of mechanistically distinct inhibitors such as IFN-α or an HCV polymerase inhibitor is unaffected by the protease inhibitor resistance mutations (81, 82). Replicon cells that are resistant to IFN-α can also be isolated; however, mapping the determinant(s) of resistance is apparently more complex, as no predominant replicon mutation is obvious from sequence analysis, and cellular as well as replicon adaptations may confer IFN-α resistance. Combinations of NS3 protease inhibitor and IFN-α in cell culture demonstrate that these two distinct drugs are effective at suppressing the emergence of resistant replicons and are predictive of useful cotherapy in the treatment of chronic HCV infection. Both VX-950 and SCH-503034 are currently in Phase II trials in combination with pegylated interferon (87, 88).

6.3 HCV Polymerase Inhibitors

The viral nucleic acid polymerase inhibitors constitute most of the currently approved antiviral drugs and cast polymerases as prime therapeutic targets for viral infections.

Consequently, the NS5B enzyme has become a major target in the search for novel inhibitors of HCV replication, and our understanding of both the structure and function of this polymerase has quickly surpassed that of other RNA-dependent RNA polymerases (RdRp). The HCV NS5B is comparable to other nucleic acid polymerases (Fig. 4) with the familiar features of fingers, palm, and thumb domains that are organized in a "right-hand" motif (89–91). A distinct feature of the HCV polymerase (and closely related RdRp) active site cavity is the protrusion of a unique β-hairpin from the thumb subdomain that apparently plays a role in the initiation of *de novo* RNA synthesis as demonstrated by both structural and biochemical studies (92–95). Another feature of the HCV polymerase is two loops that bridge the fingers and thumb subdomain and result in an encircled active site. This feature is now known to be shared by other RdRp from rhinovirus, bacteriophage phi6, rabbit hemorrhagic disease virus, bovine viral diarrhea virus, Norwalk virus, and poliovirus (96–103). Interestingly, the interface between the HCV polymerase *N*-terminal λ1 loop and the thumb subdomain is the location of a GTP binding site (92), although its precise biological role is unsolved.

Extensive research efforts by many groups have led to the discovery of a large assortment of HCV polymerase inhibitors, the most advanced of which are being evaluated in early clinical trials. The NS5B inhibitors can be broadly divided into three categories: (a) nucleoside analogs, (b) pyrophosphate mimics, and (c) non-nucleoside inhibitors reviewed in (104). Wide-ranging studies have been conducted, predominantly with the cell culture replicon model, and the latter part of this chapter will focus on those inhibitors for which resistance studies have been reported.

6.3.1 Ribavirin

Although the broad-spectrum guanosine nucleoside analog ribavirin is a component of the current gold standard combination therapy for chronic HCV infection, its mechanism of action is ill defined (26). Ribavirin has been postulated to work by at least one of the following mechanisms: (a) inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH), resulting in lower cellular GTP levels that reduce viral RNA production; (b) enhancement of the immune response which

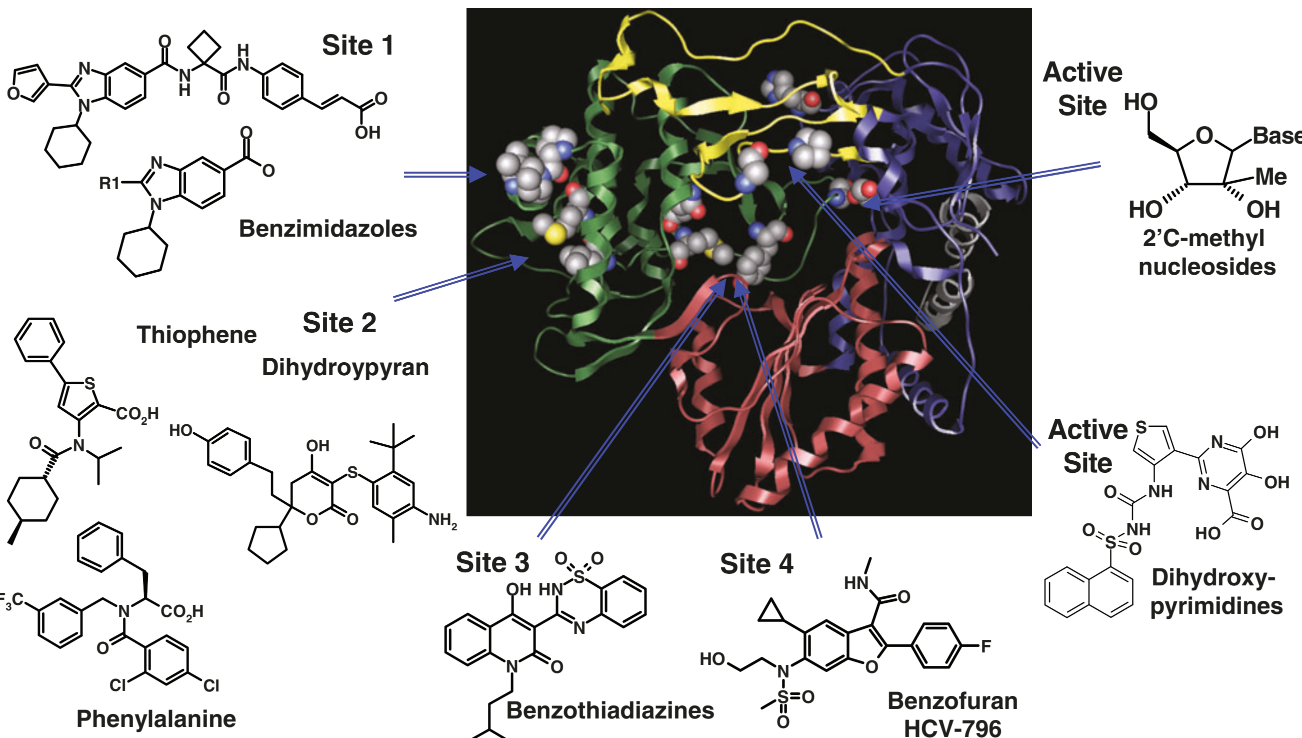

Fig. 4 Three-dimensional structure of NS5B locating the binding sites and resistant mutants to NS5B polymerase inhibitors. The structure of the NS5B polymerase highlights the *red palm* subdomain; *purple* and *grey* delineate the fingers, whereas *green* depicts the thumb subdomain; *yellow* represents the segments that comprise two loops interconnecting the fingers and thumb subdomains. Three amino acids, P495, P496, V499 (in space-filling mode), in the thumb domain identify the site 1-resistant mutants. The L419 and M423 thumb residues characterize the site 2-resistant mutants. Benzothiadiazines have selected for changes at positions that encircle the allosteric site 3: H95, M414, Y448, C451, and G558, whereas HCV-796 selects for substitutions at C316, S365, and M414. The dihydroxypyrimidine compound selects for mutants with changes in the λ2 finger loop at G152 or P156. Lastly, 2′-*C*-methyl nucleosides are sensitive to S282 substitutions in the finger domain (*See Color Plates*)

may be related to its effect on IMPDH and purine pools in immunoregulatory cells; (c) inhibition of the NS5B polymerase; and (d) error catastrophe as a consequence of the increased mutation rate during incorporation of ribavirin monophosphate into viral RNA. Although ribavirin's immunomodulatory effect may have a role *in vivo*, it cannot account for antiviral activities against a variety of viruses in cell culture (105, 106). *In vitro* biochemical assays show that ribavirin triphosphate does not inhibit NS5B enzyme activity, but it is a pseudo-substrate that is recognized and incorporated, with a lower affinity and rate, as monophosphate into the growing RNA chain (107, 108). These studies are further bolstered by the demonstration that ribavirin is also incorporated by the poliovirus RdRp and capable of base-pairing equally well with either uridine monophosphate (UMP) or cytidine monophosphate (CMP), thereby potentially causing transition mutations (30); the anti-poliovirus activity of ribavirin correlates directly with this mutagenic effect (29), suggesting that the primary mechanism of action in this system may be "error catastrophe." Subsequently, ribavirin was also suggested to increase HCV mutation frequencies in the replicon system as well (109, 110).

In further support of these *in vitro* findings, ribavirin has been reported to have a modest mutagenic effect on HCV *in vivo* (111, 112), and a mutation encoding a genotype 1a NS5B F415Y substitution may decrease sensitivity to ribavirin (111). In the genotype 1b replicon system, residue 415 is a Y and may explain why these replicons are relatively insensitive to ribavirin. The location of amino acid 415 on the inner surface of the thumb domain suggests that the F to Y substitution may alter the RNA binding pocket. However, there is no direct biochemical evidence that the F415Y substitution, a naturally occurring polymorphism, confers resistance to ribavirin.

6.3.2 2′-Modified Nucleosides

The advent of cell-culture HCV replicons accelerated the discovery of nucleoside analogs as HCV antivirals. As prodrugs, these inhibitors require conversion to the active triphosphate from, a demanding synthetic procedure that can be biologically catalyzed by the cell's nucleotide metabolic pathways. The first nucleoside inhibitors (NI) of the HCV NS5B validated in cell-culture inhibition studies included a modification at the 2′ position of the ribose sugar (113). A pyrimidine nucleoside, 2′-*C*-methyl cytidine and its 3′ valine ester derivative (NM283, Idenix pharmaceuticals), is the first NI to be evaluated for anti-HCV activity in chronically infected humans (114, 115). The 3′ valine ester improves oral bioavailability and is rapidly converted to the 2′-*C*-methyl cytidine. Treatment with NM283 alone results in a dose-proportional antiviral activity reflected by a drop in mean viral load of approximately 1 log10, which was reported with up to a 800 mg daily dose in a 15-day trial (114). These results have prompted the evaluation of NM283 in combination with pegylated interferon in Phase II trials.

A number of other 2′-modified NIs, such as 2′-*C*-methyl adenosine, 2′-*C*-methyl guanosine, and 2′-*O*-methyl cytidine (113, 116), have emerged from discovery efforts. The triphosphate derivatives of all of these 2′-modified NIs are incorporated by the HCV NS5B as the monophosphate product into RNA. The 2′ modification is thought to alter the ribose ring conformation and consequently prevent the 3′OH group from participating in a nucleophilic attack on the incoming triphosphate which results in premature termination of RNA synthesis. The 2′-modified NIs have a broader spectrum of activity and inhibit replication of genetically related viruses such as bovine diarrhea virus, yellow fever, and West African Nile viruses (117), despite the extremely limited homology among their NS5B polymerases. Moreover, these NIs also inhibit a panel of NS5B and replicons derived from HCV non genotype-1 and indicate that they may be effective against all HCV strains (118). Despite their broad activity, *in vitro* selection of replicons resistant to 2′-*C*-methyl NIs identified a single mutation in the NS5B polymerase that encodes for a Ser 282 substituted by threonine (Fig. 4). Resistance to the 2′-*C*-methyl NIs conferred by the S282T substitution is manifested in two ways. The mutant NS5B enzymes have a reduced affinity for the 2′-*C*-methyl NIs and only a modest reduction in affinity for natural substrates (117, 119); the 2′-*C*- methyl group is proposed to be in close proximity to the side chain of residue 282, allowing for an improved discrimination between the substrate and inhibitor by the mutant enzyme. In addition, the S282T mutant enzyme can apparently add a nucleotide beyond the incorporated 2′-*C*-methyl NI and maintain RNA chain elongation, albeit with significantly lower efficiency. Re-engineered replicons that only encode the S282T substitution replicate poorly in transient assays, and the selection of additional compensatory mutations is required to sustain high levels of RNA replication (117). As a potential fitness-modifying mutant, the selection of the S282T substitution, particularly with an authentic virus replication system such as the JFH-1 genotype 2a virus (68–70) or in chronically infected individuals undergoing treatment with this drug class, remains to be documented.

6.3.3 Non-nucleoside Active-Site Inhibitors

A series of diketo acids (120) and hydroxypyrimidine carboxylic acids (121) exemplify the pyrophosphate mimics that selectively bind the NS5B active-site divalent cations and act as product-like inhibitors of the polymerase reaction. Pairwise combination studies of these inhibitors with the pyrophosphate analogs such as phosphonoacetic acid and Foscarnet have revealed a mutually exclusive mode of inhibition (122).

The precise manner in which these compounds bind the polymerase is unknown; however, the acid-containing moieties are thought to chelate the Mg^{2+} ions bound in the enzyme active site, whereas the distal aromatic substituents are proposed to contact the active-site periphery of the λ2 finger loop (Fig. 4). The selection of HCV replicons resistant to a hydroxypyrimidine carboxylic acid compound identifies changes at either P156 (substituted to S or L) or G152 substituted with glutamate in this finger loop region that encircles the active site (123).

A class of NS5B polymerase inhibitors characterized by a benzothiadiazine core that was discovered from screening small molecule libraries (124) is noncompetitive with nucleotides and inhibits RNA synthesis prior to the formation of a stable elongating replication complex (124–126). The compounds associate with enzyme-bound RNA, apparently arresting the polymerase in a preinitiation complex (125). The role of these compounds in arresting the initiation of RNA synthesis was further bolstered by replicon resistance studies, which identified the location of NS5B amino acid substitutions that altered the activities of the inhibitor and enzyme. Six independent NS5B substitutions have been selected with the benzothiadiazines: H95R, M414T, N411S, Y448H, C451R, and G558R (126–128). Three of these amino acids, N411, M414, and Y448, cluster in the active site at the junction of the thumb and palm domains (also referred to as allosteric inhibitor site 3; Fig. 4). The NS5B polymerase initiates RNA synthesis *de novo* by binding the initiating nucleotide in a cavity shaped by the thumb–palm junction (129). Replicons harboring any of the three individual NS5B mutants at residues 411, 414, or 448 decrease the potency of benzothiadiazine by more than 25-fold (128). The replicons encoding the C451R or G558R mutations have a slight reduction in fitness and also suppress benzothiadiazine inhibition by more than 20-fold; the surprising observation with these latter two resistant mutants is that replicon fitness is *enhanced* in the presence of the inhibitor (126). The C451 and G558 residues lie on the thumb β-hairpin and *C*-terminus, respectively, and the inference from these studies is that the benzothiadiazines can form a ternary complex with the "inhibitor-dependent" mutant enzymes and RNA template to enhance the *de novo* initiation of replication.

The chemically distinct non-nucleoside inhibitor, HCV-796, binds in a proximal location adjacent to the palm domain and active site. Resistant replicons have also identified substitutions at M414 that moderately reduce sensitivity (less than tenfold) to HCV-796. The major HCV-796-resistant mutations that were selected in replicon studies encode for changes at C316 (to F, Y, or N) or S365 (to A or T). The importance of the C316Y mutant has been substantiated in short-term (14-day) clinical trials with HCV-796, which were selected for viruses encoding this NS5B variant (130).

6.3.4 Allosteric Inhibitors Site 1

Benzimidazole 5-carboxamide inhibitors have also been extensively pursued as non-nucleoside inhibitors (126, 131–135). These compounds inhibit an initiation phase of the reaction, and are also noncompetitive with respect to NTP incorporation, but unlike the benzothiadiazines inhibit productive binding of RNA template (136). The compounds have a unique resistance profile, and replicon resistance studies with a truncated benzimidazole 5-carboxylic acid identified substitutions in P495 of the thumb domain (137), which reflect an extensive interaction of the core benzimidazole-cyclohexyl moiety with this residue. Substitutions at P495 to either S, L, A, or T have the most profound effect on inhibitor potency by shifting IC_{50}'s up to 100-fold (138). The use of optimized benzimidazoles with extensions from the 5-carboxamide portion provides for additional interactions with the binding pocket and was used to select replicons with substitutions at P496 and V499 (138); these resistant mutants are consistent with a model in which the right-hand side carboxamide appendage interacts with the 496 and 499 residues of the binding site. The right-hand side extension of these inhibitors tolerates a higher degree of chemical diversity than the core (131, 133); consequently, changes in the amino acids of the binding pocket that constitute the recognition elements for this appendage have less of an effect on inhibitor potency than substitutions that affect core recognition. The P496 and V499 mutations shift compound potency less than 25-fold and threefold, respectively.

The location of the binding site for the benzimidazole class of inhibitors is a region that forms the interface between the *N*-terminal λ1 loop and the thumb domain (Fig. 4). Site-directed mutations in a highly conserved leucine at position 30 in the λ1 loop that anchors the loop to a pocket in the thumb alter the conformation of the NS5B polymerase and inactivate the enzyme (139). Recently derived structures from X-ray crystallography indicate that this class of inhibitors bind the thumb and displace the λ1 loop and mechanistically mimic the loop-inactivating mutants (140, 141).

6.3.5 Allosteric Inhibitors Site 2

A distinct solvent-exposed pocket on the peripheral surface of the thumb domain promiscuously binds at least three different classes of non-nucleoside inhibitors: the thiophene 2-carboxylic acids, phenylalanine derivatives, and cyclopentyl dihydropyran-2-ones (142–144). Crystallization data indicate that all three compounds, despite their diversity, occupy overlapping portions of a crevice at the outer base of the thumb (Fig. 4). Mechanistically, these compounds resemble the site 1 inhibitors in that they are not competitive with NTP and affect an early step in polymerase catalysis. The selection of replicons resistant to a thiophene 2-carboxylic

acid compound identified one of the two substitutions, L419M or M423T, that directly shift the compound potency by at least tenfold (123). The M423T substitution that shifts the potency of site 2 compounds is fully consistent with the importance of a hydrophobic pocket around residue 423 that complements a lipophilic portion of all site 2 inhibitors.

Site 1 and site 2 are spatially distinct, and the non-nucleosides that bind these pockets select for distinct resistant mutants that display no cross-resistance. Despite these differences, structural evidence indicates that these compounds may have similar modes of action. Recent crystallization of the thiophene 2-carboxylic acid class of inhibitors with NS5B polymerase indicates that the compounds disrupt an important regulatory role for the thumb (144); although these compounds do not directly bind the λ1 loop–thumb interface, the proximal location of site 2 within the thumb apparently elicits subtle conformational changes at the loop–thumb interface upon binding, which in turn prevents a conformational transition required for productive RNA binding (144).

7 Conclusions and Prospects

RNA viruses replicate with poor fidelity, and this characteristic has been exploited in intensive drug discovery efforts with the HCV sub-genomic replicon system to select for variants that confer resistance to a diverse spectrum of NS3 protease and NS5B polymerase inhibitors, in addition to other incompletely characterized targets in the HCV nonstructural region. These studies have proven useful in the genetic mapping of potential sites of action for newly discovered compounds. However, the selection for cell-culture-fit replicons does not emulate the selective pressures on HCV in chronically infected humans. In humans, HCV replicates at extremely high rates (11), which in part may account for its high chronicity and variable response to current therapy. There is a high probability for the selection of potentially unique drug-resistant viral variants during chemotherapy with the newly emerging drug classes. Indeed, in early clinical trials with NS3 protease inhibitor, resistant virus appeared within the first 2 weeks of treatment (87) and has impacted the clinical development pathways for the first generation of HCV-specific antivirals. These drugs may initially progress in combination with pegylated-IFN and ribavirin. However, with the discovery and early classification of antivirals that target distinct pockets and provide for unique resistance profiles, it is conceivable that multidrug combinations can be based entirely on highly selective anti-HCV drugs. With the rapid progress in the treatment of chronic HCV infection, we can be optimistic about the dual prospects for a significant increase in sustained viral response rates and a reduction in the severity of side effects.

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Section F
Parasitic Drug Resistance – Mechanisms

Chapter 39
Drug Resistance Mechanisms in *Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis,* and Opportunistic Anaerobic Protozoa

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1 Introduction

Resistance of organisms to toxic agents is a survival mechanism fundamental for adaptation and evolution of life. As a counterpart, drug resistance is a medical problem in cancer and infectious diseases, with not many alternatives available. *Entamoeba histolytica*, *Giardia lamblia* (syn. *duodenalis* or *intestinalis*), and *Trichomonas vaginalis* (Fig. 1) are anaerobic and microaerophilic pathogens capable of developing drug resistance. Over one billion individuals worldwide harbor these and other anaerobic protozoa such as *Blastocystis hominis*, *Cryptosporidium parvum*, *Isospora* spp., *Cyclospora* spp., and *Microsporidia* (1). Most infected people live in poor countries. Unhygienic sanitary conditions and poor health education are the causes for infectious protozoan prevalence, and they can be eradicated by implementing drainage, parasite-free water supply, and sexual education for all people.

E. histolytica and *G. lamblia* (Fig. 1a, b) enter humans by ingestion of cysts that come out with feces from infected individuals. Host factors induce transformation of cysts into trophozoites which cause the diseases. A high percentage of infected people do not present symptoms, but spread the parasite. The cysts, highly resistant to atmospheric conditions, are formed in the intestine and excreted with feces. They contaminate water and food, their vehicles to infect other hosts. *E. histolytica* mainly invades gut and liver, but also the brain, lungs, skin, and genitals. *T. vaginalis* (Fig. 1c) is the causative of the most common nonviral human sexually transmitted disease (2). Between 25% and 50% of infected people are asymptomatic, but the infection provokes vaginitis with inflammatory discharge and predisposition to cervical neoplasia, and it causes complications during pregnancy and results in low weight of newborns, preterm delivery, and respiratory diseases. In men, it produces urethritis, orchitis, oligoathenosteratospermia, and hypogonadism (3). Trichomonosis is linked to an increased risk of cytomegalovirus (CMV) (4) and human immunodeficiency virus (HIV) transmission (5).

Anaerobic protozoa emerged very early in evolution, and parasites have gained many characteristics through coevolution inside the host. They share some features, but also present striking differences. *E. histolytica* has a cytoplasm full of vacuoles, and except for the nucleus, organelles are difficult to distinguish in the highly phagocytic trophozoites (Fig. 1a). *Giardia* has eight flagella, two nuclei, and a ventral disk formed by giardins and other cytoskeleton proteins that allow parasite adherence to epithelia (Fig. 1b). *T. vaginalis* has four anterior flagella and a recurrent flagellum incorporated into an undulating membrane (Fig. 1c). It can form pseudopodia to phagocyte epithelial cells. The three parasites use adherence molecules and cysteine proteases to colonize and damage tissues (6–8). They do not have mitochondria and peroxisomes, organelles found in most eukaryotes, or canonical mitochondrial processes. *E. histolytica* has a double membrane-limited organelle called EhkO, which contains DNA and pyruvate: ferredoxin oxidoreductase (PFOR) (9). Additionally, mitochondria-like enzymes have been found in others organelles called mitosome and crypton (10, 11). Similarly, it has been reported that *G. lamblia* contains mitosomes that function in iron–sulfur protein maturation (12). *T. vaginalis* has the hydrogenosome, where both decarboxylation of pyruvate by PFOR and energy generation take place (13). Phylogenetic analysis suggest that *E. histolytica* and *G. lamblia* iron-hydrogenase genes were derived from a common eubacterial ancestor, distinct from the *T. vaginalis* iron-hydrogenases genes ancestor (14). Similarity in their metabolism allows the use of common drugs, such as the 5-nitroimidazoles, to kill them.

Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), the preferred drug for the three mentioned parasites, enters the cell by passive diffusion in an inactive form. It has a lower redox potential (−460 mV) than ferredoxin (Fd) (−320 mV), gains electrons transferred by PFOR to Fd to be

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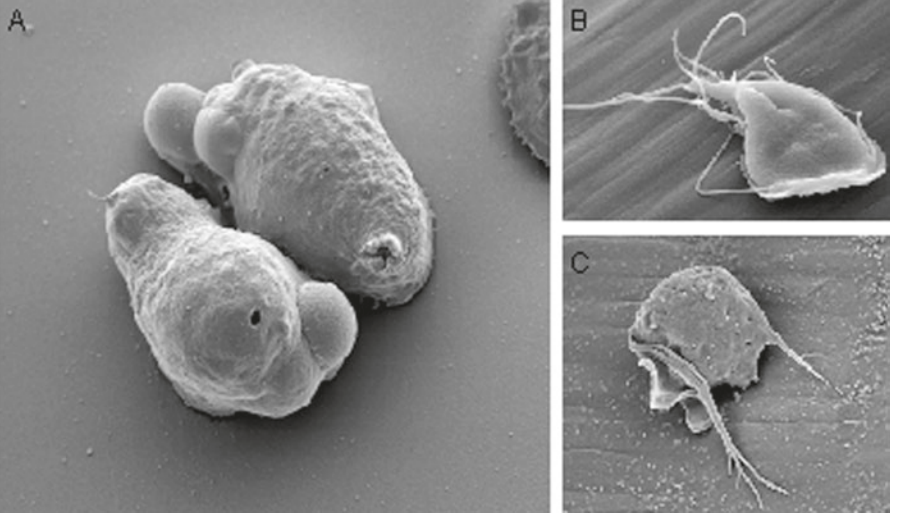

Fig. 1 Scanning electron microscopy images of (**a**) *E. histolytica*, (**b**) *G. lamblia*, and (**c**) *T. vaginalis* trophozoites. (Micrographs were kindly provided by Dr. Arturo González Robles, Departamento de Patología Experimental, CINVESTAV IPN, México)

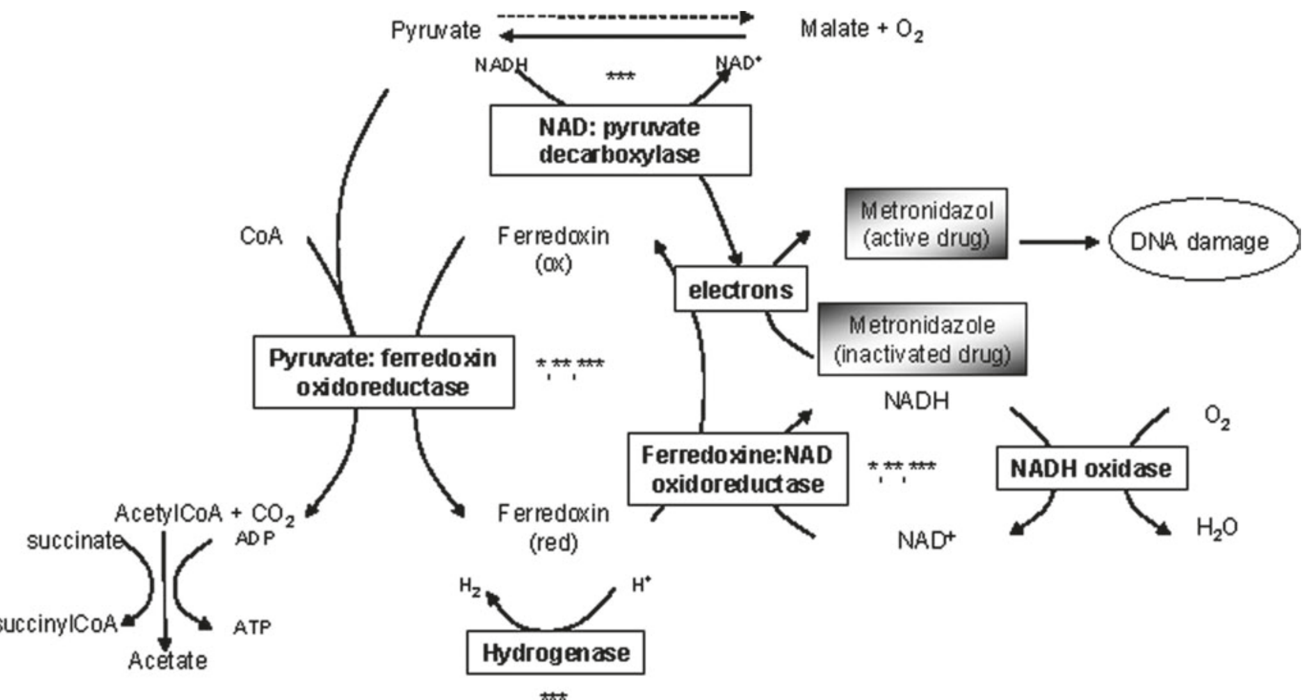

Fig. 2 Terminal part of the glycolytic pathway of anaerobic protozoa and its relation with metronidazole activation. *Represents the current knowledge on enzymes involved in drug activation. **E. histolytica*, ***G. lamblia*, ****T. vaginalis*. (Adapted from Refs. 1 and 66)

converted to toxic nitro or nitroso anions or intermediates, such as hydroxylamines (Fig. 2), and creates a concentration gradient favoring drug accumulation in the cell (15, 16). Reduced metronidazole binds DNA and interferes with respiration and motility (15–17). However, because of its toxicity and the emergence of metronidazole-resistant protozoa (18–21), new efficient drugs are needed.

Protozoa use various drug-resistance mechanisms, including DNA mutations, modulation of enzymes, and pump-like protein expression, such as the P-glycoproteins (PGPs) involved in the multidrug resistance (MDR) phenotype, described in many organisms (22). As poor countries cannot implement public health measures to prevent the spread of parasites, it is very important to understand parasite drug resistance mechanisms and find the way to bypass them, as well as to generate new drugs and vaccines. Here we review the current knowledge on drug susceptibility in the anaerobic protozoa causative of human diseases.

2 *Entamoeba histolytica*

E. histolytica infects 500 million people, provoking 50 million cases of dysentery and liver abscesses and killing 100,000 persons each year (23). Individuals harboring *E. histolytica* are asymptomatic or present diarrhea, bloody stools with mucus, colon abscesses, and dysentery. Liver abscesses, which may be lethal if not treated, occur in 3–9% of infected patients (24). Causes for the varied clinical symptoms lie in both parasite and host. Many authors have proposed that asymptomatic amoebiasis is due to *Entamoeba dispar*, whereas the invasive disease is provoked by *E. histolytica* (25). However, several studies have shown that *E. dispar* is able to destroy culture cells (26) and certain *E. histolytica* strains and clones have poor virulence, being almost unable to damage target cells (27). Whether individuals harboring nonvirulent *E. histolytica* or *E. dispar* should be medically treated, since they excrete potentially injurious cysts, is an unsolved question, because there are reports on asymptomatic carriers infecting other people who developed severe invasive amoebiasis (28).

2.1 Antimicrobial Mechanism of Action in E. histolytica

The microaerotolerant trophozoites of *E. histolytica* grow in the gut lumen and in the richly oxygenated epithelia. Agents currently used against amoebiasis are divided into tissue and luminal amoebicides. Tissue amoebicides, such as metronidazole, tinidazole, and emetine, kill trophozoites in tissues but have no effect against cysts. Luminal amoebicides, such as iodoquinol, diloxanide furoate, and paromomycin, are only active in the intestinal lumen because they are poorly absorbed. Current chemotherapy of dysentery or extra-intestinal abscesses consists of metronidazole or tinidazole, followed by a luminal amoebicide.

Trophozoites use fermentative metabolism for adenosine triphosphate (ATP) production, which involves pyruvate decarboxylation by PFOR to acetyl CoA. *E. histolytica* has one 2-oxoacid reductase, and biochemical assays have identified the PFOR activity in cytoplasm (29), whereas antibodies against the recombinant PFOR localize it in cellular membranes and EhkO organelles (30). Concomitantly with pyruvate decarboxylation, Fd is reduced. *E. histolytica* has two Fds: Fd1 and Fd2, and their amino acid sequences resemble clostridial-type Fds. They have cysteine arrangements characteristic of the coordination of 2[4Fe–4S] clusters (31). Inside the cell, an electron is transferred from Fd to the 5-nitro group of metronidazole to activate the drug and kill parasites.

Metronidazole provokes nausea and headache and is potentially carcinogenic in in vitro studies, but it has not been conclusively linked to the development of human malignancy. Emetine inhibits protein synthesis, affecting ribosome movement along mRNA. It produces cardiac arrhythmia, gastrointestinal toxicity, and skin and neuromuscular reactions (32). Diloxanide fuorate is structurally related to chloramphenicol, whereas paromomycin, an aminoglycoside antibiotic, causes flatulence and diarrhea; both drugs inhibit protein synthesis. Iodoquinol is a halogenated hydroxiquinoline that chelates ferrous ions; its toxicity is associated with the iodine component producing neuropathy and blindness after prolonged administration (33).

2.2 Mechanisms of Drug Resistance in E. histolytica

2.2.1 *E. histolytica* Metronidazole Resistance

Resistance of amoebiasis to metronidazole treatment has been reported, mainly in patients with liver abscesses (34). In vitro, metronidazole resistance has been induced by stepwise exposure to increasing drug amounts. Mutant lines growing in 40 μM metronidazole, a concentration almost fourfold the dose tolerated by sensitive amoebae (1, 20), overexpress the iron-containing superoxide dismutase (SOD) and peroxiredoxin; and they have a decreased expression of Fd1 and flavin reductase, but they do not exhibit PFOR downregulation or upregulation of PGPs involved in MDR. Additionally, overexpression of SOD and peroxiredoxin by transfection assays produced cells with increased metronidazole resistance (20). Of the three major anaerobic protozoa, *E. histolytica* shows the lowest capacity to develop metronidazole resistance at high drug concentrations.

2.2.2 Multidrug Resistance in *E. histolytica*

The MDR phenotype is a challenge in medicine and a barrier to breast cancer and protozoan infections. In *E. histolytica*, 10 μM emetine usually kills trophozoites, but emetine-resistant mutants grow in 220 μM emetine and show cross-resistance to colchicine, diloxanide fuorate, and iodoquinol (35). *E. histolytica* has six *EhPgp* (*mdr*) genes (36). As in mammalian MDR cells, mutant trophozoites accumulate less amount of drug in their cytoplasm than wild-type cells, their drug resistance is reverted by verapamil, and transfection with *EhPgp1* confers the MDR phenotype (37). The *EhPgp1* gene transcript is in a basal amount in wild-type trophozoites, and it is overexpressed in mutants independently of drug

concentration. In contrast, *EhPgp5* and *EhPgp6* gene expression is induced by emetine and the amount of *EhPgp5* transcript is related to drug concentration (38). Thus, transcription regulation is involved in *E. histolytica* MDR, but few transcription factors have been cloned in this parasite, making it difficult to establish their precise role in this phenotype. A C/EBP-like nuclear protein forms a complex with the *EhPgp1* gene promoter, and deletion of the DNA motif to which the protein is bound abolishes the promoter function (38, 39). In addition, at 225 μM emetine, *EhPgp* genes amplification occurs (36). Interestingly, the half-life of *EhPgp5* mRNA is higher in trophozoites grown in 225 μM emetine than in parasites grown in 90 μM or without drug. The *EhPgp5* mRNA 3′ UTR length is heterogeneous, with different poly (A) tail lengths, which may influence mRNA half-life. Trophozoites grown without emetine could have factors that inhibit *EhPgp5* gene expression and maintain short poly (A) tail lengths; this may contribute to a shorter *EhPgp5* mRNA half-life. Some factors could be nonexpressed in the presence of emetine, and other emetine-responsive proteins could stimulate *EhPgp5* gene transcription and induce an enhanced polyadenylation of *EhPgp5* mRNAs (40).

Intriguingly, trophozoites secrete EhPGPs, which are located in the plasma membrane and in vesicles (41). EhPGP secretion is probably related to one of the multiple functions of this protein. The MDR phenotype does not seem to be involved in metronidazole resistance in *E. histolytica*, but it is a barrier for the use of alternative agents against amoebiasis.

3 *Giardia lamblia*

G. lamblia has a prevalence of 2–7% in industrialized countries and 20–60% in developing countries. It infects more than 200 million people and there are about 500,000 new cases per year, contributing to 2.5 million deaths annually by diarrheal diseases (42). It is also the most common cause of chronic diarrhea in travelers. Trophozoites live in the small intestine and graze on the mucosa through the giardins, and reproduce by binary fission covering the epithelia and avoiding nutrient absorption by the host. Owing to microenvironment conditions such as a low cholesterol level (43), trophozoites develop into cysts that pass through the feces to other hosts. Gardiosis produces chronic diarrhea, vomiting, malabsorption, and growth retardation in children, but many infected individuals are asymptomatic. This variability is attributed to the parasite strain, the host, and host–parasite interactions. Current treatments for giardiosis include nitroheterocyclic drugs, mainly metronidazole (the antigiardial drug of choice); its derivatives tinidazole, secnidazole, ornidazole and nimorazole; furazolidone; and albendazole, mebendazole, paromomycin, and quinacrine (1). Quinacrine, the first antigiardial drug, has advantage over nitroimidazoles because it produces a decreased excystation in in vitro-derived and patient-derived cysts (44).

3.1 *Antimicrobial Mechanism of Action in G. lamblia*

As in *E. histolytica* and *T. vaginalis*, the selective toxicity of metronidazole relies on the unique biochemical properties of *Giardia* that allow metronidazole activation (Fig. 2). The protozoan has two 2-oxoacid reductases: PFOR and another called BOR (45). Pyruvate is their preferred substrate, but PFOR also decarboxylates oxaloacetate and α-ketobutyrate, and BOR utilizes α-ketoglutarate and phenyl-pyruvate. *G. lamblia* PFOR is a homodimer of 135 kDa subunits, whose activity is 75–80% membrane associated. It resists low temperatures (−70°C) and transfers electrons to purified FdI but not to either NAD^+ or $NADP^+$. *Giardia* has three Fds with iron–sulfur clusters: FdI, FdII, and FdIII (46). FdI, the major one, differs from other protozoan Fds in size, amino acid sequence, and iron–sulfur cluster. Its molecular mass was calculated to be 5.7 and 5.9 kDa by mass spectrometry and amino acid sequencing, respectively. Consistent with the amino acid profile of other Fds, methionine, arginine, histidine, and tyrosine residues are absent from *Giardia* FdI, but it has 16.4% acidic residues whereas other Fds have about 30%. The *N*-terminus contains a potential iron–sulfur binding motif (-C1-X-X-A-X-X-C3…C4-), with a nonconservative substitution of alanine for the second cysteine. *Giardia* FdI contains a $[3Fe–4S]^{(1+, 0)}$ cluster, while Fd from *E. histolytica* has two $[4Fe–4S]^{(2+, 1+)}$ clusters, and *T. vaginalis* Fd has a single $[2Fe–2S]^{(2+, 1+)}$ cluster (46). Only FdI is involved in metronidazole activation in vitro, and neither FdII nor FdIII transfers electrons to metronidazole, suggesting that they do not interact with PFOR (46). Reduced metronidazole binds to DNA, altering the helical structure, breaking the strands, provoking DNA cross-linking, and interfering with mitosis as well as making DNA unable to segregate or modifying genes involved in mitosis, arresting cell cycle in G2 + M phase (47). It would be of interest to know whether the electron transfer from PFOR to Fd is performed in mitosomes, recently reported in *Giardia* (12).

On the other hand, furazolidone is activated inside the cell by NADH oxidase to generate toxic products that interfere with DNA processes. NADH oxidase, a 46 kDa monomeric flavoprotein, contains flavin adenine dinucleotide in a 1:1 molecular ratio with the polypeptide and it is responsible for the high level of NADPH–NADH turnover in *Giardia* (48). NADPH and NADH donate electrons to NADH oxidase, which also accepts electrons from oxygen to produce H_2O as

an end product, but not from reduced Fd (48). Thus, NADH oxidase is not involved in metronidazole activation. At the cellular level, furazolidone arrests the trophozoites in the S phase, although a few cells go to the G2 + M phase where they become blocked. DNA synthesis and cell cycle completion are prevented, possibly because of DNA damage (47).

Quinacrine also intercalates in DNA, inhibiting nucleic acids synthesis. However, there are studies showing no quinacrine accumulation in trophozoite nuclei exposed to the drug; instead, the plasma membrane appeared fragile after overnight drug exposure, suggesting that it may be a drug target (49). In addition, quinacrine inhibits NADH oxidase and cholinesterase activities. Benzimidazoles bind to β-tubulin in the same site as colchicine, altering the cytoskeleton. After mebendazole or albendazole exposure, trophozoites detach from the substrate, exhibiting striking modifications of their overall morphology, including ventral disk disassembly (50). Paromomycin interferes with *Giardia* 16S-like small-subunit (SS) RNA, causing mRNA codon misreading and protein synthesis inhibition. In *Giardia,* the 3′ end of the SS RNA has the base pair C_{1409}–G_{1491} in a conserved location with respect to secondary structure. These bases are involved in paromomycin susceptibility in higher eukaryotes (51).

3.2 Drug Resistance Mechanisms in G. lamblia

Therapeutic failure in giardiosis is occurring more and more frequently with all the compounds used because of (i) low compliance with drug therapy, (ii) immunosuppression, (iii) reinfestation, (iv) post-*Giardia* lactose intolerance, the most common of the disaccharide deficiencies associated with giardiosis, and (v) development of parasite drug resistance. Clinical metronidazole resistance prevalence levels are as high as 20% with recurrence rates up to 90%, and the average success rates of albendazole are 62–95% (1). Stepwise exposure to increasing drug concentrations have allowed the generation of distinct *Giardia* lines resistant to 8.5 μM metronidazole (52), 0.8 μM albendazole (53), and 20 μM quinacrine (49).

3.2.1 Metronidazole Resistance in *G. lamblia*

In the metronidazole-resistant mutant BRIS/83/HEPU/106-2ID$_{10}$, the level of 2-oxoacid reductase activity is half of the parental drug-sensitive BRIS/83/HEPU/106 strain. Interestingly, this activity is mainly given by BOR, which does not require FdI but acts to facilitate the energy production for parasite survival (45), suggesting that BOR does not participate in metronidazole activation. Metronidazole resistance is also associated with chromosomal rearrangements. The G6/1 marker (3 kb) of the chromosome 3, identified in BRIS/83/HEPU/106-2ID$_{10}$ line, was found in chromosomes 3 and 4 in sensitive trophozoites. However, after enzymatic DNA cleavage, the G6/1 marker was located in a single band (54). Other probes hybridized with chromosomes 3 and 4 in both sensitive and resistant lines, indicating a partial duplication of chromosome 3 to generate chromosome 4 (55). Allozymic analysis of cytosolic enzymes, M13 DNA fingerprinting, random amplified polymorphic DNA studies, polymerase chain reaction (PCR) assays, and polymorphism analysis showed that isolates with distinct drug sensitivity have different genotypes (1). However, the *G. lamblia* genome shows high diversity, which can rise up to 30% in genes and up to 50% in intergenic regulatory regions (56), hindering the association of DNA changes with drug resistance. Additionally, the decreased level of metronidazole in *Giardia* cytoplasm is consistent with changes in uptake, transport, and efflux of fluorescent analogs observed in MDR (54), but MDR has not been detected in this parasite.

3.2.2 Resistance Mechanism to Other Compounds in *G. lamblia*

Interestingly, furazolidone resistance correlates with reduced drug entry and increased levels of thiol-cycling enzymes which defend *Giardia* against toxic radicals, suggesting that efficient thiol cycling may be involved in furazolidone reduction (57). Additionally, quinacrine is actively excluded from resistant trophozoites (49), and albendazole resistance is associated with cytoskeleton changes, particularly in the ventral disk. However, resistant trophozoites do not have the mutation in phenylalanine at position 200 in β-tubulin, commonly found in albendazole-resistant helminthes and fungi (53).

3.3 Cross-Resistance in G. lamblia

In patients with treatment failures in giardosis, switching to a distinct drug is not always effective. Brasseur et al. (19) reported two patients who did not respond to successive treatments with metronidazole, albendazole, and quinacrine, showing the existence of clinical cross-resistance in *Giardia*. The BRIS/83/HEPU/106-2ID$_{10}$ line is also resistant to albendazole and tinidazole (53), and furazolidone-resistant *Giardia* strains adapt more readily to quinacrine (49) and albendazole (53). There are various mechanisms involved in cross-resistance in giardiosis, since drugs target different parasite pathways and molecules. No *mdr* gene has been reported yet in *Giardia*, but genome sequence completion will certainly help in identifying genes involved in drug resistance.

4 *Trichomonas vaginalis*

T. vaginalis infects 180 million people each year worldwide and there are about 50% asymptomatic carriers (2). Trophozoites are the unique stage in its life cycle and no cysts have been identified, so transmission occurs via trophozoites only by sexual contact. However, cyst-like cells have been found in *T. vaginalis* under environmental stress (58). Eradication of trichomonosis is considered as an effective means for controlling HIV transmission, because 24% of HIV infections are attributable to *T. vaginalis* infection (59). Trichomonosis is controlled by metronidazole, although other 5-nitroimidazoles are also dispensed and used as prophylactic agents in gynecological surgery and topical intravaginal treatments, using a single 1.5–2 g metronidazole in oral dose to 500 mg twice daily over seven days (60).

4.1 *Antimicrobial Mechanism of Action in T. vaginalis*

In *T. vaginalis*, glycolysis occurs in the cytosol producing pyruvate. In hydrogenosomes, pyruvate is decarboxylated by PFOR to form acetyl-CoA that is transferred to succinate to produce acetate; then, it is released by the succinate thiokinase resulting in ATP production. Electrons are finally given by hydrogenase to hydrogen ions forming hydrogen as an end product (13) (Fig. 2). This metabolic chain produces acetate, CO_2, and molecular hydrogen under anaerobic conditions. Hydrogenosomes contain PFOR, one [2Fe–2S] Fd of approximately 12 kDa which is similar to mitochondrial Fds (61), and three [Fe] hydrogenases (62). Metronidazole activation by PFOR electrons results in poor hydrogen production and increase of intracellular hydrogen peroxide (13). Under aerobiosis, oxygen radicals oxidize the reduced metronidazole, partially inactivating the drug; therefore, higher drug concentrations are needed to kill the parasites.

4.2 *Drug Resistance Mechanisms in T. vaginalis*

4.2.1 Metronidazole Resistance in *T. vaginalis*

Two types of *T. vaginalis* metronidazole resistance have been described: (i) aerobic resistance that occurs in parasites isolated from patients with treatment failures, where oxygen competes with metronidazole for electrons, inactivating the drug (63); (ii) anaerobic resistance that has been demonstrated only in cultured strains (64). It relies on elimination or inactivation of enzymes responsible for reductive activation of drug. Additionally, targeted Fd gene replacement does not lead to metronidazole resistance (65), and lines deficient in PFOR only showed low levels of metronidazole resistance (66), suggesting the presence of alternative parasite pathways to activate the drug. Parasites grown in anaerobic conditions at relatively low drug concentrations (3 µg/mL) develop first aerobic resistance up to 200 µg/mL of drug. Longer exposure to increasing drug concentrations allowed the generation of anaerobic metronidazole-resistant mutants being able to grow at 1,120–1,425 µg/mL of drug (67). However, these strains do not multiply under anaerobiosis. In these experiments, some anaerobic drug-resistant strains presented high decrease of PFOR and hydrogenase activities, while malic enzyme (ME) and NADH:Fd oxidoreductase decreased only slightly. Lactate and other main end products of carbohydrate metabolism increased in drug-resistant cells, whereas hydrogenosomal metabolites such as acetate and hydrogen dramatically decreased. In these studies, PFOR, ME, and Fd were present in drug-sensitive and aerobic drug-resistant strains, but in anaerobic resistant strains PFOR and Fd were missing and ME decreased progressively when the anaerobic level of resistance increased. Additionally, a marked decrease of ME mRNA steady-state levels at the late phases of anaerobiosis resistance showed a downregulation of ME gene expression (67). All these findings revealed two unexpected phenomena in *T. vaginalis*: (i) aerobic and anaerobic resistance, considered to be unrelated, are developed in a common continuous process and (ii) the lack of PFOR activity, the major electron donor for drug reduction, did not result in total anaerobic resistance. ME could be transferring electrons to Fd, activating metronidazole. It seems that generation of metronidazole resistance occurs through a multistep process, suggesting that several mutations in various hydrogenosomal proteins involved in drug activation might occur (60, 67).

4.2.2 Multiple Drug Resistance in *T. vaginalis*

The *Tvpgp1* gene encodes a 589-amino-acid protein with an amino terminal hydrophobic region, a carboxy-terminal ATP binding site, and six transmembrane segments, which correspond to half the size of mammalian PGPs. *T. vaginalis* genome has two *Tvpgp1* copies, but only one was detected in four of seven drug-resistant strains studied. Moreover, several clinical metronidazole-resistant isolates overexpress *Tvpgp1* mRNA to levels ranging from 2- to 20-fold more than the wild type. However, no correlation was found between the *Tvpgp1* mRNA amount and *Tvpgp1* gene copy number with drug resistance levels (68). This does not necessarily mean that *Tvpgp1* gene is not involved in drug resistance. In *E. histolytica* drug-resistant mutants, the *EhPgp1* mRNA overexpression confers drug resistance but the transcript

amount and the gene copy number do not correlate with drug-resistance levels (36). Additionally, resistance of a metronidazole-resistant *T. vaginalis* strain is reverted by verapamil (60). Therefore, more studies are necessary to define the role of *Tvpgp1* gene in *T. vaginalis* drug resistance.

4.3 Cross-Resistance in T. vaginalis

Although metronidazole is the drug of choice for *T. vaginalis*, cross-resistance to different 5-nitroimidazoles is common. Evaluation of 104 clinically metronidazole-resistant isolates showed that almost all samples were cross-resistant to tinidazole in aerobic conditions. Interestingly, isolates with higher metronidazole resistance have decreased sensitivity to tinidazole, suggesting that increased metronidazole resistance may correlate with increased tinidazole resistance. However, metronidazole-refractory cases have finally been cured by very high doses of tinidazole (69). Several cases of metronidazole resistance were reported to be also resistant to ornidazole (1).

5 Other Anaerobic Opportunistic Protozoan Parasites

B. hominis, *C. parvum*, *Isospora* spp., and *Cyclospora* spp. invade preferentially the gastrointestinal mucosa. *Microsporidia*, amitochondriate intracellular parasites closely related to fungi, produce intestinal, pulmonary, ocular, muscular, and renal diseases. Five *Microsporidia* genera (*Enterocytozoon* spp., *Encephalitozoon* spp., *Septata* spp., *Pleistophora* spp., and *Nosema* spp.) and one unclassified genus (referred to as *Microsporidium*) are associated with human diseases. These microorganisms are considered as emerging opportunistic parasites, causing diarrhea, lethal wasting, and other symptoms in immunocompetent and immunocompromised hosts, mainly in HIV patients and malnourished infants (1).

5.1 Antimicrobial Treatments Against Opportunistic Protozoan Parasites

Metronidazole is the drug of choice against *B. hominis* (70), but iodoquinol, emetine, pentamidine, quinacrine, trimethoprim-sulfamethaxazole, furazolidone, and 5-nitroimidazole derivatives are also used. Paromomycin, in combination with azithromycin, lasalocid, or geneticin, is somewhat effective against *Cryptosporidium* (71), but nitazoxanide, indinavir, spiramycin, halofunginone lactate, eflornithine, and sinefungin also appear to hold some promise against *C. parvum. Isospora belli*, and *Cyclospora cayetanensis* are controlled by trimethoprim-sulfamethoxazole and ciprofloxacin in HIV patients. Albendazole and its sulfoxide and sulfone metabolites are the drugs of choice against most *Microsporidia,* but albendazole is ineffective against *Enterocytozoon bieneusi*, which is controlled by fumagillin. Albendazole, fumagillin, 5-fluorouracil, sparfloxacin, oxibendazole, and propamidine isethionate inhibit *Encephalitozoon cuniculi* growth in vitro. Synthetic polyamine analogs bind to nucleic acids and are effective antimicrosporidial agents in vitro and in vivo (72).

5.2 Drug Resistance in B. hominis, C. parvum, and Microsporidia

There are case reports of metronidazole treatment failures in *Blastocystis* infections, and *B. hominis* isolates from different geographical origins have distinct levels of metronidazole resistance (73). *C. parvum* contains a family of ABC transporters that resemble the PGPs described in other organisms, and the membrane protein CpABC is located in the host–parasite boundary (74), suggesting a possible role in drug resistance. However, its ability to efflux drugs has not been fully addressed. In addition, the *C. parvum* dihydrofolate reductase (DHFR) contains amino acid residue changes at positions analogous to those at which point mutations produce antifolate resistance in other parasites, suggesting that *C. parvum* DHFR may be intrinsically resistant to antifolate DHFR inhibitors. This can explain why it is refractory to treatment with common antibacterial and antiprotozoal antifolates (75). *Encephalitozoon* infections resistant to conventional treatments with albendazole and fluconazole have been cured by the antifungal itraconazole and fumagillin (76), but *Nosema bombycis* is resistant to itraconazole and metronidazole in vitro, while it is sensitive to fumagillin (77). Itraconazole and trimethoprim-sulfamethoxazole resistance has been reported in hosts infected by *Isospora*, but it is not clear how resistance occurs.

6 Spread of Resistance in Anaerobic Protozoa

Entamoeba, *Giardia, Trichomonas*, and opportunistic anaerobic parasites regulate their metabolism under drug pressure, allowing the development of resistant strains, which can easily be transmitted to other hosts, provoking their dispersion. However, there are no studies on the spread of drug resistance in anaerobic protozoa: there are only reports on treatments failures, but uncontrolled use of anti-protozoa agents

in prophylaxis and chemotherapy results in development of drug-resistant parasites. In addition, the possible transmission of *Giardia* and *Cryptosporidium* from animals to humans (78) may participate in the spread of drug-resistant strains. The existence of virus in *T. vaginalis* and *G. lamblia* (79) and the *Giardia* mini-circular plasmid, which could be a transposable element, may favor drug resistance genes transfer in these parasites. Intriguingly, it has been suggested that drug resistance may debilitate *T. vaginalis* and impede its transfer among sexually active partners, leaving the resistance a problem for the carrier alone. However, in two clinics in the United States, the number of highly resistant cases increased from 1 per 2,000 trichomonosis in 1996 to 17 in 1998, and some cases appear incurable. Therefore, it can be hypothesized that 5–10% of *T. vaginalis* isolates show some level of resistance (80).

Comparative studies of genetic polymorphisms and clinical phenotypes in *T. vaginalis* isolates show concordance between polymorphisms and in vivo and in vitro metronidazole resistance (81). Strain distribution in a phylogenetic tree reflected their level of metronidazole susceptibility in in vitro tests. Interestingly, different geographical origins of strains with increased drug resistance that cluster in a common branch suggest that only one lineage of metronidazole-resistant strains was spread around the world. However, it is not possible to know whether this lineage had spread because of the selective pressure of the drug (81). Recognition of drug resistance is crucial to achieve effective parasite control. Broad studies of genetic polymorphisms using DNA fingerprinting, genes detection, electrophoretic karyotyping, and isoenzyme typing techniques should be useful in distinguishing strains with different phenotypes and differentiate truly resistant isolates from those that are derived from cases where treatment failures occurred because of other causes.

7 Alternative Drugs Against *E. histolytica, G. lamblia, T. vaginalis,* and *Opportunistic Protozoa*

The search for new antiparasite agents relies on three major strategies:

1. Evaluation of chemically modified known products and design of novel molecules more efficient and less toxic than metronidazole. Several compounds are currently under experimentation. New 5-nitroimidazole derivatives and a lactone-substituted nitroimidazole seem to be as effective as metronidazole against *Giardia* and *Trichomonas* (82). 5,6-Dichloro-2-(4-nitrobenzylthio)-(benzimidazole) has activity against *Entamoeba* and *Giardia* because it binds DNA and inhibits the topoisomerase II (83). Additionally, several 5,6-dinitro-1-(aminoethyl)benzimidazoles are effective against *Trichomonas*, probably acting via nitro group reduction by Fd in the same way that metronidazole acts, but not via inhibition of β-tubulin polymerization as albendazole does (83), and S-substituted 4,6-dihalogeno-2-mercaptobenzimidazoles are also more active against *G. lamblia* and *T. vaginalis* than metronidazole (84); thus, benzimidazole could be a lead compound for new anti-anaerobic protozoan agents.
2. Evaluation of natural products. For many years, amoebiasis and other infections were treated by natural products, whose knowledge came from ancestral practices. Nowadays, there is increasing interest in rediscovering these compounds. As examples, a flavonoid isolated from *Rubus coriifolius* and *Helianthemum glomeratum*, two medicinal plants used in Mayan medicine for the treatment of bloody diarrhea, suppresses *E. histolytica* and *G. lamblia* growth in culture (85). On the other hand, formononetin, an isoflavone from the bark of tulipwood *Dalbergia frutescens*, appears a more potent antigiardial agent than metronidazole (86). Moreover, allyl alcohol and allyl mercaptan from garlic (*Allium sativum*) extracts exhibit potent antiprotozoan effects probably owing to interactions with nucleic acids, transcription factors, and enzymes (87).
3. Comparative study of parasite and host metabolism. The study of parasite metabolism leads to the identification of specific molecules that can be good targets. As examples, the RNA polymerase II (RNAP II) of *G. lamblia* has a high resistance to amanitin, probably due to mutations in the amanitin motif of the Rpb1 subunit (88). Additionally, in *T. vaginalis*, the methionine gama-lyase, which has no counterpart in mammals, appears as a good drug target (89). PFOR would also be an excellent target to kill anaerobic protozoa, which has not been explored yet. Few studies have been done on novel drugs against opportunistic protozoa, but several of the mentioned compounds could be tested against them.

8 Concluding Remarks

Metronidazole, the drug of choice for amoebiasis, giardiosis, trichomonosis, and other opportunistic diseases produced by anaerobic protozoan parasites can soon become outdated because of its excessive use in chemotherapy and prophylaxis. The emergence of metronidazole resistance is a serious challenge to eradicate these infections, and protozoa are able to develop resistance to most antiparasitic drugs. Our knowledge on the cellular pathways used by protozoa to bypass drug effects and survive inside the host is still limited. Although several genes, proteins, and cellular pathways

Table 1 Drugs, target molecules, genes, and proteins involved in drug resistance in anaerobic protozoa

Parasite	Drug	Target molecule	Target location	Altered pathway	Genes and proteins involved in drug resistance
E. histolytica	Metronidazole	DNA	Nucleus	Electron transport	PFOR, SOD
	Emetine	Ribosome	Cytosol	Protein synthesis	EhPgp1, EhPgp5, EhPgp6
	Iodoquinol	DNA	DNA	Electron transport	ND
	Diloxanide furoate	ND	Cytosol	Protein synthesis	ND
T. vaginalis	Metronidazole	DNA	Nucleus	Electron transport	PFOR, Fd, NADP-dependent ME
G. lamblia	Metronidazole	DNA	Nucleus	Electron transport	PFOR, FdI
	Quinacrine	DNA, plasma membrane	Nucleus	ND	ND
	Tinidazole	DNA	Nucleus	Electron transport	Thiol-cycling enzymes
	Benzimidazole	β-Tubulin	Cytosol	Microtubule polymerization	NADH oxidase
	Furazolidone	DNA	Nucleus	Protein synthesis	ND
	Paramomycin	SS RNA	Cytosol	Protein synthesis	ND

ND not determined, *PFOR* pyruvate:ferredoxin oxidoreductase, *SOD* superoxide dismutase

involved in drug action and drug resistance have been discovered (Table 1), it is urgently required to continue with the study of mechanisms responsible for the emergence of resistant parasites to overcome this problem and design new chemotherapeutic strategies. We also need to know the prevalence of resistance in specific geographical areas to look for better alternatives and avoid the use of toxic and obsolete drugs for patients infected with resistant protozoa. The current advances and novel genomics and proteomics as well as high-throughput tools to detect genes and proteins involved in parasite virulence and drug resistance mechanisms may help in finding new and efficient therapeutic alternatives, such as vaccines and better drugs.

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Chapter 40
Mechanisms of Antimalarial Drug Resistance

Giancarlo A. Biagini, Patrick G. Bray, and Stephen A. Ward

1 Introduction

It has been estimated that in 2002 there were 515 (range 300–660) million episodes of clinical *Plasmodium falciparum* malaria (1). It is very difficult to estimate the number of deaths caused by malaria, but a figure of approximately 2.7 million per year has been widely accepted, with over 75% of these deaths occurring to African children (2). Unfortunately, these figures are on the increase largely as a result of parasite multi-drug resistance (3). The introduction of chloroquine (CQ), shortly after World War II, had a tremendous impact on the global health; however, today resistance to the drug has been observed in every region where *P. falciparum* occurs (4). Resistance developed from a number of independent foci including South America, Southeast Asia and Papua New Guinea (5, 6). Gradually over the next 20 years, resistance spread throughout South America and Southeast Asia and started occurring in East Africa in the late 1970s. CQ resistance has since spread across all of sub-Saharan Africa. As drug resistance is genetically determined, it will spread by active malaria transmission, as gametocytes from resistant isolates will produce resistant offspring. Many African countries switched their first-line drug to sulfadoxine–pyrimethamine (SP); however, resistance to SP has grown and spread very quickly, especially in Southeast Asia, South America (4, 7) and more recently in many areas of Africa (8). Drug resistance in *P. falciparum* is not confined to CQ and SP. Amodiaquine (AQ) is an active analogue of CQ used in chemotherapy for cases of treatment failure but is also subject to resistance-mediated failures (9–12). More worrying though is that parasite resistance to the newer class of antimalarials, such as mefloquine (MQ), was reported as early as 5 years after its introduction as a prophylactic treatment in parts of Thailand (4). In some regions of Thailand, cure rates for MQ have now dropped to below 41% (13). Resistance to atovaquone was even more rapid, emerging in the same year as its launch (4). A number of initiatives have been introduced to stem the number of drug resistance-mediated clinical failures such as the introduction of combination therapy and the deployment of new drugs (e.g. LapDap). However, it is clear that a better understanding of resistance mechanisms to currently used drugs is required to support the development of such strategies and help prevent the development of resistance to new therapies.

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2 Resistance Mechanisms to 4-Aminoquinolines

The quinoline antimalarials such as CQ, AQ, quinine (Q) and MQ have been the defensive bastions against malaria for many years (Fig. 1). The success of these drugs is based on their excellent clinical efficacy, limited host toxicity, ease of use and cost-effective synthesis. Although it has taken over 20 years to appear, resistance to quinoline antimalarials is now one of the biggest challenges facing malaria-endemic countries (14, 15). The exact mode of action of quinolines has not fully been elucidated, but it is accepted that a crucial step is the binding of the drug to ferriprotoporphyrin IX (FP or heme), a by-product of haemoglobin degradation, which occurs in the parasite digestive food vacuole (DV). The uncertainty of the mode of action of quinolines, together with a poor understanding of parasite physiology, leaves the mechanism of CQ resistance in *P. falciparum* for the most part enigmatic.

2.1 *Access to Haematin Is the Biochemical Basis of CQ Resistance*

The exquisite specificity of CQ for malaria parasites stems from the parasite-specific accumulation of the drug to levels far greater than that seen with mammalian cells. CQ is a weak base and depending on the surrounding pH can

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Fig. 1 Chemical structure of the 4-aminoquinoline antimalarials: (**1**) chloroquine, (**2**) amodiaquine, (**3**) quinine and (**4**) mefloquine

take the form of the un-protonated (CQ), singly protonated (CQ^{+}) or doubly protonated (CQ^{++}) species, owing to the protonatable diethylamine nitrogen side chain (p*K*a 10.2) and the quinoline-ring heteroatom nitrogen (p*K*a 8.1 (16)). The un-protonated (uncharged) species of CQ is membrane permeable and is able to distribute equally across all cellular compartments, whilst the protonated species is relatively impermeable to membranes (17). In the parasite DV (estimated pH ~ 5.2–5.8 (18, 19)), a high concentration of CQ^{++} is trapped in its doubly protonated and membrane impermeable form (17). The "proton-trapping" of CQ potentially results in this drug accumulating several thousand-fold with concentrations possibly reaching mM levels in the food vacuole of the parasite (20). However, many mammalian cells contain large acidic vacuoles yet accumulate much less CQ than malaria parasites. These data indicate that proton trapping cannot be the only mechanism driving CQ uptake into the parasite. To elucidate the full mechanism of CQ uptake, we also have to consider the role of the DV in digesting host cell haemoglobin. The process of haemoglobin digestion releases large quantities of heme or FP which is toxic in its free form. In the malaria parasite, FP is oxidized and dimerized to beta-haematin before biocrystallization into haemozoin or malarial pigment, which is non-toxic to the parasite (21, 22). One hypothesis is that protonated CQ binds to FP inhibiting the haemozoin biocrystallization process and causing a build-up of free FP and/or CQ–FP complex, leading ultimately to parasite death (23, 24). CQ binds to FP with high affinity, both in the test tube and in the intracellular parasite (24–26). The consensus view is that the parasite-specific hyper-accumulation of CQ is probably due to a combination of proton trapping in the acid DV and binding to FP in the same organelle. For all species of *Plasmodium*, CQ-resistant parasites are observed to accumulate much lower levels of CQ than their CQ-sensitive counterparts (25, 27–32). This observation, together with the demonstration that CQ-resistant and CQ-sensitive parasites contain similar amounts of the FP target (33), limits the potential mechanisms of CQR to those that reduce the access of CQ to its haematin target (25).

Many hypotheses have been proposed to account for the observed reduction of CQ uptake in CQ-resistant parasites. It was thought for a long time that CQ-resistant parasites have a smaller ΔpH (DV_{IN} vs. DV_{OUT}), e.g. a more alkaline DV lumen compared to CQ-sensitive parasites, reducing the level of trapping of the charged CQ species (CQ^{+} and CQ^{++}). Because each molecule of CQ can potentially associate with two protons, relative small changes in the DV pH can have a dramatic effect on the concentration of CQ in this organelle. For example, increasing the pH of the DV from 5.2 to 5.7 will decrease the amount of protonated CQ tenfold, explaining the reduced susceptibility of CQ-resistant parasites (31). Although at first an appealing theory, it has subsequently received no evidence to support it. Measuring the pH of the DV is technically very demanding (34–36) and until recently this hypothesis had not been tested. Ironically, the first reports of a comparison of DV pH of CQ-sensitive and CQ-resistant parasite lines suggested that CQ-resistant parasites may have a *more acidic* DV than CQ-sensitive parasites (19, 37, 38). Several workers (36, 39) have expressed reservations with regard to the experimental design adopted in the initial studies purporting to report the DV pH values (37, 38); however, a more recent study using more robust pH measurement techniques has again reported a more acidic DV pH in CQ-resistant lines (19). CQ-sensitive lines were reported to have a DV pH of around 5.7 and this was found to fall to around 5.2 in CQR lines. If so, this would be expected to increase the amount of CQ accumulated in the DV of CQ-resistant parasites by about tenfold. In an attempt to reconcile these physiological data with the sixfold reduced CQ uptake that is actually measured, it was proposed that increased aggregation of μ-oxo-dimeric FP at lower pH causes a reduction in CQ accumulation because of the lower affinity of binding of CQ to aggregated vs. soluble species of FP (40). This hypothesis would predict that CQ-resistant parasites have an altered steady-state CQ-binding capacity compared to CQ-sensitive lines. However, the reverse is true; a study comparing CQ-resistant and CQ-sensitive lines revealed an equal number of CQ-binding sites (25). CQ uptake into *P. falciparum* consists of both a saturable

and a non-saturable component (25, 28). The saturable component of CQ uptake is evident at drug concentrations, which are pharmacologically relevant, suggesting that this is the component that is relevant to the antimalarial activity of the drug. Detailed analysis of equilibrium CQ uptake in several lines of CQ-sensitive and CQ-resistant parasites was performed and modelled (25). It was demonstrated that no significant differences were found in either the non-saturable component of CQ uptake or in the capacity (B_{max}) of the saturable component. Notably however, the sensitivity of parasites to CQ (as measured by the IC_{50}) was found to be directly proportional ($r^2 = 0.93$) to the apparent affinity (K_d) of the saturable uptake component (Fig. 2a, b). In lay terms, this study established that the concentration of the drug target haematin does not change between CQ-sensitive and CQ-resistant parasites and instead the *apparent* affinity for the target changes. This was subsequently confirmed in a study that directly measured the haemozoin content of a panel of isolates with a large variation in sensitivity to CQ. Only small differences in the rate of haemozoin generation were observed, and these small differences were unrelated to the CQ sensitivity of the isolates (41). These data are very difficult to reconcile with a reduced DV pH that would accelerate the rate of haemozoin (malarial pigment) generation in CQ-resistant isolates (40). Further experiments to measure DV pH and clarify this issue are keenly awaited.

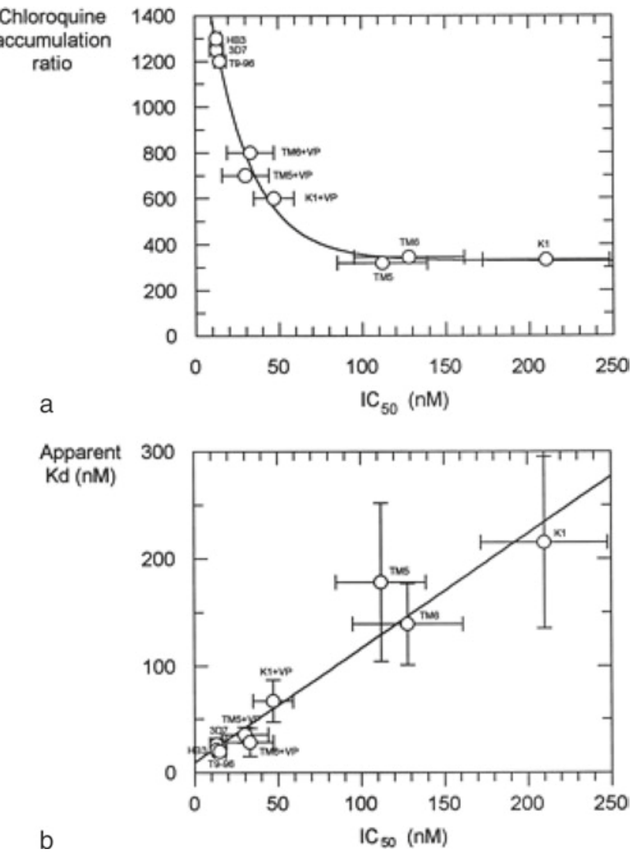

Fig. 2 Analysis of the antimalarial activity and saturable binding characteristics of chloroquine (CQ). The antimalarial activity of CQ is linearly related to the *apparent* receptor K_d (**b**) rather than the total cellular CQ accumulation (**a**)

Rather than changes in DV pH reducing the amount of target molecules, it is far more likely that CQR results from a transport process that reduces the local concentration of the drug available to bind the FP target (25, 42, 43). This hypothesis has been supported by independent analysis of CQ-binding parameters (42, 44) and subsequent molecular studies which have pinpointed the genetic determinant for CQ resistance.

2.2 *pfcrt* Is the Genetic Determinant of CQ Resistance

In the late 1980s, an intricate study was initiated to localize the molecular loci which harbours gene(s) responsible for CQ resistance. This approach involved crossing a cloned CQ-resistant (Dd2) and a cloned CQ-sensitive (HB3) parasite populations during the asexual stage of the *P. falciparum* life cycle in the mosquito host and isolating the recombinant progeny during the asexual cycle in the primate host (45). Phenotypic typing of progeny and mapping of loci using RFLP and microsatellite markers (46–48) localized a key determinant to a region on chromosome 7. An open reading frame, which termed *cg2* for "candidate gene 2", was identified as a possible candidate for CQ resistance (49); however, subsequent transfection studies showed that the *cg2* gene did not confer CQ resistance in transformed parasites (50). Further analysis of the 36 kb region on chromosome 7 eventually yielded a highly fragmented (13 exons) open reading frame, named *pfcrt* for CQ-resistance transporter, which showed highly significant linkage to over 40 CQ-resistant parasite lines examined (51). Genetic mutations in *pfcrt* were reported to be associated with reduced in vitro susceptibilities to CQ in laboratory lines and field isolates (51–54). Subsequent allelic exchange experiments have now shown without doubt that polymorphisms in *pfcrt* confer CQ resistance (55).

Point mutations have been observed in 10 codons of the *pfcrt* gene of CQ-resistant parasite isolates from various regions. These include mutations at amino acid positions 72, 74, 75, 76, 97, 220, 271, 326, 356 and 371 (Fig. 3). Broadly speaking, the CQ-resistant parasite isolates from Southeast Asia and Africa have *pfcrt* genes with seven to nine mutated codons, and their mutated codons are represented by the amino acid residue pattern of CIETH(L)SEST(I)I, from

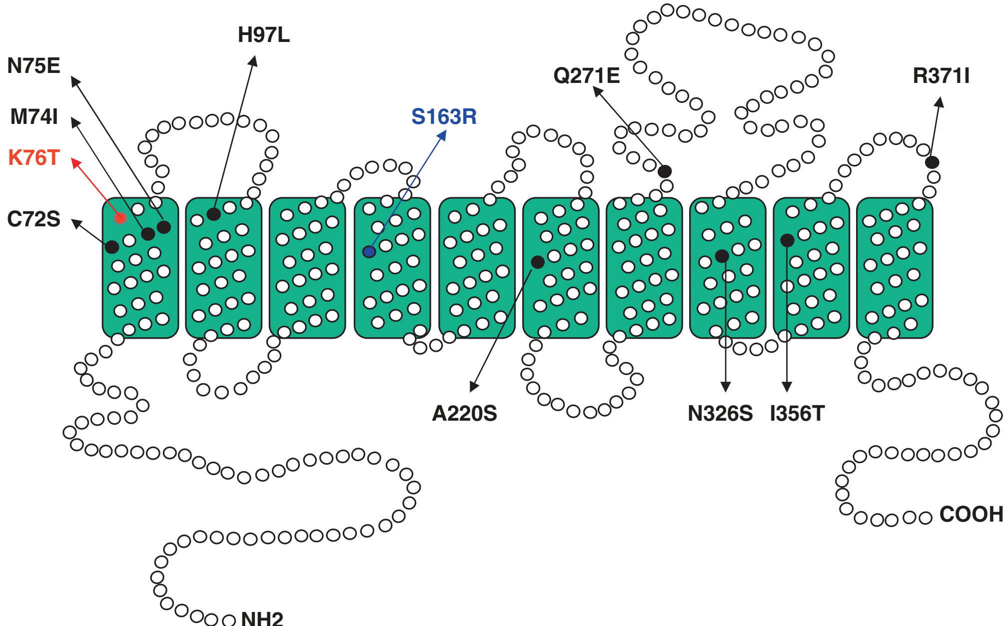

Fig. 3 Schematic representation of the protein structure of PfCRT. The scheme highlights the 10-transmembrane domains with known polymorphisms conferring chloroquine (CQ) resistance represented by the *black dots*. The vital K76T mutation found in all known CQ-resistant isolates is shown in the *red dot* and the novel S163R mutation, which can confer CQ sensitivity and the loss of the verapamil effect, is shown in *blue*. (Adapted and reproduced with permission from Elsevier Science)

positions 72 to 371 (5, 51). The CQ-resistant parasites from South America and Papua New Guinea possess *pfcrt* genes with four to five mutated codons forming patterns of S(C) MNTHSQDLR (5, 51, 53).

The minimum number of mutations, previously reported in *pfcrt* of CQ-resistant parasites, is four: C72S, K76T, N326D and I356L (5). Mutation K76T is found in all CQ-resistant parasites and A220S is observed in most CQ-resistant isolates, signifying their essential role in CQ resistance. Recently, two novel mutations, A144T and L160Y, were identified outside of the ten known mutations in *pfcrt* in Morong isolates (6). These novel mutations were identified only in parasites with K76T and N326D but without the common A220S mutation found in most CQ-resistant isolates. Confirmation of these novel mutations by other study groups is yet to be reported. The role of the remaining *pfcrt* mutations in CQ resistance remains unclear. It should be noted, however, that mutations in the *P. vivax* homologue of *pfcr*, are not associated with CQ resistance (56), suggesting a genetic basis for CQ resistance in *P. vivax* that is different from that in *P. falciparum*.

In summary, the epidemiological evidence to support the theory that *pfcrt* is the critical determinant of CQ resistance is strong. It would appear likely that *pfcrt* is critical for parasite survival with *pfcrt* knockout experiments so far proving lethal. In addition, the fitness of *pfcrt* mutants appears to be reduced. Detailed studies from Malawi have revealed a progressive loss of the mutant allele over a decade since the replacement of CQ with Fansidar as first-line treatment and the effective elimination of CQ usage within that population (57).

2.3 Proposed Functional Roles for PfCRT in CQ Resistance

Although localized to the DV membrane (51), the physiological role of the PfCRT transporter in *P. falciparum* physiology is currently unknown, and for this reason the role of PfCRT in CQ-resistance mechanisms remains elusive. This deficiency, however, has not deterred assiduous workers in proposing a variety of putative resistance mechanisms. Three main theories have evolved; the first proposes that PfCRT influences CQ distribution indirectly, by altering ion gradients such as chloride across the DV membrane (33, 38, 58). The second hypothesis proposes that CQ is effluxed out of the DV by an ATP-dependent primary active transport process (42, 59, 60). The final hypothesis, known as the “charged drug leak model”, proposes that PfCRT facilitates the movement of protonated CQ (CQ^{++}) down its concentration gradient out of the DV (43, 61).

In support of the first hypothesis, studies which have heterologously expressed PfCRT into yeast (*Pichia pastoris* (33)) and *Xenopus oocytes* (62) indicate that PfCRT is able to modulate host transport systems. In the yeast, PfCRT is

reported to function in the passive movement of Cl^- (33), whilst in the *Xenopus* system, PfCRT-expressing oocytes exhibit a depolarized resting membrane potential (ψ_m) and a higher intracellular pH (pH_i), compared to control oocytes (62). However, the fact that PfCRT "modulates" other transport process is somewhat vague. There is considerable distinction to be drawn between the scenario whereby PfCRT actively regulates other transporters and whereby it merely acts consequentially on other transport processes by the perturbation of ion (e.g. Ca^{2+}, Cl^-, K^+, Na^+, H^+) homeodynamics. A further problem faced by these studies is that, because of the high A/T of *P. falciparum* genes, the coding content of the *pfcrt* gene had to be reconstructed to allow for protein translation. It is not known therefore how these changes affect the function of the heterologously expressed protein.

Evidence for an energy-dependent CQ transporter, as described in the second hypothesis, was first proposed by Krogstad et al. (59, 60). It was demonstrated that steady-state accumulation of CQ by CQ-resistant parasites is reduced by adding glucose to the medium. By contrast, adding glucose to suspensions of CQ-sensitive parasites markedly stimulated the accumulation of CQ (60). The simplest interpretation of these data is that CQ-sensitive parasites have an energy-dependent CQ uptake mechanism (energy is required both to maintain the DV proton gradient and to traffic and digest haemoglobin, releasing FPIX) and CQ-resistant parasites have an additional energy-dependent CQ efflux mechanism. In addition, a recent study has demonstrated that CQ uptake can be trans-stimulated and that in CQ-resistant parasites this effect is energy dependent (42). On the basis of these observations, these authors suggested that an ATP-dependent primary active efflux transporter is responsible for CQ resistance (42). There are, however, other explanations for these data and currently this theory as yet is to be widely accepted.

The "charged drug leak" hypothesis (43, 61) has recently gained support from two independent studies, indicating that PfCRT is a member of the drug/metabolite transporter superfamily (63, 64) that may therefore be able to transport CQ directly. Transporters of this class are not directly energized by ATP and transport is often modulated by the transmembrane ψ_m. The charged drug leak hypothesis provides a potential explanation on how polymorphisms in *pfcrt* may directly mediate CQ resistance. The critical mutations associated with the development of CQ resistance are located on the food vacuole side and in the membrane (Fig. 3). These mutations are associated with a loss of basic and hydrophobic residues. Since CQ is diprotonated at the pH of the food vacuole, the loss of a basic residue at the opening of the channel in mutated PfCRT may allow the positively charged CQ to diffuse through an aqueous pore into the parasite cytoplasm. The release of CQ will be aided by both the protonated CQ (CQ^{++}) concentration and proton gradients across the food vacuole membrane (Fig. 4). In addition, it provides a potential explanation for the observed "reversal" of CQ resistance by a wide variety of structurally unrelated compounds whose only common features are hydrophobicity and positive charge (65). It is predicted that such compounds at high concentrations could sit in the hydrophobic core of the transporter, replace the positive charge and block the leak of charged CQ (e.g. verapamil, Fig. 4). In support of this, a recent study shows that a novel mutation in PfCRT (S163R) replaces a positive charge inside the barrel of the PfCRT transporter (43) returning the parasites to a CQ-sensitive status and abolishing verapamil reversibility while retaining all of the mutations, including K76T and A220S, associated with resistance.

2.4 *pfmdr1* and Resistance Mechanisms to Mefloquine and Quinine

It was hypothesized that analogous with mammalian tumour cells exhibiting multi-drug-resistance (mdr) phenotypes by virtue of the up-regulation of ATP-dependent P-glycoproteins, it was possible that drug-resistant *P. falciparum* lines may also harbour similar multi-drug-efflux transporters. Subsequently, two genes showing homology with human *mdr*-type genes were identified and named *pfmdr1* and *pfmdr2* (66, 67). Further analysis of *pfmdr2* indicated that there was no up-regulation or polymorphisms which correlated with *P. falciparum* drug resistance (67, 68) and in addition it was shown that structurally this gene product differed significantly from mammalian *mdr*-encoded proteins (69). Polymorphisms in *pfmr1*, however, were shown to correlate with CQ-resistant parasite (70), although further surveys did not always show such a good correlation (71–73). Nevertheless, the localization of the *pfmdr1* gene product, Pgh1 (for P-glycoprotein homologue) in the membrane of the parasite DV (74) suggested an involvement in the quinoline drug resistance.

The polymorphisms found in the *pfmdr1* gene, which correlate with drug resistance, include N86Y, Y184F, S1034C, N1042D and D1246Y. The mutation N86Y shows an association with CQ resistance; however, it is absent from a large number of South American CQ-resistant strains (e.g. 70, 75). The discrepancies surrounding the involvement of *pfmdr1* in resistance to CQ and related quinolines were resolved in a study by Cowman and colleagues using allelic exchange techniques (76). Variant *pfmdr1* genes from a drug-resistant line (7G8) carrying the mutations 1034C, 1042D and 1246Y were transfected into a CQ-sensitive *P. falciparum* strain (D10) carrying the wild-type sensitive residues (1034S, 1042N and 1246D). The variant *pfmdr1* genes from the drug-resistant line did not confer resistance to CQ but did confer

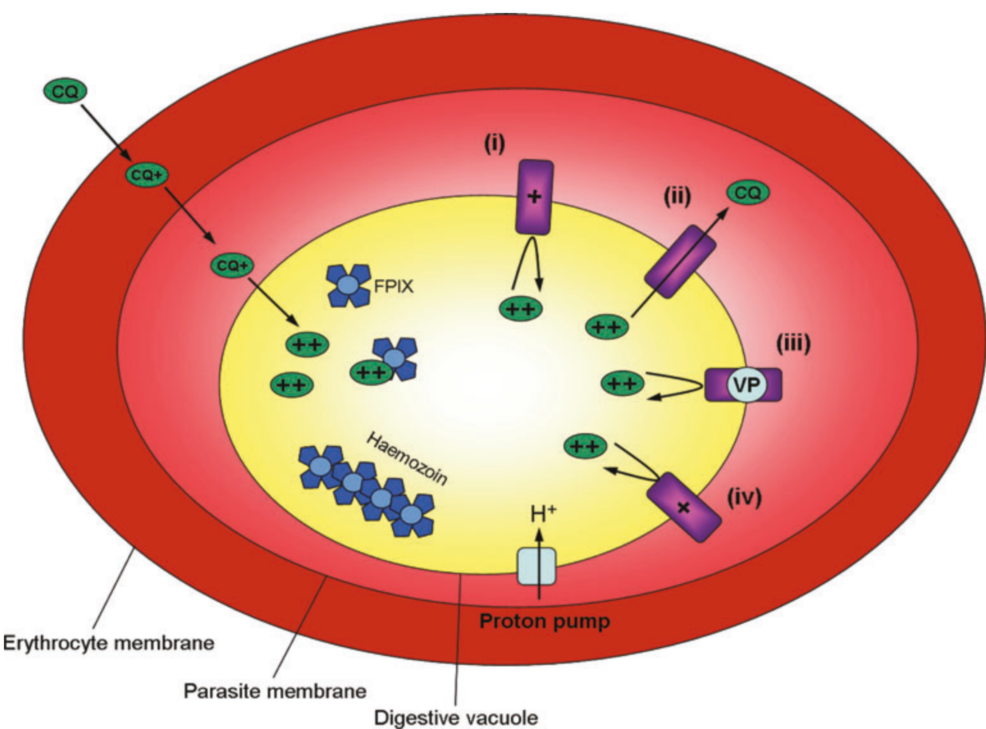

Fig. 4 The "charged drug leak" model for chloroquine (CQ) resistance. Allelic exchange studies have shown a definite role for PfCRT in CQ resistance. (i) In the wild type state (CQ-sensitive, K76), the positive charge on the K (lysine) residue may prevent the movement of the di-protonated CQ (CQ^{++}) through PfCRT. (ii) Replacement of this residue in the CQ-resistant parasites by the K76T mutation (replacement with the neutral residue threonine) might allow the flux of CQ^{++} through PfCRT, thus lowering the concentration of CQ in the digestive vacuole (DV) away from the heme target. (iii) Verapamil (VP) may work by reintroducing the positive charge to the barrel of the PfCRT protein, thus preventing the flux of CQ out of PfCRT, resulting in an increased sensitivity to CQ. (iv) The selection for the novel S163R mutation potentially mimics the effects of both VP and the normal K (lysine) residue at codon 76 by introducing a positive charge to the barrel of PfCRT, thereby preventing the flux of CQ through PfCRT. (Reproduced with permission from Johnson et al. (43))

resistance to quinine (76). However, removal of the *pfmr1* mutations from the CQ-resistant strain did increase sensitivity to CQ and confer resistance to MQ and halofantrine. These data conclusively demonstrated that *pfmdr1* was a genetic determinant for MQ, quinine and halofantrine but not for CQ. In order to explain the "CQ-modulation" effect of Pgh1, it was proposed that Pgh1 can act in concert with another system (now known to be PfCRT) which confers CQ resistance.

In addition to polymorphisms arising from point mutations, gene amplification of *pfmdr1* has also long been suggested as a possible cause for antimalarial drug resistance (77), and a casual link between halofantrine, MQ and quinine resistance was inferred (71, 78). Recently, gene amplification of *pfmdr1* was correlated to MQ resistance in vivo (79). It was concluded that increased copy number of *pfmdr1* was the most important determinant of MQ resistance. Interestingly, single nucleotide polymorphisms in *pfmdr1* were only associated with increased MQ susceptibility in vitro, and not in vivo.

Quinine remains effective against *P. falciparum*, but decreasing efficacy has been reported in the main malaria-endemic areas (80–82). It is assumed that quinine resistance shares some of the mechanisms associated with CQ and MQ resistance. As described above, it was shown that polymorphisms in *pfmdr1* increase resistance to quinine (76), and in addition mutations in PfCRT and in particular K76T also confer a quinine-resistant phenotype (83). Interestingly, it was observed that the K76I mutation greatly increased sensitivity to quinine but reduced sensitivity to its enantiomer quinidine, indicative of a unique stereospecific response not observed in other CQ-resistant lines (83). A recent search of genetically crossed *P. falciparum* lines for quantitative trait loci (QTL) associated with quinine resistance has identified three main loci on chromosomes 5, 7 and 13 (84). The mapped segments on chromosomes 5 and 7 are consistent with the involvement of *pfmdr1* and *pfcrt*, respectively; however, the chromosome 13 segment implies the involvement of a novel genetic determinant. Several candidate genes have been analysed and some correlation has been demonstrated between quinine resistance and polymorphisms in *pfnhe-1*, a putative Na^+/H^+ exchanger; however, it should be stressed that this work is still at a preliminary stage.

3 Resistance Mechanisms to the Antifolates

Folate is an essential vitamin which cannot be synthesized by humans. The *de novo* folate synthetic pathway is, however, present in the malaria parasite and for this reason it has been a most attractive drug target for decades (Fig. 5). In particular, two enzymes in the *P. falciparum* folate biosynthetic pathway have been targeted for antimalarial chemotherapy; the first is dihydropteroate synthase (DHPS) and the second is dihydrofolate reductase (DHFR). DHPS is not found in mammalian cells, and in *P. falciparum* it is the C-terminal domain of a bi-functional protein combined with 7,8-dihydro-6-hydroxy-methylpterin pyrophosphokinase (or pfPPPK-DHPS (85, 86)). The DHFR in *P. falciparum* is quite unlike that of mammalian cells and more akin to that found in other protozoa and plants in that it is only one domain of a bi-functional enzyme that contains the thimidylate synthase (or TS (87)). DHPS is susceptible to sulfonamides such as sulfadoxine (SD) and dapsone (DDS), whilst DHFR is susceptible to antimalarials such as pyrimethamine (PYR) and biguanides such as proguanil (PG) and chlorproguanil (CPG).

Combinations of DHPS and DHFR inhibitors act synergistically (88), a fact that has been successfully exploited clinically by combining PYR and SD in the drug Fansidar™ (or SP) and more recently by combining CPG with DDS, known as LapDap™ (89–91). Inhibition of these enzymes leads to a depletion of parasite intracellular folates which further interferes with methionine and pyrimidine biosynthesis eventually leading to death (Fig. 5).

As a result of growing resistance to CQ treatment, SP became a first-line drug of choice for the treatment of uncomplicated malaria in many countries with an existing burden of CQ resistance. However, resistance to SP has grown and spread very quickly, especially in Southeast Asia, South America (4, 7) and more recently in many areas of Africa (8).

3.1 *Resistance Caused by Mutations in DHPS and DHFR*

A number of resistance mechanisms have been attributed to the resistance of antifolates by *P. falciparum*; the principle mechanisms involve point mutations in DHPS and DHFR. Point mutations in the DHPS domain of the *pppk-dhps* gene confer resistance to sulfadoxine and dapsone (8, 85, 92–99), whilst point mutations in the DHFR domain of the *dhfr-ts* gene confer resistance to pyrimethamine and the biguanides (100–103).

Variations of five amino acids in the *P. falciparum dhps* have been shown in the laboratory and in the field to be associated with increased IC_{50} values for sulfadoxine and to a number of other sulfonamides as well as dapsone. Among these, the A437G mutation has been shown by transfection experiments to be the initial mutation, causing a fivefold increase in IC_{50}. Higher IC_{50} levels were associated with the

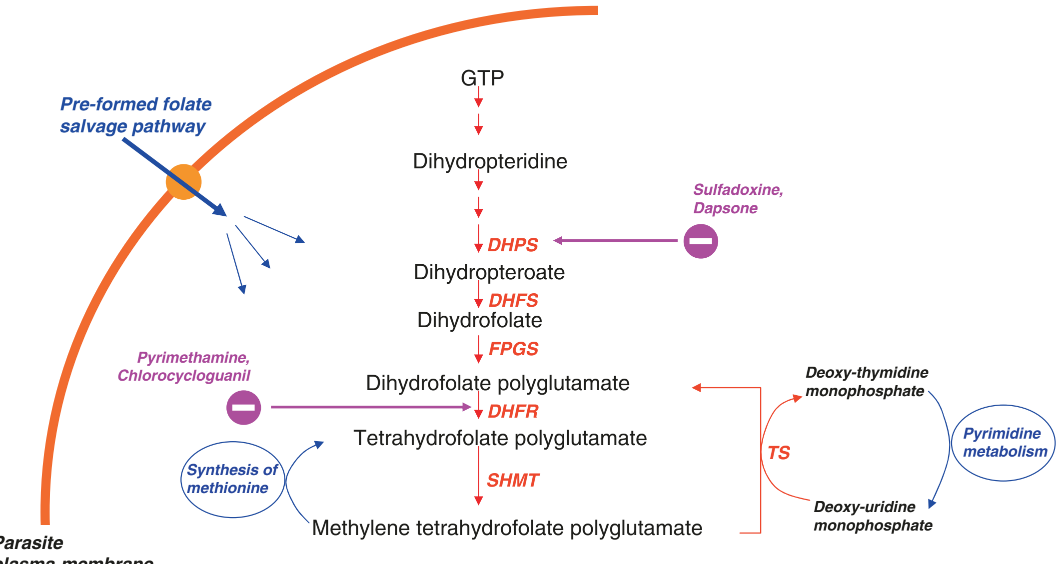

Fig. 5 The de novo folate synthetic pathway of *P. falciparum*. Enzymes: *DHPS* (dihydropteroate synthase), *DHFS* (dihydrofolate synthase), *FPGS* (folyl polyglutamate synthase), *DHFR* (dihydrofolate reductase), *SHMT* (serine hydroxymethyl transferase), *TS* (thymidylate synthase). DHPS is susceptible to sulfanomides such as sulfadoxine (*SD*) and dapsone (*DDS*) whilst DHFR is susceptible to pyrimethamine (*PYR*) and biguanides proguanil (*PG*) and chloroproguanil (*CPG*)

additional mutations S436F/A, K540E, A581G and A613S (94). Mutations affecting positions 436, 437 and 540 can each occur singly; however, the A581G variation is always associated with A437G and similarly the A613S/T alteration is always coupled with changes in either residue 436 or 437 (98), apparently reflecting steric constraints of the enzyme. All of the five polymorphic residues are believed to form a part of a solvent-accessible channel connecting the catalytic centre (98, 104); however, detailed structural knowledge remains elusive until the DHPS can be crystallized.

Studies on *P. chabaudi* performed over 20 years ago first suggested that alterations in the DHFR enzyme led to reduced pyrimethamine binding and consequently resistance (105). The *dhfr* gene point mutations that confer resistance to pyrimethamine were subsequently described (100, 101, 106) with conclusive proof linking the point mutations and pyrimethamine resistance provided by transfection studies (102). Compared with the wild-type *dhfr*, S108N increases resistance to pyrimethamine by about 100-fold (100, 101). Succeeding mutations N51I, C59R and I164L progressively increase resistance to pyrimethamine up to a further order of magnitude to about 1000-fold compared to the wild type (8). It has been observed in the field that the mutations N51I, C59R and I164L do not occur alone in the absence of the S108N mutation. Recent structural studies on the crystallized *P. falciparum* DHFR-TS (107) have shown that the mutations occur in the active site of the DHFR domain (Fig. 6). These *dhfr* mutations also confer resistance to the active cyclic metabolites of proguanil (PG) and chlorproguanil (CPG), namely, cycloguanil and chlorcycloguanil, respectively. Fortunately, the recently launched CPG–DDS drug LapDap™ is effective at clearing patients with triple-mutant *dhfr* infections which are commonly found in Africa (108); however, laboratory studies suggest that quadruple mutations of *dhfr* will also lead to clinical resistance to LapDap™ (8). There are, however, other inhibitors of DHFR which may be useful for the future such as the pro-drug PS-15 which is metabolized into the active triazine known as WR92210. This inhibitor is potent against quadruple *dhfr* mutants (8) and recent structural studies suggest that this is because of its flexible side chain which adopts a conformation which can still fit into the active site modified by the S108N mutation, whereas rigid inhibitors such as chlorcycloguanil and pyrimethamine cannot avoid short contacts and thus bind poorly (107, 109).

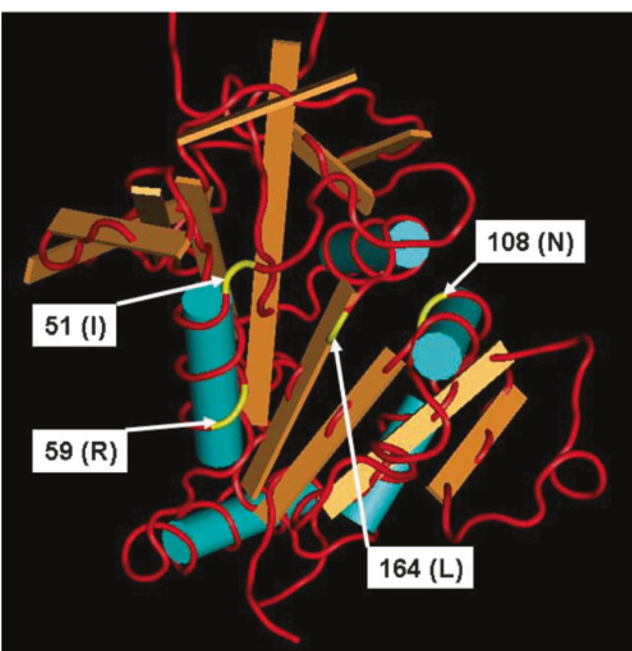

Fig. 6 Structural model of the *P. falciparum* DHFR domain. Mutations (S108N, N51I, C59R and I164L) in the active site of the enzyme alter drug binding and confer resistance. (Drawn from the crystal structure submitted by Yuvaniyama et al. (107)) (*See Color Plates*)

3.2 Further Putative Antifolate Resistance Mechanisms

Studies on *P. falciparum* have shown that the addition of folic acid or folate derivatives decrease the activity of antifolate drug both in vitro and in vivo (110, 111). Similarly, lowering the folate concentration enhances the in vitro activity of antifolate drugs (112). Taken together, these observations indicate the presence of a folate salvage phenotype in *P. falciparum* (Fig. 5). Recently, it was shown that a range of antifolates could be potentiated by probenecid (believed to be an inhibitor of the salvage pathway) at therapeutically relevant concentrations (113) highlighting the potential of the folate salvage pathway as an auxiliary drug target.

Several studies have also explored on whether *P. falciparum* is able to increase expression of target proteins (DHPS and DHFR) in order to “dilute” the effect of the antifolates. Increased expression can occur by either up-regulation at the transcriptional level and/or the translational level. No evidence of a drug-induced increase of expression at the transcriptional level has been observed (114); however, recently it has been reported that translational up-regulation of the DHFR-TS can be induced by antifolates and inhibitors of TS (115). It is not clear at this stage, however, on how much these observed changes in translation contribute to antifolate resistance and whether they have any clinical relevance.

4 Resistance Mechanisms to Naphthoquinones

Atovaquone is a naphthoquinone developed to selectively compete for ubiquinone (CoQ) in the mitochondrial electron transport chain of *P. falciparum* (Fig. 7) (116). Atovaquone, which is 1000-fold more active against parasite compared with

mammalian mitochondria (117), specifically acts by binding to the CoQ oxidation site in the cytochrome bc_1 complex (116, 118). When used as a single agent, resistance to atovaquone was quickly observed both in vitro (119, 120) and in mice models (121). Initial clinical trials demonstrated a 30% treatment failure within 28 days of treatment (122, 123). Significant synergy in antimalarial activity was achieved when atovaquone was combined with the biguanide proguanil (Malarone™). This synergistic effect is unrelated to the inhibition of folate metabolism (124) and was shown to be successful in significantly reducing the number of treatment failures (125). Nevertheless, atovaquone-resistant parasites are equally resistant to atovaquone/proguanil combinations (124). In the species of plasmodium, resistance to atovaquone is associated with missense mutations around the Q_o (CoQ oxidation site) region of the cytochrome bc_1 gene, especially near the highly conserved PEWY sequence (118, 121, 126, 127). Atovaquone-resistant *P. falciparum* lines, generated in the laboratory, were polymorphic at codons 133, 272 and 280 (126). Whilst in vivo, the first cases of Malarone-treatment failure were associated with mutations at codon 268, namely Y268N (127) and Y268S (128). These mutations were subsequently considered useful tools for the surveillance of Malarone resistance (129, 130); however; recent reports indicate the presence of Malarone resistance in the absence of the 268 mutation (131).

5 Resistance Mechanisms to Artemisinin

Artemisinin and its derivatives (artesunate, artemether, arteether and dihydroartemisinin) represent a very different class of antimalarial compounds (Fig. 8) developed from an ancient Chinese herbal remedy from the sweet wormwood *Artemisia annua* or "qinghao" (132). Artemisinins are endoperoxides (containing a peroxide bridge) and this feature is believed to be the key to their mode of action. Ferrous iron (Fe^{2+}) from heme (FP) or other sources of Fe^{2+}, catalyse the cleavage of this bridge, forming highly reactive free radicals (132, 133). A wide range of different reactive intermediates can be generated by the cleavage of the peroxide bridge (133); however, the exact species and the biological target(s) of the reactive intermediates have been the subject of much debate.

It has been suggested that the Fe^{2+}-induced decomposition of artemisinin produces an oxyl radical that subsequently re-arranges into more than one carbon-centred radical species. Alkylation of macromolecules such as haemoglobin-derived heme (134) and parasite proteins such as falcipain (135), histidine-rich protein (136) and Ca^{2+}-ATPase (PfATPase6 (137)) have been proposed to lead to parasite death. Alternatively, it has been proposed the artemisinins act as a masked source of hydrogen peroxide. Heterolytic, rather than homolytic cleavage of the peroxide bridge and subsequent Fenton-like degradation of the hydrogen peroxide results in the formation of the highly toxic hydroxyl radical, leading to cell death (138).

Artemisinin and derivatives are widely used in antimalarial combination therapy; however, currently there is no evidence for clinically relevant artemisinin resistance. This observation leads many scientists to believe that artemisinins do not exert their activity by affecting a single biological target but rather simultaneously hit several targets, known as the "cluster-bomb" effect (139). In the laboratory, strains showing reduced artemisinin sensitivity have been generated both for *P. falciparum* (140) and for the mouse plasmodium, *P. yoelii* (141). In *P. yoelii*, it has recently been reported that up-regulation of the mouse multi-drug-resistance gene, *mdr1*, is responsible for a decrease in artemisinin susceptibility (142); there are as yet no reports of similar studies performed with *P. falciparum*.

Some clinical failures have been reported as a result of artesunate monotherapy, but generally this is considered not to depend on inherent parasite resistance but due to rapid drug elimination or possibly due to variations in the parasite burden (143).

(a) (b)

Fig. 7 Chemical structure of (**a**) ubiquinone (CoQ) and (**b**) atovaquone. Atovaquone is a competitive inhibitor of CoQ and selectively inhibits the electron transport chain of *P. falciparum* mitochondria

Artemisinin

Sodium artesunate R = α-OC(O)$CH_2CH_2CO_2Na$
Artemether R = β–OMe
Arteether R = β–OEt
Dihydroartemisinin R = β + α OH

Fig. 8 Chemical structures of artemisinin and derivatives (artesunate, artemether, arteether and dihydroartemisinin)

6 Conclusion

Here, we have attempted to summarize the principal mechanisms of antimalarial drug resistance for the major groups of drugs currently deployed in malaria-endemic countries. The protozoan parasite has been shown to deploy an array of mechanisms of escapism including reducing the concentration of intracellular drug concentration by the action of altered or increased transport away from target sites as well as altering the target site to reduce drug binding. Our understanding of these mechanisms has been radically improved by pioneering genetic and biochemical advances notwithstanding the completion of the malaria genome project. Understanding the mechanisms underpinning drug resistance remains paramount for the development of effective global malaria chemotherapy; however, there are many other factors which can reduce the efficacy of antimalarial drugs. These include drug pharmacodynamics, host immunity, malaria transmission and drug effectiveness (e.g. compliance). It may transpire that only a holistic approach will win the race against antimalarial drug resistance and pacify this terrible disease affecting poor countries.

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Chapter 41
Drug Resistance in *Leishmania*

Hiranmoy Bhattacharjee and Rita Mukhopadhyay

1 Introduction

Leishmaniasis is a parasitic disease caused by the obligate intracellular protozoa of the genus *Leishmania*. At least 21 of the 30 species of *Leishmania* are known to be infectious to humans. The parasite exists in two forms. The promastigote form of the parasite resides in the intestinal tract of the insect vector and appears as a slender, spindle-shaped structure with an anterior flagellum. The amastigote forms of the parasite are small, oval-shaped structures that reside in macrophages and other mononuclear phagocytes in the mammalian host. The female phlebotomine sandflies are solely responsible for the transmission of *Leishmania* parasites amongst vertebrate hosts. Transmission of leishmaniasis could be anthroponotic, that is, transmission from human to human through the sandfly vector, where humans are the sole reservoir host. The disease can also spread from animals to humans (zoonosis); in this case, domestic animals (dogs) and wild animals (foxes, jackals, rodents, hyraxes) serve as the reservoir hosts.

Leishmaniasis is endemic in parts of 88 countries across five continents – the majority of the affected countries are in the tropics and subtropics. Approximately 12 million people worldwide are affected by leishmaniasis, while a total of 350 million people are at a risk of contracting the disease (http://www.who.int/tdr/diseases/leish/). The disease in humans has been classified into three different forms, each having a broad range of clinical manifestations. Visceral leishmaniasis (VL) is the most severe form of the disease and is fatal if left untreated. VL is caused by *Leishmania donovani*, *Leishmania infantum,* or *Leishmania chagasi* and is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia. Approximately 90% of the 500,000 new cases of VL reported annually occur in Bangladesh, Brazil, India, Nepal, and Sudan. Cutaneous leishmaniasis (CL) is caused by a variety of species including *Leishmania major*, *Leishmania tropica*, *Leishmania mexicana*, and *Leishmania panamensis*. CL is characterized by skin lesions on the exposed parts of the body, such as the face, arms, and legs, causing serious disability and leaving the patient permanently scarred. It is the most common form of the disease with 1–1.5 million new cases reported annually worldwide, and 90% of all CL cases are reported from Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria. Mucocutaneous leishmaniasis (MCL) due to *Leishmania braziliensis* infection produces lesions which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth, and throat cavities. Ninety percent of MCL cases occur in Bolivia, Brazil, and Peru.

The last decade has recorded a sharp increase in leishmaniasis along with a significant expansion of *Leishmania*-endemic regions (http://www.who.int/inf-fs/en/fact116.html). This geographical spread is due to several factors. Widespread rural–urban migrations for business ventures bring non-immune urban dwellers into endemic rural areas. Projects having considerable environmental impact, like dams, irrigation systems as well as extensive deforestation also contribute to the spread of the disease. Civil wars and regional conflicts leading to mass exodus, accompanied by collapse of public health have also increased the number of *Leishmania*-infected patients. Additionally, *Leishmania*/HIV co-infection is currently emerging as an extremely serious, new disease and is being considered a real threat in various parts of the world. VL has been widely recognized as an opportunistic infection among persons who are immunosuppressed, particularly in patients infected with human immunodeficiency virus (1, 2).

The first-line compounds against all forms of leishmaniasis are the two pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) (Fig. 1). However, clinical resistance to this treatment is becoming prevalent (3, 4). In fact, more than 50% of VL cases in Northeast India are resistant to pentostam (5).

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Leishmania resistant to trivalent antimony has also been reported (6). The second line of anti-leishmanial drugs includes amphotericin B and pentamidine (Fig. 1). Recently, alkyl-lysophospholipids (ALP) such as miltefosine (Fig. 1) and edelfosine, originally developed as anticancer drugs, have shown significant antiproliferative activity against *Leishmania* (7). Miltefosine is the first oral drug that has been used against VL in India, including antimony-resistant cases (8). Other drugs in various stages of clinical trials include allopurinol, atovaquone, fluconazole, paromomycin, and sitamaquine (Fig. 1).

Either clinical or laboratory-induced drug resistance have been observed against many of these drugs. Consequently, prevention and circumvention of resistance are important medical priorities. Understanding the mechanism of drug resistance will help in the development of tools towards recognition of resistance early in the infection process. This in turn would allow the clinicians to start alternate or combination therapy at an early stage of infection and to minimize the development of resistance. Additionally, identification of intracellular drug targets and parasite defense mechanisms will lead to rational drug design thereby providing much

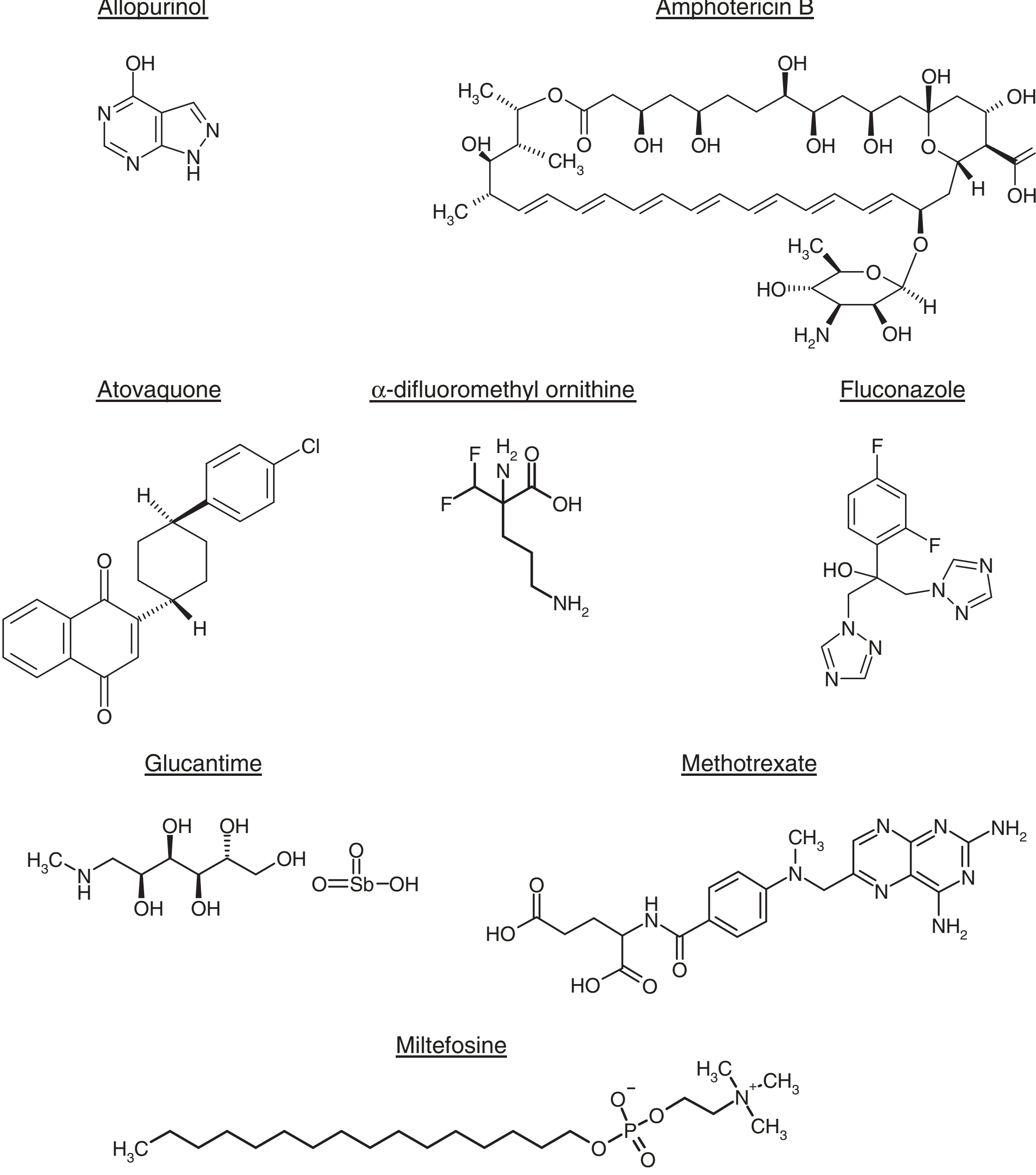

Fig. 1 Chemical structure of anti-leishmanial agents. Chemical structures were drawn from the National Library of Medicine, Specialized Information Services ChemIDplus database (http://chem.sis.nlm.nih.gov/chemidplus/)

Fig. 1 (continued)

effective treatment of the disease. Multiple biochemical mechanisms have been employed by *Leishmania* in conferring drug resistance (9). These include (a) downregulation of the uptake system(s) for the drug, (b) intracellular sequestration, (c) drug inactivation or modification, (d) modification of the drug target to prevent binding of the drug or overproduction of the target so that drug concentration becomes limiting, (e) more efficient repair of drug damage and, (f) bypassing a blocked target.

2 Mechanisms of Drug Action and Resistance

2.1 Antimonials

Pentavalent antimonials have been used for the treatment of leishmaniasis for over half a century. The recommended regimen consists of daily injection of 20 mg/kg of either sodium stibogluconate or meglumine antimonate for 20–28 days (10).

2.1.1 Mechanisms of Action

Despite being used for several decades, the mode of action of pentavalent antimonials is poorly understood. The possibility of in vivo metabolic conversion of pentavalent [Sb(V)] to trivalent [Sb(III)] was suggested more than 50 years ago (11). This hypothesis was supported by the observation that hamsters infected with *Leishmania garnhami*, and then treated with Glucantime [Sb(V)], showed similar serum concentrations of Sb(III) and Sb(V) (12). Reduction of Sb(V) to Sb(III) was suggested to be associated with decreasing size and healing of the leishmanial ulcers (12). Several investigators have shown that Sb(III) is more toxic than Sb(V) to either the promastigote or amastigote forms of different *Leishmania* species (13–15). Sereno et al. demonstrated that axenically grown amastigotes of *L. infantum* were more susceptible to Sb(III) than to Sb(V) (15). However, these amastigotes were found to be poorly responsive to meglumine [Sb(V)], compared to amastigotes grown in human macrophages (16). These results strongly suggested a putative reductase residing within the macrophage, which catalyzes the conversion of Sb(V) to Sb(III).

Since arsenic and antimony are related metalloids, and arsenical-resistant *Leishmania* strains are frequently cross-resistant to antimonials, we considered the possibility that Sb(V) is reduced by a leishmanial As(V) reductase. The *Saccharomyces cerevisiae* arsenate reductase (ScAcr2p) sequence (17) was used to identify and clone the *L. major* homologue, LmACR2 (18). LmACR2 was able to complement the arsenate-sensitive phenotype of either *Escherichia coli* or *S. cerevisiae* arsenate reductase disrupted strains. Transfection of *Leishmania infantum* with *LmACR2*

augmented Pentostam sensitivity in intracellular amastigotes. LmACR2 was purified and shown to reduce both As(V) and Sb(V) in vitro. We propose that LmACR2 is responsible for the reduction of pentavalent antimony in Pentostam to the active trivalent form of the drug in *Leishmania* (18) (Fig. 2). Denton et al. (19) have recently identified and characterized a thiol-dependent reductase (TDR1) from *L. major* that can catalyze the reduction of pentavalent antimonials to the trivalent form using glutathione as a reductant. TDR1 is a trimer of two-domain monomers – each domain having some similarity to omega glutathione transferases. The higher abundance of the enzyme in mammalian stages of the parasite might explain the greater susceptibility of this parasite form to the drug.

Ultrastructural changes in *Leishmania tropica* within human macrophages exposed in vitro to Pentostam have been reported by Langreth et al. (20). Pentostam-treated macrophages demonstrated loss of membrane definition. It was suggested that impaired macrophage membrane function may contribute towards the effect of this drug against macrophage-contained *Leishmania*. To understand the antileishmanial effects of antimonial agents, Roberts et al. synthesized complexes of tri- and pentavalent antimony with mannan (14). They observed that macrophages accumulated antimony after a 4-h exposure with potassium antimony tartrate, trivalent antimony-mannan, or pentavalent antimony-mannan, which was retained intracellularly for at least 3 days. Amastigotes inside macrophages had higher antimony content 6 days after a single 4-h treatment suggesting that macrophages serve as a reservoir and prolong parasite exposure to antimonial agents.

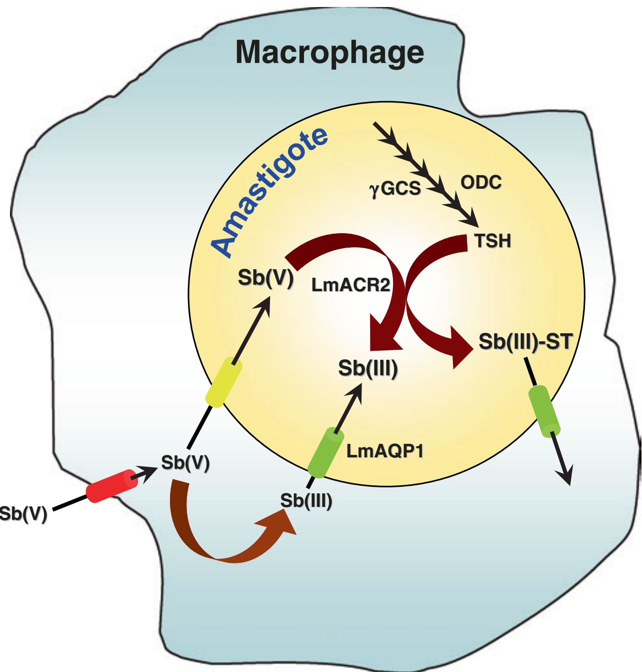

Fig. 2 Model of Pentostam uptake and resistance in macrophage-associated amastigotes of *Leishmania*. Sb(V) is taken up by macrophages, and a portion is reduced to Sb(III), which is then transported into the amastigote by LmAQP1. The other portion of the Sb(V) is taken into the amastigote and reduced to Sb(III) by LmACR2 and perhaps other enzymes such as TDR1. Trypanothione (TSH) is overproduced in *Leishmania* by the higher activity of the rate-limiting enzymes γ-glutamyl cysteine synthetase (γGCS) and ornithine decarboxylase (ODC). Resistance is conferred when the plasma membrane pump extrudes As/Sb–TSH complex along with sequestration inside intracellular vesicles by an MRP homologue PGPA. The relative contributions of the two pathways to drug action would depend on the relative rates and expression of their respective components in both the human host and parasite. This could be different in different strains of *Leishmania*, as well as in different infected individuals, leading to variability in drug response

Berman et al. (21) have shown that the viability of *Leishmania mexicana* promastigotes and amastigotes were decreased by 40–61% following a 4-h exposure to 500 μg/ml of sodium stibogluconate. Such an exposure also resulted in a 56–65% decrease in incorporation of label into purine nucleoside triphosphate along with a 34–60% increase in incorporation of label into purine nucleoside monophosphate and diphosphate. Further experiments suggested that inhibition of glycolysis and the citric acid cycle might be partly responsible for the inability to phosphorylate ADP. An apparent decrease in ATP and GTP synthesis was therefore proposed to contribute to decreased macromolecular synthesis and decreased *Leishmania* viability.

Chakraborty and Majumder (22) reported that one possible mode of action of antimonials may be in their ability to inhibit *Leishmania* topoisomerase I. These authors demonstrated that *L. donovani* topoisomerase I catalyzed relaxation of supercoiled plasmid pBR322 was specifically inhibited by sodium stibogluconate. Dose-dependent inhibition suggested that antimonials interact with *Leishmania* topoisomerase I rather than the DNA. Calf thymus topoisomerase I and *E. coli* DNA gyrase was not inhibited by Sb(V).

Demicheli et al. investigated the ability of pentavalent antimonials to form complexes with adenine nucleosides and deoxynucleosides in aqueous solution (23). Circular dichroism (CD) titration suggested that adenosine and adenosine monophosphate, but not 2′-deoxyadenosine, form 1:2 Sb(V)–nucleoside complexes. NMR analysis indicated that Sb(V) binds to the sugar moiety at the 2′ position. Upon incubation of meglumine antimonate with adenosine, transfer of Sb(V) from its original ligand to the nucleoside molecule was observed at an acidic pH. Similar formation of Sb(V)–nucleoside complexes within the phagolysosome of *Leishmania* bearing macrophages was proposed as a possible mechanism of anti-leishmanial activity of antimonials.

2.1.2 Mechanisms of Resistance

The study of drug resistance in the field isolates of *Leishmania* is often complicated by two factors – first, the host response and bioavailability of the drug to an intracellular parasite and second, removal of parasites from the host and adaptation in the culture medium spontaneously selects for a population of the microorganisms that are best suited for growth in that medium. Additionally, clonal selection is difficult in the field isolates along with the fact that isogenic parental sensitive population is impossible to find in the field. Thus, dissection of drug-resistance mechanisms is mostly limited to in vitro stepwise selection of *Leishmania* species with antimonials and the related metalloid arsenicals.

L. tarentolae antimonial-resistant mutants exhibited cross-resistance to various concentrations of arsenite (24), but not to other anti-leishmanials like miltefosine, paromomycin and amphotericin B (25). However, Prasad et al. reported that an arsenite-resistant strain of *L. donovani* displayed low levels of cross-resistance to structurally unrelated drugs such as doxorubicin and pentamidine (26).

Metalloid resistance in *Leishmania* appears to be multifactorial, involving transport and cellular thiol levels (Fig. 2). The ATP-binding cassette (ABC) protein PGPA has been assumed to play a major role on metal resistance in *Leishmania* (27). PGPA is a member of the multidrug-resistance protein (MRP) family, a large family of ABC transporters, several of which are implicated in drug resistance (28). The *PGPA* gene has been shown to be frequently amplified in *Leishmania* cells that are selected for resistance to arsenite- or antimony-containing drugs (29–34), and its transfection as well as its disruption prove that PGPA is involved in metal resistance (33–37). We have shown that arsenite-resistant *L. tarentolae* promastigotes overproduce trypanothione (TSH), a unique glutathione-spermidine conjugate found only in trypanosomatids (38). We have also shown that trypanothione overproduction is mediated by increased activity of two rate-limiting enzymes in the polyamine and glutathione biosynthetic pathways, namely ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (γ-GCS), respectively (39, 40) (Fig. 2). Increased levels of TSH was not sufficient to observe metal resistance, but the modulation of TSH levels by using specific inhibitors of γ-GCS or ODC could revert the resistance in mutants (40). Co-transfection of the *GSH1* gene that codes for the heavy subunit of γ-glutamylcysteine synthetase (γ-GCS) or *ODC* and *PGPA* genes in partial revertants, but not in wild-type cells, lead to synergistic levels of resistance strongly suggesting that PGPA recognizes metals conjugated to TSH (39, 40). Experiments by Legare et al. indicate that PGPA is localized in small vesicles near flagellar pocket and these are responsible for intracellular sequestration of arsenic/antimony–thiol conjugates, thereby conferring arsenite and antimonite resistance (41).

Transport experiments in arsenite-resistant *L. tarentolae* mutants indicated the presence of an active efflux system that did not correlate with *PGPA* gene amplification (24, 42). An analysis of *L. tarentolae PGPA* transfectants did not show a marked difference in the steady-state accumulation of arsenite (37), although a decrease in the uptake of antimony was proposed to explain the resistance in *L. major PGPA* transfectants (43).

Resistance mechanisms to pentavalent antimonials have been explored in the field isolates of *Leishmania* (44, 45). No amplified *PGPA* sequence could be detected, suggesting that *PGPA* amplification is not involved in As(V) resistance in these strains (44). Instead, Singh et al. observed that a novel gene was amplified in these drug-resistant parasites whose locus is on chromosome 9 (45). These authors speculate that after drug exposure, protein phosphorylation may play a role in signal transduction pathway in the parasite thereby conferring resistance (45).

Brochu et al. (46) have quantified the accumulation of Sb(V) and Sb(III) in *Leishmania* by using inductively coupled plasma mass spectrometry (ICP-MS). The accumulation was studied in three *Leishmania* species at various life stages that were either sensitive or resistant to antimony. Both the promastigote and amastigote forms of the parasites accumulated Sb(III) and Sb(V). Competition experiments with arsenite indicated that the routes of entry of Sb(V) and Sb(III) into the parasites were most likely different. However, the level of accumulation of either Sb(III) or Sb(V) did not correlate with the susceptibility of wild-type *Leishmania* cells to antimony. In contrast to metal susceptibility, resistance to Sb(III) correlated well with decreased antimony accumulation. This phenotype was energy dependent and highlighted the importance of transport systems in drug resistance of this protozoan parasite.

Microbes often become resistant to drugs by mutation or downregulation of uptake systems. Uptake systems for As(III) and Sb(III) have been identified in microbes and in mammals, but the identities of such system(s) in parasitic protozoans including *Leishmania* are unknown. In bacteria (47), yeast (48) and mammals (49) aquaglyceroporins have been shown to be the uptake systems for trivalent metalloids. We have recently reported the identification and characterization of aquaglyceroporins from *L. major* (LmAQP1) and *L. tarentolae* (LtAQP1), respectively (50) (Fig. 2). These *Leishmania* aquaglyceroporins have the conserved signature motifs of mammalian aquaglyceroporins (51). *LmAQP1* was transfected into three different species of leishmania – *L. tarentolae*, *L. infantum*, and *L. major*. Each transfectant became hypersensitive to both As(III) and Sb(III). We have also shown that the drug-resistant parasites, with various mutations leading to resistance mechanisms, became hypersensitive to both metalloids after overexpression of LmAQP1.

Increased rates of uptake of either As(III) or Sb(III) correlated with metalloid sensitivity of the wild-type and drug-resistant transfectants (50). We have recently constructed a heterozygous knockout of *L. major* by disruption of one of the two alleles. This single knockout is 10-fold more resistant to Sb(III) than the homozygous wild-type parent. This supports our hypothesis that the amount of AQP1 in the plasma membrane is the rate-limiting step in the uptake of the activated form of the antimonial drugs. It also strengthens the argument that point mutations in the *AQP1* gene could give rise to clinical resistance. This is an important observation, because it was not clear whether a single mutation would result in a sufficient reduction in uptake to produce resistance. Future studies will focus on the appearance of mutations in the *AQP1* gene and the levels of AQP1 in the plasma membrane of the field isolates of drug-resistant parasites.

It has been suggested that the anti-leishmanial activity of pentavalent antimony is dependent on its reduction to Sb(III) (52). ICP-MS measurements indicated that Sb(V) was reduced to highly toxic Sb(III) in *L. donovani* amastigotes. Furthermore, one of the Pentostam-resistant mutants lacked this reducing activity, suggesting a novel mechanism of Sb(V) resistance (52). Since *Leishmania* thrives inside macrophages, it has always been an open question whether Sb(V) is reduced in the parasite or macrophages, or in both. As discussed earlier, we have identified an arsenate reductase gene (*LmACR2*) in *L. major* (18). This protein complements the arsenate-resistance phenotype when overexpressed heterologously in *E. coli* and the purified protein shows arsenate and antimonate reductase activity in vitro. LmAcr2 also confers pentostam hypersensitivity to *Leishmania* amastigotes, sensitive and resistant to pentostam (18).

2.2 Amphotericin B

Amphotericin B has been often used as a second line of treatment against leishmaniasis in India. In an uncontrolled study, 93% cure was observed in antimonial unresponsive patients with short-course treatment of amphotericin B fat emulsion (five alternate day infusions of 2 mg/kg) (53). Although the drug is effective, its use is limited by toxicity problems, for example, renal impairment, anemia, fever, malaise, and hypokalemia. Liposomes have been proposed as an effective way to target drugs at macrophages. In animals, Amphotericin B incorporated into liposomes is highly effective against experimental leishmaniasis, with low toxicity. Davidson et al. reported the successful treatment of a patient with multiply drug-resistant visceral leishmaniasis with a commercially prepared formulation of liposomal amphotericin B (54).

2.2.1 Mechanisms of Action

The primary site of action of amphotericin B on *L. donovani* promastigote cells appears to be membrane sterols, which results in the loss of permeability barrier to small metabolites (55). Uptake of [^{14}C]glucose was inhibited quickly while inhibition of respiration by the drug was comparatively a slower process (55).

2.2.2 Mechanisms of Resistance

Amphotericin B-resistant *L. donovani* promastigotes were selected by increasing the drug pressure (56). The resistant cells had 2.5 times longer generation time along with decreased uptake and increased efflux of the drug. The drug-resistant promastigotes showed increased membrane fluidity. Analysis of lipid composition showed that saturated fatty acids were prevalent in resistant cells, with stearic acid as the major fatty acid, and the major sterol was an ergosterol precursor, the cholesta-5, 7, 24-trien-3β-ol and not ergosterol as in the amphotericin B-sensitive strain (56). This study suggested that resistance of *L. donovani* promastigotes to amphotericin B involved the substitution of another sterol for ergosterol in the cell membrane, change in membrane fluidity, and a weak affinity of the drug for such modified membranes (56).

Singh et al. (57) reported step-by-step selection of two amphotericin B-resistant *L. tarentolae* cells. One of the mutants was also cross-resistant to ketoconazole. DNA amplification was observed in both mutants. Gene transfection experiments indicated that the link between the locus amplified and the resistance levels were not straightforward and several mutations were possibly responsible for amphotericin B resistance.

2.3 Pentamidine

Pentamidine isothienate, an aromatic diamidine, has been used as a second-line anti-leishmanial drug in antimony unresponsive visceral leishmaniasis patients. The recommended dosage is 4 mg/kg by intramuscular route on alternate days for 6 weeks (53, 58, 59). The drug achieves poor response rates (around 75%) and is associated with side effects like myalgia, nausea, headache, and hypoglycemia, with an exceptional risk of developing irreversible diabetes (53).

2.3.1 Mechanisms of Action

The mechanism of action of pentamidine is poorly understood. The uptake process for pentamidine in *L. donovani* and *L. amazonensis* promastigotes and axenic amastigotes

is saturable, carrier-mediated and energy-dependent (60). Pentamidine was found to be a competitive inhibitor of arginine transport (61, 62) in *L. donovani*, and a noncompetitive inhibitor of putrescine and spermidine transport in *L. infantum* (63), *L. donovani*, and *L. mexicana* (64). The physiological roles of the carrier proteins that accumulate pentamidine are still unknown. When treated with low concentrations of pentamidine for 24 h, both *L. donovani* and *L. amazonensis* cells showed significant decrease in ornithine decarboxylase activity, the rate-limiting enzyme in the polyamine biosynthetic pathway (65). Therefore, the polyamine biosynthesis pathway might be a target of pentamidine in *Leishmania*.

The mitochondrion has also been implicated in the action of pentamidine against leishmania (66). *L. tropica* amastigotes exposed in vitro to pentamidine demonstrated swollen kinetoplasts and fragmentation of the kinetoplast DNA core (20, 67). A rapid collapse of the mitochondrial inner membrane potential of *L. donovani* promastigotes was also observed upon treatment of these parasites with the drug (68).

2.3.2 Mechanisms of Resistance

Resistance to pentamidine has been described for *L. donovani* and other *Leishmania* species (66). The mechanism of resistance to pentamidine is not well understood. Coelho et al. (69) used a genetic strategy to search for loci able to mediate pentamidine resistance (PEN^r) when overexpressed in *L. major*. A shuttle cosmid library containing genomic DNA inserts was transfected into wild-type promastigotes and screened for PEN^r transfectants. Two different cosmids identifying the same locus were found, which differed from the other known *Leishmania* drug-resistance genes. The PEN^r gene was mapped by deletion and transposon mutagenesis to an open reading frame belonging to the P-glycoprotein/MRP ATP-binding cassette transporter superfamily and was named pentamidine resistance protein 1 (PRP1). PRP1-mediated pentamidine resistance could be reversed by verapamil and overexpression of PRP1 showed cross-resistance to Sb(III) but not to Sb(V). Although a 1.7- to 3.7-fold pentamidine resistance was observed, this may be significant in clinical drug resistance given the marginal efficacy of the drug against *Leishmania*.

Basselin et al. (66) compared the uptake of [^{3}H]pentamidine into wild-type and drug-resistant strains of *L. mexicana*. A substantial decrease in the accumulation of the drug accompanied the resistance phenotype, although the apparent affinity for pentamidine by its carrier was not altered. Their experiments indicate that diamidine drugs accumulate in the *Leishmania* mitochondrion. The development of resistance phenotype is accompanied by the lack of mitochondrial accumulation of the drug and its exclusion from the parasites.

2.4 Alternate Agents

Alternate agents are used mostly in antimony unresponsive cases. Miltefosine, allopurinol, atovaquone, and paromomycin are commonly used either alone or in combination with antimonials against different forms of leishmaniasis.

2.4.1 Miltefosine

Miltefosine (hexadecylphosphocholine) is the first drug approved for the oral treatment of VL. Miltefosine was originally developed as an antineoplastic agent and later found to be highly active against *Leishmania* in vitro and in animal models (70, 71). The recommended dose of miltefosine for the treatment of visceral leishmaniasis is approximately 2.5 mg/kg per day for 4 weeks (72). Initial clinical trials showed miltefosine to be approximately 90% effective in combating childhood visceral leishmaniasis (8, 73).

Mechanisms of Action

The molecular mechanisms that contribute to the anti-leishmanial activity of miltefosine are still unknown. Preliminary studies on *Leishmania mexicana* promastigotes suggested that miltefosine might cause perturbation of ether lipid metabolism, glycosylphosphatidylinositol (GPI) anchor biosynthesis and leishmanial signal transduction (74, 75). Later, Lux et al. reported that miltefosine inhibits the glycosomal alkyl-specific-acyl-CoA acyl transferase in a dose-dependent manner (76). It was proposed that a perturbation of ether lipid remodeling might be responsible for the anti-leishmanial activity of miltefosine (74). Zufferey and Mamoun (77) reported that choline transport into *Leishmania* is inhibited by miltefosine. Miltefosine has also been reported to induce an apoptosis-like death in *L. donovani* wild-type promastigotes (78).

Mechanisms of Resistance

Miltefosine-resistant *L. donovani* strains have been raised in vitro (79). The promastigotes were cross-resistant to edelfosine, but not to standard anti-leishmanial drugs. The resistant mutants were found to be deficient (>95%) in their ability to take up [^{14}C]miltefosine. Binding of the drug to the plasma membrane and efflux from the cells were similar in the resistant and sensitive lines. The resistant promastigotes were also unable to take up other short-chain phospholipid analogs, independent of their polar head group, even though endocytosis remained unaltered. This suggested that a short-chain phospholipid translocase might be downregulated or mutated in the resistant promastigotes (79).

Recently, a putative miltefosine transporter (LdMT) has been cloned by functional rescue using a resistant *L. donovani* strain defective in the inward-directed translocation of both miltefosine and glycerophospholipids. LdMT is a novel P-type ATPase belonging to the partially characterized aminophospholipid translocase (APT) subfamily. Resistant parasites transfected with LdMT regained their sensitivity to miltefosine and also the ability to normally take up [^{14}C] miltefosine and fluorescent-labeled glycerophospholipids. LdMT was shown to localize to the plasma membrane, and its overexpression in *L. tarentolae*, a species nonsensitive to miltefosine significantly increased the uptake of [^{14}C]miltefosine, strongly suggesting that this protein behaves as a true translocase. Both *LdMT*-resistant alleles contained single but distinct point mutations, each of which impaired the transport function thereby explaining the resistant phenotype. These results clearly demonstrate the direct involvement of LdMT in miltefosine and phospholipids translocation in *Leishmania* (80). It has been also observed that *L. tropica* cells overexpressing a P-glycoprotein like transporter are cross-resistant to alkyl-lysophospholipids like miltefosine and edelfosine (81). Thus, greater efflux of the drug by MDR-like transporters could be another mechanism by which *Leishmania* cells get resistant to miltefosine.

2.4.2 Allopurinol

Allopurinol (20 mg/kg per day) in combination with antimonials have been used with some efficacy against VL (82–84). Das et al. report a randomized clinical trial of a combination of pentamidine (half dose) and allopurinol (15 mg/kg) in the treatment of antimony unresponsive cases of VL (59). The combination therapy was found to be more effective in achieving ultimate cure with an added advantage of reduced toxicity in unresponsive cases as compared to full pentamidine dose.

Allopurinol riboside is not phosphorylated by the kinases normally found in mammalian cells and shows little or no toxicity. *Leishmania* on the other hand have a nucleoside phosphotransferase that can catalyze conversion of allopurinol riboside to its 5′-monophosphate. Subsequently, allopurinol riboside 5′-monophosphate is sequentially acted upon by adenylosuccinate synthetase and lyase to form the corresponding adenosine nucleotide analogs, which are incorporated into RNA, thereby conferring anti-leishmanial activity (85, 86). Clinical resistance has not been reported, since this drug has not been used widely.

2.4.3 Atovaquone

Atovaquone, a hydroxynapthoquinone, has been shown to have anti-leishmanial effect in murine models and has been suggested for use as an adjunct to conventional antimony treatment in visceral leishmaniasis (87, 88).

The mechanism of action of atovaquone against *Leishmania* is not known. In *Plasmodium*, atovaquone appears to act by selectively affecting mitochondrial electron transport resulting in inhibition of nucleic acid and ATP synthesis (89, 90). Atovaquone-resistant *L. infantum* promastigotes were selected in vitro by stepwise drug pressure, and showed no cross-resistance to other anti-leishmanial drugs (91). The resistant promastigotes showed decreased ergosterol biosynthesis, increased membrane cholesterol content, and decreased membrane fluidity that were most likely responsible in blocking the passage of atovaquone through the membrane (91).

2.4.4 Paromomycin

Paromomycin (also called aminosidine) is an aminoglycoside antibiotic that is active against *Leishmania* species. A number of clinical studies have been performed in India to test the effectiveness of injectable paromomycin against VL, where the proportion of patients unresponsive to antimonial treatment has steadily increased (92).

Little is known about the mechanism of action of paromomycin. The drug has been shown to affect the RNA synthesis and modify membranous polar lipids and membrane fluidity in *L. donovani* promastigotes (93). *L. donovani* promastigotes resistant to 800 μM of paromomycin were selected by exposing them to gradual increments of the drug (94). These promastigotes did not acquire multidrug resistance. Paromomycin resistance was stable in the absence of the drug in the culture and also remained stable in amastigotes isolated after a passage in mice. The major mechanism of resistance seemed to be due to decreased drug uptake, probably as a consequence of altered membrane composition (94).

2.4.5 Other Drugs

Fluconazole

Treatment with fluconazole, an orally active antifungal azole (200 mg daily for 6 weeks), has been found to be safe and effective in treating cutaneous leishmaniasis caused by *L. major* (95). The mechanism of action of fluconazole against *Leishmania* is not known. In *Trypanosoma cruzi*, fluconazole inhibits the cytochrome P450 enzyme, sterol 14α-demethylase, with consequent loss of normal sterols and accumulation of 14α-methyl sterols (96).

Sitamaquine

Sitamaquine (1 mg/kg per day for 2 weeks) has shown promise as an orally effective agent for the treatment of visceral

leishmaniasis (97) and is currently undergoing phase III trials (98). *L. tropica* amastigotes showed cytoplasmic condensation when exposed to sitamaquine (20) and oxidation of hemoglobin has also been reported (99).

α-Difluoromethyl Ornithine

It has been observed that modulation of the polyamine biosynthetic pathway by inhibitors like α-difluoromethyl ornithine (DFMO) can be leishmanicidal (100, 101). DFMO is a suicide inhibitor of ornithine decarboxylase, the rate-limiting enzyme in the polyamine biosynthetic pathway (102). Although DFMO was used successfully against sleeping sickness (103), it has never been used clinically against leishmaniasis. In vitro resistance to this ornithine analog has been reported (101, 104). DFMO resistance has been shown to be associated with increased ornithine decarboxylase activity (104) and unstable amplification of two extrachromosomal elements (105). Bis(benzyl)polyamine analogs, like MDL27695, that condense DNA were able to inhibit *L. donovani* promastigote growth in vitro (106). Thus, drugs that manipulate the polyamine biosynthetic pathway might be used in alternate therapy for leishmaniasis.

Antifolates

Although antifolates are not used clinically against leishmaniasis, in vitro study shows that the anticancer drug methotrexate (MTX) has considerable potential against leishmaniasis. Also new drug targets have been identified by studying MTX resistance in vitro.

Mechanisms of Action

Antifolate, like MTX, is a specific inhibitor of dihydrofolate reductase (DHFR). DHFR is the key enzyme for providing reduced folates to the cell. Reduced folates are utilized as cofactors in a variety of one carbon transfer reactions, e.g., in the synthesis of thymidylate. In plants and protozoans, DHFR is fused to thymidylate synthase (TS) resulting in a bifunctional DHFR-TS enzyme (107, 108). DHFR reduces DHF to tetrahydrofolate (THF) that is next converted to dTMP by TS. MTX is an analogue of dihydrofolate (DHF). Competitive inhibition of DHFR by MTX allows TS to run continuously, depleting the cells of THF, thereby inhibiting growth.

Pteridine reductase (PTR1) of *Leishmania* reduces pteridines such as biopterin and folate (109) and it has the potential to act as a bypass and/or modulator of DHFR inhibition under physiological conditions. This suggests why antifolate chemotherapy has not been very successful in leishmaniasis. Therefore, successful antifolate chemotherapy in *Leishmania* will have to target both DHFR and PTR1.

Mechanisms of Resistance

The mechanisms of MTX resistance have been dissected by analyzing MTX resistant strains raised by stepwise selection in vitro (108, 110). It has been observed that *L. major* promastigotes, resistant to structurally unrelated drugs like primaquine or terbinafine that produce H-region amplification, are highly cross-resistant to MTX (111). A second MTX-resistant *L. donovani* strain was cross-resistant to aminopterin but just as sensitive to pyrimethamine, trimethoprim, and cytotoxic purine and pyrimidine analogs (112). The first mutation observed was the amplification of the DHFR-TS gene as a part of the R-locus (113). Later, another locus was found to be amplified in response to MTX resistance, which was named the H locus (114). PTR1 was present in the H locus and when overexpressed could reduce DHF to THF, thereby providing resistance to MTX. PGPA overexpression has also been observed in *Leishmania* (115). Another gene *ORF G* was identified by functional cloning in LD1/CD1 genomic locus that is frequently amplified in several *Leishmania* stocks. Overexpression of *ORF G* provided MTX resistance by increasing the uptake of pterins and selectively increasing the uptake of folic acid, but not MTX. This compensated for the mutations in the high-affinity folate/MTX transporter of the resistant mutants. Amplification of DHFR-TS in MTX-resistant *Leishmania* was only observed in *L. major*, but not in any other species. On the other hand, the PTR1 amplification and reduced uptake were observed in all the species selected for MTX resistance in vitro (116). The reason for this discrepancy is unknown.

It has also been shown that *Leishmania* has several folate transporter genes as it is a folate auxotroph. FT5 is a high-affinity folate transporter and MTX transport in a resistant mutant (FT5 null) is inhibited only at low substrate concentrations (50 nM) (117). At other times, it has been observed that MTX-resistant *L. donovani* is genetically deficient in other folate-MTX transporter(s) (112). Polyglutamylation of folates and MTX is an important determinant of MTX susceptibility. Modulation of the folylpolyglutamate synthase (FPGS) responsible for polyglutamylation has recently been found to be responsible for the MTX resistance in *Leishmania*. However, FPGS transfectants were much more sensitive to MTX in folate-deficient medium (118). It has also been observed that presence of shorter glutamate chains on MTX correlated with resistance (118).

Modification of drug targets by point mutations has been implicated in MTX resistance. MTX-resistant *L. major* exhibits an amplification of DHFR-TS along with structurally altered DHFR-TS (119). The altered DHFR revealed a Met53 to Arg substitution. This resulted in a 30-fold increase in the K_i for MTX in the mutant enzyme when compared to the wild type.

3 Spread of Resistance

Leishmaniasis is primarily a zoonotic disease with dogs and wild canids, carnivores, rodents, sloths, anteaters as reservoir hosts. However, VL caused by *L. donovani* in India, China, and East Africa, as well as the CL caused by *L. tropica* in the Mediterranean countries, is anthroponotic. There are no known animal reservoir hosts other than humans for these two parasite species. Therefore, when the parasite becomes resistant to a drug, the resistance spreads quickly and efficiently.

Pentavalent antimonial unresponsiveness is an emerging problem in endemic areas. Therefore, information on factors which could modulate the transmission of drug-resistant phenotypes and parasites during life cycle is necessary. Bhattacharyya et al. (120) reported that wild-type parasites isolated from VL patients, who were clinically cured after treatment with Sb(V), were a mixture of resistant and sensitive cells. The resistant promastigotes were also resistant as amastigotes in vivo. It was further observed that Sb(V)-sensitive parasites could be made resistant to the drug by repeated passages in experimental animals followed by incomplete treatment with suboptimal doses of the drug. These results suggested that the steady rise in Sb(V) unresponsiveness of VL patients in India is because of infection with the resistant parasites, generated as a result of irregular and often incomplete treatment of the patients (120).

Using a continuous drug pressure protocol, Sereno et al. (121) induced pentamidine-resistant *L. mexicana* amastigotes. Two clones with different levels of resistance to pentamidine were selected in vitro – LmPENT5 resistant to 5 μM and LmPENT20 resistant to 20 μM of pentamidine. During in vitro infectivity experiments, axenically grown LmPENT20 amastigotes remained pentamidine resistant, whereas LmPENT5 amastigotes lost their ability to resist pentamidine. These results indicate that the level of pentamidine tolerated by resistant amastigotes after the life cycle was dependent on the induced level of resistance. This fact could be significant in the in vivo transmission of drug-resistant parasites by sand flies. These observations demonstrate that different factors could modulate the transmission of *Leishmania* drug resistance during the parasite's life cycle.

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Chapter 42
Drug Resistance in African Trypanosomiasis

Thomas Seebeck and Pascal Mäser

1 Introduction

Human sleeping sickness has been with mankind from its earliest beginnings, and may actually have contributed to the evolutionary split of the hominids from their primate relatives. Drugs against sleeping sickness were among the first targets of the new art and science of chemotherapy that was initiated by Paul Ehrlich and his collaborators at the turn of the last century. Ironically and tragically, sleeping sickness drugs have steadily fallen back on the list of priorities of drug development ever since. In contrast, the disease has rebounded, from its comparative obscurity in the sixties of the last century, to the rampant epidemics that devastate several African countries and threaten scores of others today. The treatment of this re-emerging killer disease is still completely dependent on drugs that were developed between 30 and 80 years ago, none of them satisfactory, all of them toxic, all of them impractical – but all of them in daily use because of the sheer lack of alternatives. The chemotherapy of human sleeping sickness with all its plights has been extensively reviewed in many excellent articles (e.g. (1–6)). The current text, besides summarizing the status quo, also intends to touch on the ever-increasing problem of drug resistance, and will give an outlook into a future that might harbour some justifiable hope for improvement.

2 Mechanisms of Action of Currently Used Drugs

2.1 *Suramin (Germanin®, Bayer)*

Suramin is a derivative of trypan red, the dye originally used by Paul Ehrlich in his pioneering experiments on trypanosome chemotherapy (7). The compound was first clinically tested against human sleeping sickness in 1921 and has stayed the drug of choice against East African *T. rhodesiense* infections ever since.

Suramin (Fig. 1) has a very long elimination half-life (44–54 days), and it is excreted predominantly via the urine. It is almost completely bound to plasma proteins (>99%), and its concentration in the cerebrospinal fluid (CSF) is vanishingly small even at high plasma levels (8). Suramin is the drug of choice for treating stage I of *T. rhodesiense* infections. It is usually given by intravenous injections at 20 mg/kg body weight in a series of five injections at 5–7 day intervals. While generally effective, suramin treatment suffers from a significant relapse rate (9). Its use is inconvenient because it has to be given by intravenous injection, and it is plagued by numerous side effects such as fever, emesis, mucocutaneous eruptions, polyneuropathy, haematological toxicity and, in extremis, renal failure that can be fatal. A disconcerting observation is that large-scale suramin use for veterinary purposes in the Sudan has induced a solid and genetically stable resistance to this drug in *T. evansi* (10). While a direct genetic transfer of resistance from *T. evansi* to *T. brucei* is unlikely, the phenomenon highlights that trypanosomes are able to acquire suramin resistance given the appropriate selective pressure, and that this resistance remains genetically stable even when the drug pressure disappears.

The mechanism of action of suramin against *T. brucei* has not been pinpointed. This is not for the lack of trying, but may reflect the fact that suramin does very many things to a cell. Its lethal effect on trypanosomes most likely is due to the sum of many, often rather non-specific interactions with many different subcellular systems. The full scale of this pleiotropism of suramin has become evident as the compound was scrutinized for clinical applications other than the treatment of sleeping sickness. Suramin is now a well-studied antagonist for both the P2X and the P2Y adenosine receptor families (11–14). It interacts with the α- subunit of heterotrimeric G proteins and interferes with signal transduction through $G_{(s)}$, $G_{(i)}$, $G_{(o)}$ and $G_{(t)}$. Suramin binding slows down

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Suramin (Bayer 205, Germanin)
1917

Pentamidine
1939

Melarsoprol (MelB, Arsobal)
1947

Melarsen oxide

α-difluoromethyl-ornithine (eflornithine, DFMO)
1978

Fig. 1 Drugs currently in use for the treatment of human sleeping sickness. For each compound, the year of its first publication is given

the interaction of the G-protein with G-protein coupled receptors, thus acting as a receptor antagonist (15, 16). Another mode of action of suramin is that it binds to the calmodulin-binding sites of the ryanodine receptor of skeletal muscle cells and induces a voltage-gated potentiation of Ca^{2+} release (17). The effect of suramin as an inhibitor of reverse transcriptases has given the drug, at least temporarily, the prominence of a potential anti-HIV drug (18). While more effective and specific drugs have since been synthesized, the effect of suramin as a reverse transcriptase inhibitor may explain why sleeping sickness patients occasionally develop neuropathies after suramin treatment. Inhibitors of reverse transcriptase activity affect mitochondrial functions (19) which might be the underlying reason for the marked apoptosis in dorsal root ganglia that suramin can induce (20). In mammalian cells, suramin was shown to accumulate in the lysosomes to >150 μM and may eventually induce lysosomal storage pathology (21). Furthermore it inhibits nucleoside triphosphate binding to the ribosomal protein L3 (22) and a human brain ganglioside sialidase (23). Recently, suramin has gained interest as a potential antifertility agent through the observation that it binds to sperm proacrosin and inhibits the interaction of the sperm with the zona pellucida glycoproteins of the egg (24, 25). Intense research activity on suramin has also been triggered by the observation that it acts as an anti-angiogenic compound with carcinostatic activity (4, 26, 27). Here, the mode of action is a tight binding of suramin to several growth factors such as fibroblast growth factor 2 (FGF2), preventing their interaction with the appropriate receptors on the target cell (28–30). By similar mechanisms, suramin also ‘normalizes’ the phenotype of many tumour cell lines by preventing the FGF-mediated downregulation of lysyl oxidase expression (31), and it offers the potential to be a locally applied drug to prevent restenosis of human arteries (32). Interestingly, suramin similarly inhibits a peptide factor (systemin) that is involved in signalling in tomato cells. It binds to the peptide and inhibits its interaction with the respective cell surface receptor (33).

After this brief, and with certainty incomplete, outline of the many actions of suramin, it is up to the reader to pick his or her favourite mechanism of action of suramin against trypanosomes.

2.2 Pentamidine (Pentamidine Isethionate, Aventis)

Pentamidine (Fig. 1) was introduced as a trypanocide in 1941 and remains the drug of choice against stage I *T. gambiense* infections (34). It is also effective against *T. rhodesiense*, but the efficacy is much more variable. This might be explained by the fact that the CNS appears to be invaded in *T. rhodesiense* infections much earlier than in *T. gambiense* infections. The drug is currently supplied by the producer, Bayer AG, free of charge to WHO in ampoules containing 200 mg pentamidine isethionate per vial. A similar formulation is sold under the brand name of Pentacarinat® for infections other than sleeping sickness. The drug is given by intravenous or intramuscular injection at 2–4 mg/kg/day. Injections are given daily for 7–10 consecutive days. As maximal plasma levels are reached already 1 h after intramuscular injection and the elimination half-life is very long (weeks), the drug accumulates considerably during the injection regime. The slow clearance of pentamidine may be due to an intrinsically low turnover rate by the microsomal cytochrome P-450 oxidases (35–37). Pentamidine is also found in the CSF, though at much lower levels (0.5–0.8%) than in the plasma. Despite this poor penetration of pentamidine through the blood-brain barrier, old and largely forgotten observations have reported considerable success of the drug against early stage II-trypanosomiasis (38, 39), and renewed efforts are currently under-way to re-explore this (6, 40, 41). In the plasma, about 70% of the drug is bound to plasma proteins. The compound is taken up by the trypanosomes via membrane transporters (42–45), but other routes of entry may also exist. Intracellularly, the drug accumulates to millimolar concentrations (43), which might lead to the more or less non-specific interference of the drug with many different subcellular systems. Interestingly, and unlike other clinically used trypanocides, pentamidine has never (up to the present) posed a problem of drug resistance, despite the fact that it was used on a very large scale as a prophylactic in the sixties of the last century (46, 47). However, from an epidemiological standpoint, this may just represent a case of temporary good luck, since trypanosomes can easily be rendered resistant by genetic manipulation, e.g. by deleting the TbAT1 transporter of T. brucei (45). In addition, experiments with *Leishmania major* have demonstrated that the overexpression of the ABC transporter PRP1 leads to pentamidine resistance in this parasite (48). A second potential mechanism of pentamidine resistance was recently observed in *L. mexicana*, where pentamidine-resistant strains selectively excluded pentamidine from their mitochondria (49). Given these observations, the appearance of a mutation that confers pentamidine resistance to *T. brucei* may just be a matter of time.

The mechanism of action of pentamidine is still unclear. As pointed out for suramin, the trypanocidal effect may reflect the sum of many non-specific actions, rather than a single, specific killer mechanism. This is all the more likely as pentamidine can accumulate to high concentrations within the cells (see above). Pentamidine is a nucleic-acid-binding drug that can form tight complexes in the minor groove of AT-rich regions of double-stranded DNA (50). In hindsight, it is a case of pharmacohistorical good luck that pentamidine, unlike other minor groove binders, is not a mutagen (51). Nucleic acid binding may also be responsible for the interference of pentamidine with intron self-splicing in *Candida albicans* (52), and for its inhibitory action on yeast mitochondrial intron splicing and on translation (53). Apart from interacting with DNA, pentamidine can also interfere with other cellular mechanisms. It is a well-characterized agonist of the *N*-methyl-*D*-aspartate (NMDA) glutamate receptor and of the delta2-receptor that is related to the NMDA receptors, but is not gated by glutamate (54, 55). A very different aspect of pentamidine is that it inhibits the PRL family of oncogenic phosphatases and exerts anticancer activity. Pentamidine suppresses the growth of human melanoma cells in nude mice and may represent a lead compound for novel tumour therapeutics (56).

2.3 Melarsoprol (MelB, Arsobal®, Aventis)

Melarsoprol (Fig. 1) was introduced in 1947 for the treatment of human sleeping sickness after about 12,000 arsenical compounds had been synthesized and screened for their activity against human sleeping sickness (57). Since these pioneering days, it has remained the one and only drug for treating stage II infections of both *T. gambiense* and *T. rhodesiense* sleeping sickness. Melarsoprol has gained this prominence not by its outstanding pharmacological properties, but by the sheer lack of alternatives. In fact, melarsoprol is probably one of the most unlikely and unsuitable drugs ever to be unleashed onto mankind. Its active principle is a highly toxic trivalent arsenic compound. Trivalent arsenicals had originally been developed as vesicant war gases during World War I (e.g. 2-chlorovinylarsinedichloride, Cl(CH = CH)$AsCl_2$; Lewisite). The toxicity of the basic melaminophenylarsine core is such that it had to be moderated by inclusion of a dimercaprol residue into the final formulation, to form melarsoprol (MelB). Dimercaprol itself was developed during World War II as an antidote to Lewisite (58), but remained

problematic due to its own high toxicity (59) that can lead to convulsions and coma. To complete the picture of an ugly drug, the water-insoluble melarsoprol is supplied in propylene glycol. Propylene glycol, used otherwise as an antifreeze and engine coolant, is a powerful irritant that often causes thrombophlebitis and severe necrosis at the injection site if the liquid is injected into the tissue surrounding the vein (60). Whenever this happens, such an incident precludes the use of this site for another injection for an extended period (154). The drug is currently produced by Aventis and supplied free of charge to WHO in ampoules containing 180 mg active compound in 5 ml propylene glycol. The standard treatment scheme varies from one country to another, but is essentially based on three to four treatment series spaced by 8–10 days. Each series consists of three consecutive days of slow intraveneous injection of 3.3 mg/kg/day of melarsoprol. Recently, a promising abbreviated treatment schedule has been developed that consists of a single series of injections of 2.2 mg/kg/day for ten consecutive days. This reduces drug use by about 30%, and the treatment time from 26 to 40 days to just 10 days (61). The therapeutic success rate, as well as the extent of side effects, was comparable for both schedules. The major side effects of melarsoprol therapy are myocardial damage, hypotension, exfoliative dermatitis and, most serious of all, reactive encephalitis. This occurs in 5–10% of the treated patients, and its case fatality can be up to 50%. The incidence can occur at any time during the treatment, and no reliable advance warning signs have been identified. The countermeasures are the immediate stop of further melarsoprol treatment, plus the massive use of corticosteroids and anticonvulsants (62).

Despite all its serious side effects, melarsoprol is a fairly successful drug, and the rate of treatment failures has remained consistently low (3–9%) over many years. Disconcertingly, over the last decade, an alarming increase in the rate of treatment failures has been observed in several regions, such as Angola, Southern Sudan, the Republic of Congo, and Northern Uganda (6, 63–65).

At the present time, the underlying reasons for melarsoprol resistance are not clear. Merlarsoprol is taken up into the cells by the adenosine/adenine transport activity P2, encoded by the gene *TbAT1* (44, 66–68), and deletion of the *TbAT1* gene results in increased melarsoprol resistance both in culture and in the mouse model (45). A trypanosome strain selected in the mouse by subcurative doses of cymelarsan lacked P2 activity (66). A similar strain derived independently and by a different protocol (69) was shown to have accumulated a series of inactivating mutations within *TbAT1* (68). Subsequently, *TbAT1*^{R} alleles were shown to be significantly more prevalent in isolates from melarsoprol relapse patients than from patients newly infected with *T. gambiense* (65). In vitro, melarsoprol resistance was also obtained by the overexpression of an ABC transporter, TbMPRA (70). The relevance of these various in vitro resistance phenotypes for the increased relapse rate after melarsoprol treatment observed in the field is unclear. Many of the field isolates show no increased melarsroprol resistance when tested in cell culture.

The mode of action of melarsoprol is not clear. As already shown for suramin and pentamidine, melarsoprol action on the trypanosomes may reflect the sum of numerous more or less non-specific actions on different enzymes. Trivalent arsenic can add to sulfhydryl groups and inactivate a large number of enzymes (71). However, melarsoprol-mediated toxicity to the cells may exhibit at least some degree of specificity as suggested by a comparison of the toxic effects of melarsoprol and arsenic trioxide on human cells. Arsenic trioxide, but not melarsoprol, inhibits pokeweed-mitogen induced differentiation of B cells to plasma cells (72). Similarly, arsenic trioxide has no effect on either cell proliferation or bcl-2 expression in different lymphoid or myeloid cell lines. In contrast, melarsoprol dramatically decreases bcl-2 expression and induces apoptosis in these cell lines (73).

2.4 α-Difluoromethylonithin (DFMO, Eflornithin, Ornidyl®, Aventis)

DFMO (Fig. 1) is an irreversible inhibitor of ornithine decarboxylase. Inhibition of this enzyme blocks polyamine synthesis and disrupts the intracellular polyamine homoeostasis which is critical for cell survival (74). DFMO was first synthesized in 1978 (75), and its antiproliferative properties were recognized (76). The compound was then explored for cancer chemotherapy, and simultaneously its potential as a new trypanocidal drug was recognized (77). This represented the first new trypanocidal drug since the introduction of melarsoprol more than 30 years earlier! Despite all the problems associated with the drug (prohibitively high cost, difficult application and a host of side effects), it has remained the major (the only) back-up drug for the treatment of *T. gambiense*-infected patients who relapse after melarsoprol treatment (2, 78, 79). While very effective against *T. gambiense*, DFMO is not useful for *T. rhodesiene* infections (80). The reason appears to be a much higher ODC enzyme activity, and a much faster turnover of the enzyme in *T. rhodesiense,* a situation similar to that in mammalian cells (81).

The standard treatment schedule consists of a continuous treatment over 14 successive days. The drug is given intravenously every six hours at a dosing of 100 mg/kg. This amounts to about 300 g (!) of active substance per patient, a logistic and financial nightmare *par excellence*.

3 Cross-Resistance Analysis

The concept of cross-resistance analysis was introduced by Paul Ehrlich at the beginning of the last century. By repetitive subcurative treatment of infected mice, Ehrlich and co-workers selected drug-resistant trypanosomes for all known trypanocides of the time (82). Based on the drug sensitivity profiles of their resistant cell lines, they were able to identify three major phenotypes of drug resistance: (I) Resistance to trypan red and trypan blue (Fig. 2), two ancestors of suramin (Fig. 1); (II) resistance to the triphenylmethane dye parafuchsin (Fig. 2); (III) resistance to atoxyl, the first organic arsenical of therapeutic use (Fig. 2). Ehrlich proposed that every newly identified trypanocide be tested against drug-resistant reference strains of types I, II, and III in order to classify it. In cases where a novel compound was active against all strains, a new drug-resistant strain was to be produced for further reference. Thus cross-resistance analysis would serve as a 'therapeutic sieve' reflecting common pharmacologic properties of drugs. Such a sieve would provide a platform for the combination of compounds to formulations that minimize the risk of drug resistance. Unfortunately, a rational approach to prevent drug resistance in protozoan parasites by combinatorial chemotherapy was not implemented until resistance had become a major public health problem (83).

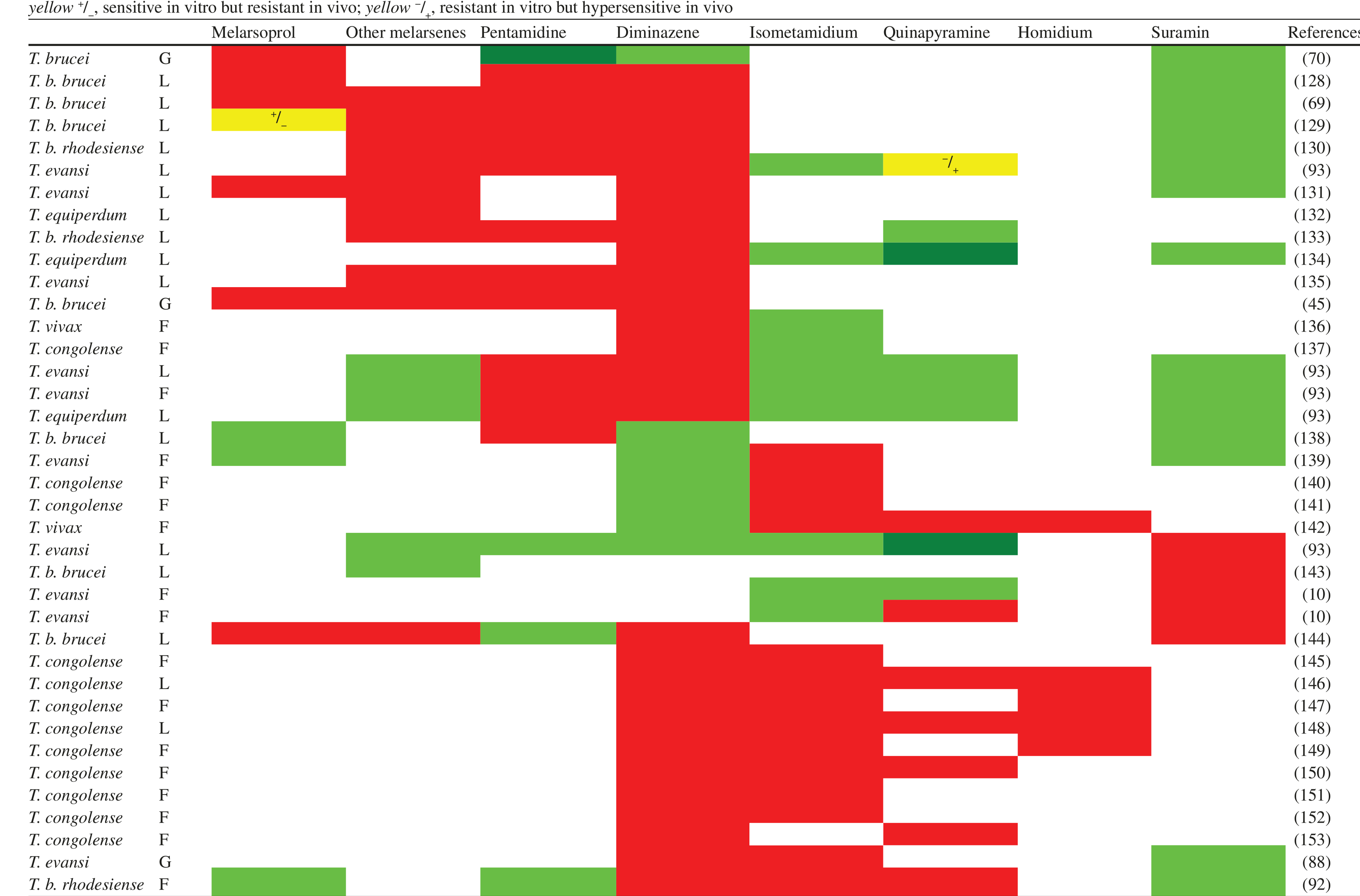
Today, cross-resistance analysis with African trypanosomes is hampered by the fact that many drugs used during the first heydays of trypanosome research at the beginning of the twentieth century are not available any more. Tryparsamide (Fig. 2) was used against late-stage west-African sleeping sickness, but eventually became useless due to the spread of resistant *T. b. gambiense*. It was replaced by melarsoprol and is no longer produced, as are atoxyl, parafuchsin, trypan red, or stilbamidine (Fig. 2). Scientifically, this cleft between old and recent studies is all the more regrettable as the former were often performed with extraordinary care and patience. Yorke and co-workers maintained arsenical-resistant *T. b. rhodesiense* continuously for more than 12 years by a series of 1,528 mouse passages, showing that resistance was stable in the absence of drug pressure (84).

Here we focus on cross-resistance analysis of drugs currently in use against African trypanosomes; for former drugs, the reader is referred to (85). Table 1 summarizes the cross-resistance phenotypes reported from drug-resistant field isolates as well as laboratory-bred stocks. The main conclusions from Table 1 are:

1. There are at least six different patterns of cross-resistance in African trypanosomes, reflecting different drug resistance genotypes. Not all of the cross-resistance patterns exhibited by laboratory-induced resistant mutants were found in field isolates, and vice versa.
2. Cross-resistance between diamidines and melamine-based arsenicals was observed repeatedly. This association is particularly important since it might explain the spread of melarsoprol resistance that is postulated for

Fig. 2 Older sleeping sickness drugs that are no longer in use

Table 1 Cross-resistance studies with African trypanosomes. *F*, field isolate; *G*, genetically engineered stocks; *L*, laboratory-bred stock; *red*, resistant; *green*, sensitive; *dark-green*, hypersensitive; *yellow* $^+/_-$, sensitive in vitro but resistant in vivo; *yellow* $^-/_+$, resistant in vitro but hypersensitive in vivo



		Melarsoprol	Other melarsenes	Pentamidine	Diminazene	Isometamidium	Quinapyramine	Homidium	Suramin	References
T. brucei	G									(70)
T. b. brucei	L									(128)
T. b. brucei	L									(69)
T. b. brucei	L	$^+/_-$								(129)
T. b. rhodesiense	L									(130)
T. evansi	L						$^-/_+$			(93)
T. evansi	L									(131)
T. equiperdum	L									(132)
T. b. rhodesiense	L									(133)
T. equiperdum	L									(134)
T. evansi	L									(135)
T. b. brucei	G									(45)
T. vivax	F									(136)
T. congolense	F									(137)
T. evansi	L									(93)
T. evansi	F									(93)
T. equiperdum	L									(93)
T. b. brucei	L									(138)
T. evansi	F									(139)
T. congolense	F									(140)
T. congolense	F									(141)
T. vivax	F									(142)
T. evansi	L									(93)
T. b. brucei	L									(143)
T. evansi	F									(10)
T. evansi	F									(10)
T. b. brucei	L									(144)
T. congolense	F									(145)
T. congolense	L									(146)
T. congolense	F									(147)
T. congolense	L									(148)
T. congolense	F									(149)
T. congolense	F									(150)
T. congolense	F									(151)
T. congolense	F									(152)
T. congolense	F									(153)
T. evansi	G									(88)
T. b. rhodesiense	F									(92)

Diminacene aceturate (Berenil)

Homidium bromide (Ethidium bromide)

Isomethamidium chloride (Samorin)

Quinapyramine

Fig. 3 Veterinary drugs used for animal trypanosomiasis

several *T. b. gambiense* foci (86). Cross-resistance between melaminyl arsenicals and diamidines can be caused by the loss of function of TbAT1 (see Sect. 2).

3. Samorin and berenil (Fig. 3), once termed a sanative pair (87), can no longer be regarded as such. Isometamidium and diminazene cross-resistant isolates of *T. brucei* as well as *T. congolense* have been reported from 1989 onwards. Recently, it has been found that RNAi-mediated silencing of the adenosine transporter gene *TevAT1* reduced the sensitivity to berenil and samorin in *T. evansi* (88).

It is regrettable that human-pathogenic trypanosome isolates are not usually tested for their resistance against veterinary drugs, and vice versa. This would be particularly important considering that in the tsetse fly midgut, human-pathogenic trypanosomes can become exposed to veterinary drugs upon blood-meals on treated cattle. Multiple cycles of infection of animals containing varying levels of veterinary trypanocides might constitute a fertile breeding ground for selecting trypanosome strains that are cross-resistant against human sleeping sickness drugs. The resistance genes acquired in this process might then spread through local or regional trypanosome populations through genetic exchange mechanisms. The entire process may completely escape observation until the silently acquired resistance suddenly turns up as an acute clinical problem.

4 Mechanisms and Spread of Drug Resistance

The spread of drug resistance can be viewed as the origination, multiplication, and dissemination of a drug resistance genotype within a population of parasites that are themselves proliferating in several populations of different hosts. In order to be successful, a drug resistance genotype has to (I) confer a selective advantage to its carrier, (II) be passed on to the carrier's descendants, and (III) confer an advantage to the descendants as well. Trivial as they are, these three conditions can limit the spread of drug resistance in parasites with multistage life-cycles involving different host species, as is the case for African trypanosomes.

4.1 Origin of Drug Resistance

The probability of spontaneous origination of a drug resistance genotype is proportional to the total number of parasites in a given focus times their genetic plasticity. The genetic plasticity of African trypanosomes in the mammalian host is very high with respect to homologous recombination of surface glycoprotein genes and antigenic variation. Concerning drug resistance, however, experimental observations suggest a

comparably low mutation rate of trypanosomes. It appears to be harder to select for drug-resistant trypanosomes than it is for other protozoan parasites. *T. brucei* have a very low propensity for episomal amplification of DNA segments, a mechanism that is frequently observed in drug-resistant *Leishmania* (89, 90). So far, the only example known from trypanosomes is the inosine-monophosphate dehydrogenase gene that was amplified tenfold in *T. b. gambiense* selected for resistance to mycophenolic acid (91). This was achieved at a high cost, since the whole chromosome carrying the *IMPDH* locus was multiplied, resulting in an increase of nuclear DNA by 70% (91). Target amplification at the level of DNA is therefore not likely to contribute to drug resistance in African trypanosomes in the field.

Point mutations in the adenosine transporter gene TbAT1 are thought to cause drug resistance in *T. brucei* (65, 68). Surprisingly, almost identical sets of coding and silent point mutations were found in resistant laboratory strains and in field isolates from different areas. This makes an independent origination of the individual mutations highly improbable; however, the origin of the mutant *TbAT1* alleles remains a mystery. Hopefully, the recent completion of the *T. b. brucei* genome project will help in solving this puzzle. Retrotransposons are an evolutionarily important factor in many aspects of genome plasticity. The *T. brucei* genome harbours numerous retrotransposons such as ingi or rime, but whether their contribution to genetic plasticity is relevant for the origination of drug resistance remains to be investigated.

The origination of drug resistance is in principal independent of the presence of a drug. However, in the special case where a drug is mutagenic, it will increase the mutation rate of the target cells and make it more prone to developing resistance. Ethidium bromide (Homidium; Fig. 3), which is prophylactically used to protect cattle from Nagana, is known to intercalate to DNA and cause mutations.

4.2 Multiplication of Drug Resistance

Multiplication of a drug resistance genotype depends on the relative selective advantage that it confers to its host cell (conditions I and III, above). This selective advantage is cogently dependent on the details of drug usage, e.g. the proportion of treated hosts in a given focus, or pharmacokinetic parameters of the respective drug, such as its half-life or tissue-distribution inside the treated hosts. A reservoir of untreated hosts strongly diminishes the selective advantage of drug-resistant parasites. *T. b. rhodesiense* cause acute sleeping sickness in humans, but also infect a large number of rodents, ruminants and carnivores without pathogenesis in

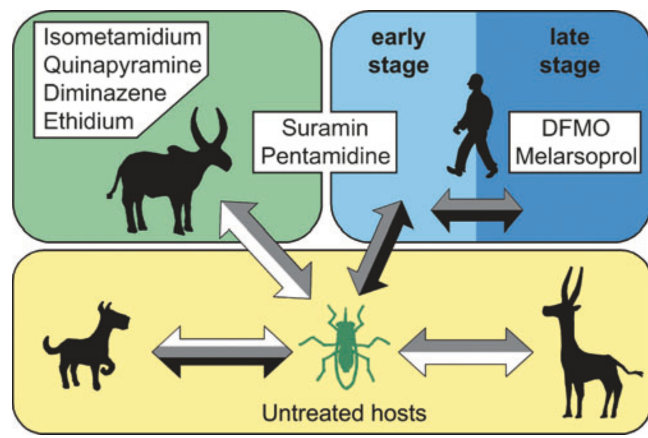


Fig. 4 Drugs and hosts of African trypanosomes. For each host range, trypanocides in current use are indicated. *Green field*: cattle and other domestic animals where trypanosomiasis is controlled by chemotherapy; *blue field*: human sleeping sickness with its early and late stages; *yellow field*: animals that are not treated with trypanocides. The tsetse fly is rendered in *green* to indicate that it too can become exposed to trypanocides, upon feeding on treated cattle. *White arrows*, animal-pathogenic trypanosomes; *grey*, *T. b. rhodesiense*; *black*, *T. b. gambiense* (*See Color Plates*)

wild animals (Fig. 4). In accordance, there has not been any indication of resistance in *T. b. rhodesiense* to human trypanocides. However, a *T. b. rhodesiense* stock resistant to veterinary trypanocides has been isolated from cattle (92).

The second important determinant of the selective advantage is the phenotype of the drug-resistant parasite itself, not only including the resistance factor, but also the cost of resistance. Resistance factors to clinical trypanocides reported for *T. brucei* were rather low. tbat1 null mutants, for instance, were only two- to threefold less sensitive to melarsoprol than wild-type trypanosomes (45). Yet even such a moderate phenotype could cause treatment failures, since the drug levels reached in the CSF of the patients lie in the nanomolar range, not far above the minimal inhibitory concentrations of susceptible trypanosomes. The cost of drug resistance may be paid in the form of chemical energy for expression of a new or enhanced function, or as a metabolic concession for a loss of function. Such costs contribute to the displacement of resistant pathogens by sensitive ones in the absence of selective drug pressure, as frequently observed for prokaryotes. In African trypanosomes, however, drug resistance appears to be remarkably stable (84). Suramin had not been used in the Sudan since 1975, when the drug became obsolete due to widespread resistance and was replaced by quinapyramine. In a survey on *T. evansi* isolates collected between 1993 and 1996, all stocks still showed reduced susceptibility to suramin, with several among them exhibiting resistance factors of over one hundred (10). Stable inheritance of trypanosomal drug resistance was also observed in untreated mice and rabbits (93).

4.3 Dissemination of Drug Resistance

The epidemiology of drug resistance in bacteria is shaped by almost unlimited vertical and horizontal flows of drug resistance genes. In protozoan parasites, the dissemination of drug resistance genotypes is not necessarily granted (condition II). Drug resistance genes may not be passed on because the treated host represents a dead end to the parasite, as for instance man to *Toxoplasma gondii*. Late-stage sleeping sickness patients who are treated with melarsoprol are hospitalized and kept under observance for eventual relapse. Since this regime also protects the patients from tsetse flies, melarsoprol-resistant trypanosomes originating in an individual patient are highly unlikely to spread (Fig. 4). Nevertheless, *T. b. gambiense* melarsoprol treatment failures occur in foci (63, 64), indicative of spreading. A possible explanation for this puzzle is that melarsoprol resistance is often accompanied by resistance to diamidines. Melarsoprol-diamidine cross-resistance genotypes have a selective advantage in hosts that they are able to leave again: Pentamidine-treated first-stage patients, and possibly tsetse flies that feed on diminazene-treated cattle (see Fig. 4). The feeding habits of the fly vectors, and the drug-treatment regime of cattle in a given geographic region may therefore be important factors for the spread of drug resistance.

5 Experimental Drugs

The severely limited array of drugs that are currently available for human sleeping sickness has stimulated a re-investigation of candidate drugs used so far for other purposes. None of them is currently licensed for human use, but limited patient trials on a compassionate basis have been undertaken. In this context, the toxicity of the veterinary drug diminazene aceturate (Berenil; Fig. 3) has been investigated, and the compound was found to be safe for human use. Efforts are also under way for improving drug delivery in humans, such as the synthesis of lipid-drug-conjugate nanoparticles (94) loaded with berenil. Another drug, Nifurtimox (Bayer 2502, Lampit; Fig. 5) is currently used against *T. cruzi* infections in South America, but has also shown activity against *T. brucei*. Its potential use against melarsoprol-refractory *T. brucei* infections through label extension is currently being discussed in detail. The nitroimidazole megazol (Fig. 5) has been used to successfully cure *T. brucei*-infected monkeys (95), and combination treatment of megazol plus suramin was successful in curing *T. brucei*-infected rats (96). New combinations of established drugs are also explored, such as the simultaneous treatment with suramine and DFMO or with pentamidine and DFMO. Older observations that pentamidine can accumulate in the CNS, albeit at low concentrations (38, 39), are being followed up again (6, 40, 41).

6 New Drug Opportunities

Considering all the breathtaking advance of drug development, for anything from curing cancer to lifestyle drugs such as Viagra® and Xenical®, one might ask about the pipeline of new drugs against a deadly disease such as sleeping sickness. The answer is all too evident: the pipeline is empty, and the prospects are mostly bleak. The most evident problem may be on the way of being at least partly resolved – the problem of financing the huge development cost. Several major initiatives have been set in motion over the last few years (e.g. the Medicines for Malaria Venture (www.mmv.org),; the Drugs for Neglected Diseases Initiative (DNDi) (www.dndi.org), the Bill and Melinda Gates Foundation (www.gatesfoundation.org), the drug development program of WHO/TDR (www.who.int/tdr), and the Global Forum for Health Research (www.globalforumhealth.org). They all work differently and have defined different foci of activity, but all are most welcome test beds for exploring the possibilities (and the limits) of public-private partnerships in drug development (97). These developments have paralleled a major shift in thinking in the pharmaceutical industry, in the health providers for the afflicted areas, and in the academic research settings. Mountains of mutual distrust have slowly but steadily been eroded, and a genuine sense of partnership is evident, a realization of a common problem that needs a common effort to be solved.

However, institutional obstacles do not represent the sole difficulties on the way to new and better drugs. Biology, by its own nature, might limit our progress just as severely. The

Megazol Nifurtimox Mycophenolic acid

Fig. 5 Examples of experimental trypanocides

implicit assumption that the elucidation of whole-genome sequences would provide us with essentially unlimited numbers of potential drug targets may not have been more than wishful dreaming. A pharmacogenomic analysis of the human genome, assumed to contain about 30,000 protein-coding genes, arrived at the sobering conclusion that only about 1,000 of these might be suitable for any kind of drug development (98). Everything else being equal, the *T. brucei* genome of about 6,000 genes would leave us with a miserly 200 potential drug targets. Of these, many might be unsuitable as targets for kinetic reasons, as recently exemplified by quantitative mathematical modelling of the glycosomal metabolism. This study concluded that of all the numerous glycosomal enzymes, only inhibition of the pyruvate transporter would significantly affect the overall rate of glycolysis in bloodstream-form trypanosomes (99).

Trypanosome pharmacogenomics will take a considerable step forward since the long-announced completion of the *T. brucei* genome project is finally coming close to realization. Another major step forward has been by the establishment of the Structural Genomics of Pathogenic Protozoa consortium (www.sgpp.org) that aims at high throughput structure determination of protozoal proteins.

6.1 Ongoing Developments

A quick and certainly incomplete glance over ongoing efforts towards drug development shows an amazing variety of approaches and potential drug targets. Though it is difficult to predict which approaches will eventually be successful, we always have to consider that no single drug can be expected to solve the problems posed even by a single parasite. Over the next decade, we will need at least 20–30 effective compounds to control only the most important protozoal diseases, and to delay the onset of resistance against the individual compounds. In principle, a safe way to do so would be to withhold a novel trypanocide from the market until a second, chemically unrelated compound is developed, then combine the pair for chemotherapy. Such concepts are integrated into release policies for transgenic pest-resistant plants. However, for a fatal human disease like sleeping sickness, this approach is not ethically feasible.

6.1.1 Inhibitors of Protein Farnesylation and Myristoylation

Farnesyl transferase inhibitors are interesting antitumour drug candidates. The *T. brucei* farnesyltransferase is an essential enzyme, and its inhibition leads to rapid death of the trypanosome (100). Based on peptide substrates for the enzyme, peptidomimetic inhibitors have recently been developed that inhibit parasite proliferation at low nanomolar concentrations (101). Interestingly, an enzyme immediately upstream of farnesyl transferase, the farnesyl pyrophosphate synthase, is also essential in trypanosomes (102). This offers the possibility of developing combination drugs that act synergistically at different steps of the farnesylation pathway. Bisphosphonates are safe and potent pyrophosphatase inhibitors that are already in extensive clinical use, e.g. against osteoporosis. Representatives of this class of compounds have shown activity against *T. brucei* (103) and may constitute a basis for the development of more potent, trypanosome-specific inhibitors.

On the level of protein acylation, the enzyme that transfers myristic acid to proteins (myristoyl-CoA: protein *N*-myristoyltransferase) was also shown to be essential in trypanosomes (104), and this enzyme may constitute a further target for lethal inhibition of protein modification.

6.1.2 Lipid and Fatty Acid Synthesis Inhibitors

T. brucei acquires its phospholipids partly via scavenging host phospholipids, and via endocytosis of low-density lipoprotein particles. The exogenously acquired phospholipids are then rapidly remodelled in the trypanosomes. On the other hand, *T. brucei* can also synthesize fatty acids de novo, using the type II fatty acid biosynthesis pathway (105). This pathway is completely absent in mammals, and specific inhibitors of this pathway have proven their value as antibacterial drugs. Indeed, *T. brucei* is sensitive to thiolactomycin (106, 107), and other type II fatty acid synthesis inhibitors may constitute a good starting point. Lethal interference with phospholipid synthesis might also be possible via phospholipid analogues, compounds that have originally been developed as anti-cancer drugs (108).

6.1.3 Phosphodiesterase Inhibitors

Phosphodiesterase (PDE) inhibitors with sometimes exquisite specificity against one of the numerous human PDE isoenzymes have been developed into potent (and lucrative) drugs. PDE inhibitors of various PDE subtype specificities are used against a broad spectrum of human ailments such as impotency, asthma, intermittent claudication, and chronic obstructive pulmonary disease, just to name a few. Similar lines of drug development may now lead to a novel class of antitrypanosomal drugs after the demonstration that *T. brucei* contains a number of PDEs that are similar to their mammalian counterparts (109–112), and that at least one of these enzymes, TbPDE2C, is absolutely essential (109).

6.1.4 Metabolic Inhibitors

This area has been extensively studied over several decades, and an enormous amount of structural, kinetic, and quantitative information on the energy-converting, biosynthetic, and redox pathways has been gathered. At the present time, the prospects are good that this wealth of information can finally be transformed into drug development (113–115).

6.1.5 Interference with Protein Glycosylation

Protein glycosylation has been recognized as an essential feature for trypanosome survival, even in the relatively sheltered world of a culture flask. Several enzymes involved in various steps of GPI anchor biosynthesis and protein glycosylation were now shown to be essential for cell survival (116, 117). High-resolution structures of the crucial enzymes are becoming available for structure-based drug development (118), and the feasibility of suicide substrate inhibitors has already been demonstrated (119).

6.1.6 New Diamidines

Pentamidine has served trypanosome treatment for well over half a century. Despite its venerable age, the diamidine class of drugs has been vigorously revived over the last few years. New efforts have been made to synthesize pentamidine congeners with more desirable properties, and with activity against both drug-sensitive and drug-resistant parasites (120, 121). However, the most significant development was the establishment of a large consortium along the lines of public-private partnership, and largely financed by the Gates Foundation, devoted to the systematic development of better diamidines. This approach has proven very successful, and a first prototype drug, DB-289 (Fig. 6), has passed phase IIa clinical trials and is slated for a larger, multicenter phase IIb trial. DB-289 represents a truly new generation of trypanocidal drugs that are administered as prodrug. In this form, it is totally inactive against trypanosomes. In the patient, DB-289 is transported across the gut wall and is then enzymatically converted into the active compound, DB-75 (Fig. 6). DB-289 also seems to be able to cross the blood-brain barrier. Most importantly, a large portfolio of even more active and specific backup drugs have been assembled that can be rapidly moved to clinical trials once DB-289 has furnished the proof of principle in human sleeping sickness patients. The availability of several different compounds with a similar spectrum of activity is also an important contribution to diminish the risk of resistance development.

6.2 *'Non-rational' Drug Finding*

All developments outlined above represent more or less rational approaches to drug development: Identification of an enzyme as a potential drug target, validating this enzyme by showing that it is in fact essential for the trypanosome, screening for a compound that inhibits it reasonably well and specifically, and then improving this lead compound by further developmental chemistry. Despite the undeniable success of this strategy (though not for trypanocides so far), one should not neglect the potential of more traditional approaches, such as screening plant extracts for trypanocidal activities (122–127). The validity of this approach has clearly been highlightened by the finding of artemisinin as an anti-malarial drug. Also, we should not forget that none of the drugs currently in use for human trypanosomiasis (with the possible exception of DFMO) has a clearly defined, single intracellular target. When considering the ever-present specter of drug resistance, a compound that interacts with a multitude of targets 'nonspecifically', to use this no-no word of modern pharmacology, might actually be a better choice than a highly target-specific designer drug.

7 Outlook

Chemotherapy of human sleeping sickness has been on the research agenda for over a century, but with a drastically changing priority status over the decades. Faced with the

In vivo activation

DB-289: Prodrug

DB-75: active compound

Fig. 6 Mechanism of activation of the diamidine prodrug DB-289

disasters caused by sleeping sickness in Africa, the founding father of pharmaceutical chemistry, Paul Ehrlich in 1906 pleaded to his fellow scientists to do their utmost to combat this ravaging disease: 'Es ist die äusserste Pflicht eines jeden der dazu in der Lage ist, diese zerstörerischste aller Pestilenzen zu bekämpfen'. The success of the early trypanocides, many of them from Ehrlich's own cuisine, combined with enormous logistic efforts of the afflicted countries gradually led to the virtual eradication of human trypanosomiasis. In the sixties' of last century, sleeping sickness had all but vanished from public perception. Ironically, this remarkable success, combined with a deteriorating political and economic environment, constituted the beginning of the disaster that sleeping sickness again presents today. Other, seemingly more pressing health priorities withdrew funding and infrastructure from sleeping sickness control projects, and endless civil wars and general insecurity destroyed what was left. In parallel, the interest of the industrialized world in developing urgently needed, more effective tryanocides was all but non-existent. Predictably, disaster struck three decades later, and today human sleeping sickness is ravaging many African countries more savagely than ever. What has remained unchanged is the lack of funding for control programs, in many regions the lack of security that makes control operation hazardous if not impossible, constant wars and banditry, and penniless public health systems overburdened with the concomitant epidemics of malaria, tuberculosis, and HIV. In this bleak outlook, the renewed interest of industry and academia alike in developing new trypanocides may constitute a silver lining on the horizon, however thin and ephemeral. None of all the ongoing research efforts will produce a magic solution to the sleeping sickness problem, but they eventually might be able to help reducing human suffering just a tiny little bit. All considered, this may not be all that little of a success.

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Chapter 43
Drug Resistance and Emerging Targets in the Opportunistic Pathogens *Toxoplasma gondii* and *Cryptosporidium parvum*

Boris Striepen

1 *Toxoplasma gondii*: An Opportunistic Pathogen

T. gondii was initially described as a tropical parasite of wild rodents at the North African dependence of the Institute Pasteur in Tunis (4). Ironically, Paris, the home of Institute Pasteur, has now (and most likely had back then) one of the highest prevalences of *T. gondii* in humans in the world. Whereas the sexual development of the parasite is limited to cats, a large variety of birds and mammals can serve as intermediate hosts. Both oocysts shed by cats with the feces as well as tissue cysts developing in the muscle and brain of the intermediate host are infective to humans (5). Recent studies point to the overall importance of foodborne tissue cysts as a predominant source of infection; however, epidemic outbreaks of toxoplasmosis have been associated with oocyst contamination of soil or drinking water (6, 7).

Despite high seroprevalence (10–55%), clinically overt disease is relatively rare (7–9). However, three conditions associated with *T. gondii* infection have significant clinical importance: toxoplasmosis in immunosuppressed individuals, ocular toxoplasmosis, and congenital toxoplasmosis. Toxoplasmosis in immunosuppressed patients is characterized by the reactivation of chronic infection, leading to significant tissue damage and necrosis due to uncontrolled parasite replication. A variety of organs can be afflicted, but Toxoplasma encephalitis is of greatest clinical importance (1). Toxoplasmosis in immunocompentent patients is most prominent as ocular disease, and has been thought to often represent a late sequel of previous congenital infection; however, evidence for postnatal infections causing ocular disease is emerging (10). Moreover, the analysis of recent waterborne outbreaks has clearly shown that ocular toxoplasmosis can develop in immunologically competent adults.

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Such epidemic outbreaks might be linked to a specific parasite strain genetically predisposed for high (ocular) virulence (11, 12). In any case, the ability of the parasite to reversibly switch from chronic to acute lifecycle stages seems to be of great importance for the development of disease in the presence or absence of a fully functional immune system (1, 13). Finally, primary infection of mothers during pregnancy can lead to the transfer of parasites to the fetus, resulting in congenital toxoplasmosis. In the absence of a mature functional immune system, the parasite can severely damage fetal tissue, and neurological manifestations are again of greatest clinical importance (14).

1.1 Antimicrobial Mechanism of Action

1.1.1 The Current Treatment of Toxoplasmosis

The treatment of choice for symptomatic toxoplasmosis is a combination of pyrimethamine and sulfadiazine, which is highly effective in killing tachyzoites in vitro and in vivo (15, 16). It is important to note that this treatment does not eradicate the chronic infection maintained by encysted bradyzoites. Atovaquone, which acts as a ubiquinone analog targeting the mitochondrial membrane potential, has received some interest for its potential as a drug that might be effective against cysts. Unfortunately, despite promising in vitro results, atovaquone does not seem to affect cysts in mouse models (17–20). The development of sulfonamide hypersensitivity can force the discontinuation of sulfadiazine. In such cases, sulfadiazine has been successfully substituted with azithromycin (21), clarithromycin (21), clindamycin (16), or atovaquone (22) (all in continued combination with pyrimethamine).

Pyrimethamine acts as a competitive inhibitor of the parasite's dihydrofolate reductase (DHFR), which, in *Toxoplasma,* is fused to thymidylate synthase (DHFR-TS) (23). Sulfadiazine interferes at an earlier step in folate biosynthesis and competitively inhibits dihydropteroate synthase

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(DHPS), which is fused to hydroxymethyl dihydropterin pyrophosphokinase in *T. gondii* and many other apicomplexans (24). The combination of both drugs shows strong synergism (15). The main antimicrobial effect of antifolates therapy lies in the starvation of the thymidylate synthase reaction for tetrahydrofolate. Lack of dTMP and the resulting nucleotide pool imbalance effectively block parasite DNA synthesis (methionine synthesis is another important pathway dependent on tetrahydrofolate).

1.1.2 Is Short-Term Treatment Beneficial in Congenital and Ocular Toxoplasmosis?

Screening programs are in place in several European countries to detect primary maternal infection as early as possible during pregnancy. Comparisons of the clinical outcome of confirmed fetal infections treated *in utero* with historic data have suggested that treatment might reduce transmission of the infection to the fetus and limit disease severity (25, 26). Prenatal treatment is initiated with spiramycin immediately after maternal diagnosis, and is followed up with the more effective pyrimethamine and sulfadiazine combination when infection of the fetus has been established (generally pyrimethamine is not used before 12 weeks' gestation, to avoid possible teratogenic effects). More recent retrospective studies have failed to detect a significant beneficial effect of prenatal treatment with respect to the rate of maternal-to-fetus pathogen transmission and fetal disease severity (27–29). The benefit of prenatal treatment remains a point of intense discussion, and a number of technical and statistical differences among the various studies have been pointed to, to explain the different conclusions (30–32) (see (14) for an extensive critical discussion of pre- and postnatal treatment of congenital toxoplasmosis). Further work is needed to resolve these differences. Large randomized, double-blind clinical trials including placebo controls would be most informative, but might face ethical challenges.

Screening and prenatal diagnosis might be beneficial by indicating the need for treatment of the infant after birth. Congenital toxoplasmosis can be inapparent at birth, but symptomatic disease develops months, years, and even decades later in a very significant number of cases (33). Long-term treatment of infants and children with pyrimethamine and sulfadiazine has been shown to be quite effective in the prevention of such sequelae of congential infection (34).

Treatment also remains challenging in the management of ocular toxoplasmosis, which shows a high propensity for relapses and recurrent disease. Relapses occur in up to 80% of all ocular toxoplasmosis patients (35), and are probably due to the local persistence of bradyzoite cysts in the retina. This view is based on the observations that active parasite foci are most often found right adjacent to scars of previous inflammation, and that recurrent disease can be limited to a single eye over many years (13, 35, 36). Pyrimethamine combined with sulfadiazine or azithromycin (which has a lower frequency of adverse drug effects) is effective in eliminating tachyzoites associated with acute episodes (37). However, randomized retrospective studies fail to detect a significant long-term protection against visual impairment by these short-term interventions (35). Longer-term schemes of intermittent treatment have been evaluated, and show modest but detectable efficacy in preventing recurrent disease within the limited follow-up time studied (38).

1.2 Mechanisms of Drug Resistance

1.2.1 Is There Drug Resistance in *Toxoplasma*?

Treatment of toxoplasmosis heavily depends on the antifolates pyrimethamine and sulfadiazine. In *Plasmodium falciparum*, the predominant agent of severe malaria, strong and widespread resistance to pyrimethamine and sulfadoxine has emerged, leading to frequent treatment failure of patients on Fansidar (39). This resistance in *Plasmodium* has been linked to a series of point mutations in the genes for the drug targets DHFR-TS and DHPS (see (39) and chapter of this book for a detailed review). Fortunately, cases of established drug resistance resulting in treatment failure have not yet been reported for *T. gondii*. However, treatment, especially in AIDS patients, is not always fully effective, which could point to a low frequency of resistance developing in individual patients (40–42).

To further investigate the possibility of emerging drug resistance, Aspinall and colleagues amplified and sequenced the DHPS and DHFR genes of numerous clinical *T. gondii* samples (32) of these isolates that originated from human infections (42). Virtually no polymorphism was detected in DHFR, whereas DHPS showed 6 polymorphic codons. One sample (Swa-20) carried an Asn407 to Asp change that was also noted in the analysis of the sulfadiazine-resistant laboratory strain R-Sul^{R}-5 (43). Furthermore, this mutation is at an analogous position to polymorphisms associated with sulfonamide resistance in *P. falciparum* (44, 45). When expressed in *E. coli*, the recombinant *T. gondii* DHPS carrying this mutation was markedly resistant to sulfadiazine inhibition when compared to all other genotypes identified in this study, suggesting that this allele might indeed be associated with resistance (42). The Swa-20 sample was obtained from a fatal case of congenital toxoplasmosis, no sulfonamides were used in the treatment of the mother, and the parasite strain was not isolated at the time (see (42) and Dr. Sims's personal communication). This clinical circumstance could imply that drug-resistant alleles are present in the parasite population at a low frequency in the absence of drug pressure, which might explain occasional treatment failure. A survey of meat

contamination by *T. gondii* from the same investigators did not detect this potentially resistant allele in any of the parasite strains identified in this study (46). It would be most interesting to further substantiate these findings. The availability of excellent reverse genetics for *T. gondii* (47, 48) should make direct experimental evaluation of mutant alleles straightforward. Transfection of the Asp407 allele, for instance, into sensitive wild type should confer sulfonamide resistance in tissue culture. The isolation of parasite lines associated with treatment failure in the clinic would be invaluable in providing firm proof of drug resistance in *T. gondii*.

1.2.2 Drug-Resistant Parasites Are Easily Generated in the Laboratory

No confirmed case of clinical pyrimethamine resistance has been detected in *T. gondii* so far. However, this is not due to fundamental differences of the *T. gondii's* folate pathway or DHFR gene. Fully drug-resistant tachyzoites have been isolated by random mutagenesis of the *T. gondii* DHFR-TS gene, followed by transfection and pyrimethamine selection in the parasite (49). Also, transfection of *T. gondii* with alleles of its DHFR-TS gene carrying point mutations associated with resistance in *Plasmodium* generated strong resistance in vitro and in vivo equally (50, 51). In the drug-resistant M2M3 DHFR allele, now widely used as selection marker (50, 52), Ser36 is changed to Arg and Thr83 to Asn, mirroring one of the most common *P. falciparum* alleles found in Fansidar-resistant strains (Arg59 and Asn108, *P. falciparum* numbering will be used in the following) (39, 53, 54). This *T. gondii* allele also confers pyrimethamine resistance to *Plasmodium falciparum* when used as heterologous transgene in transfection experiments (55). The similarity between DHFR from *Toxoplasma* and *Plasmodium* has since been exploited to model the development of apicomplexan pyrimethamine resistance in *T. gondii*, where genetic manipulation of the parasite is more convenient (56). A detailed genetic and biochemical study of *T. gondii* explored a number of mutations, either individually or in combination (57). As predicted by field data from *P. falciparum*, Arg36 on its own did not confer resistance, but considerably enhanced the initial resistance derived from the Asn83 mutation.

Interestingly, the *T. gondii* study also provided a compelling reason why Val16/Asn 108 was never observed in the field; this enzyme is not active, and fails to complement the *E. coli fol* mutant. Equally, kinetic analysis revealed that the highly drug-resistant Ser223 mutation results in an enzyme with an unfavorable K_{cat}, potentially explaining why this mutant was viable under laboratory conditions but not represented among field isolates (57, 58). This hypothesis has recently been further substantiated by allelic replacement of the DHFR-TS wild-type locus with genes carrying either the Arg59/Asn108 or the Arg59/Ser223 mutation. Both transgenics were highly resistant to pyrimethamine, and showed little difference when growth was measured over the window of time afforded by routine tissue culture assays. However, using sensitive direct competition assays following wild-type and mutant lines over longer periods of time, Fohl and Roos were able to demonstrate differences in fitness (59). Interestingly, and consistent with field observations, these effects were more pronounced in a mouse model of toxoplasmosis than in tissue culture.

Clindamycin represents a third case of a clinically used drug in which a mechanism for potential resistance has been demonstrated for *T. gondii* within the confines of the laboratory. Clindamycin is a macrolide antibiotic and interferes with transpeptidation at the large subunit of the prokaryotic ribosome. Pharmacological and genetic experiments have linked the activity of this drug and several other antibiotics to the presence of the apicoplast in *T. gondii* (60–63). The apicoplast, a vestige chloroplast, is the product of an event of secondary endosymbiosis early in the evolution of Apicomplexa (64–66). Due to their prokaryotic origin, the rRNA encoded by the plastid genome is susceptible to macrolide inhibition (60). Clindamycin-resistant parasites have emerged from previously sensitive strains in tissue culture under drug pressure, both with and without mutagenesis (67). Sequence analysis of drug-resistant clones revealed a G to U point mutation at position 1857 within the apicoplast large-subunit rRNA. Comparison with *E. coli* indicated that this region of the RNA is responsible for clindamycin binding (67–69). Interestingly, these clones also exhibited limited cross-resistance with chloramphenicol and azithromycin.

1.3 Why Is There Not More Drug Resistance in the Clinic?

Drug resistance is rampant in *Plasmodium*. If simple point mutations confer resistance in *T. gondii* to several widely used drugs, why do we not see more clinically apparent resistance in this infection? The reason probably lies in differences in numbers and lifecycles. The overwhelming majority of *T. gondii* infections are asymptomatic and will not be treated, thus limiting selection pressure at the population level. Also, human infection accounts for only a fraction of the *T. gondii* "habitat"; infection of a diverse collection of wild and domestic animals that are never treated generates vast refugia, counteracting the development of resistance. Finally, the lifecycle of *T. gondii* does not include human-to-human transmission, preventing the spread of a resistant subpopulation in an environment with high selection pressure (like the AIDS patient pool). However, the ease with which drug resistance was generated in the laboratory, suggests that resistance on the level of individual patients could emerge, which might

become important in long-term treatment and prophylaxis regimens for opportunistic and ocular toxoplasmosis.

1.4 Alternative Agents

1.4.1 The Challenge of a Dormant Foe

The most important goal in *Toxoplasma* drug development remains the identification of drugs able to fully eradicate the infection. Encysted bradyzoites, which are resistant to currently used treatments, are the source of dangerous relapses in all clinical manifestations discussed above. Not unlike the challenges faced with tuberculosis, attacking the dormant bradyzoites has proven much harder than killing the proliferating acute stages. The slow growth rate in itself might be the main obstacle; alternatively, the cyst "wall" surrounding bradyzoites could limit drug access; finally, there might be pronounced differences in the metabolism between the tachyzoite and the bradyzoite (70, 71). The finding that bradyzoites use enzymes for segments of their carbohydrate metabolism not found in the tachyzoite suggests that metabolic differentiation indeed occurs (72–75). Bradyzoites express a wider set of stage-specific genes, and expression control seems to be mostly (but not exclusively) at the transcriptional level (71). Emerging mechanistic studies interestingly link this control to elements of conserved transcriptional stress response pathways (76, 77). Microarray technology combined with the almost completed *T. gondii* genome should provide a powerful tool to map out further stage-specific pathways (78). Likewise, parasite mutants unable to differentiate into bradyzoites could provide further insights into the mechanisms behind *T. gondii* dormancy (79, 80). The identification of a "drugable" pathway critical to bradyzoite metabolism and survival could provide an important stimulus to drug development. Powerful high throughput assays are available to screen for compounds against the tachyzoites stage (81, 82), and technology that permits similar assessment of the efficacy in the dormant stage is urgently needed.

2 Cryptosporidiosis: A Widespread Disease in Developing and Industrialized Countries

Cryptosporidium parvum has emerged as one of the most troublesome waterborne infections in the industrialized world. A number of large outbreaks have occurred in the U.S., with the largest in Milwaukee causing 403,000 cases of acute gastrointestinal diseases (83, 84). The resistance of the infective oocyst stage to standard water treatment poses a significant challenge to disease prevention, and has also heightened biodefense concerns. Cryptosporidiosis is also an important factor in severe diarrheal disease in children in developing countries (85, 86).

C. parvum differs from *T. gondii* in that it has a single host lifecycle. Infection occurs through ingestion of oocysts. After passage through the stomach, 4 sporozoites emerge from the oocyst and invade epithelial cells in the middle and lower small intestine. Two cycles of asexual schizogony lead to the formation of micro- and macrogametes and fertilization. Meiosis of the zygote finally results in oocysts, which are shed with the feces (87). *C. parvum* can complete this lifecycle in a variety of mammals, including humans. Human infection occurs by oocyst contamination of food and, more importantly, of drinking and recreational water. These oocysts can originate from animals (mainly cattle) and humans. Extensive genetic work has now firmly established that human infection results from two distinct genotypes of *C. parvum* (88): genotype one only infects humans, whereas genotype two infects a large variety of mammals, including cattle and humans, and is the source of zoonotic infections. Recently, it has been proposed that the two genotypes are actually two different species, and several authors now use *C. hominis* as a new name for the parasites restricted to humans (89). The two-species model has been challenged by pointing to patterns of polymorphisms in two genes, which were interpreted as signs of genetic recombination and hence sex between the genotypes (88). For the moment, the taxonomy of *C. parvum* remains contentious and in flux, and the complete genome sequence for both genotypes/species should be an important resource to resolve this issue (90, 91) (see (88, 92) for further discussion and reference on *C. parvum* taxonomy and population genetics).

Cryptosporidiosis is a gastrointestinal disease, characterized by watery diarrhea, abdominal cramps, nausea, and fever (93). The disease is self-limiting, with symptoms usually subsiding after 2–3 weeks. Some longer-term sequelae have been reported (94). In contrast, immunosuppressed patients suffer from prolonged chronic disease resulting in severe dehydration and weight loss, which can become life-threatening (2, 95). It is these patients for whom effective antimicrobial therapy is most urgently needed.

2.1 Antimicrobial Mechanisms of Drug Resistance

2.1.1 What Is the Basis of Cryptosporidium's Formidable Drug Resistance?

A wide variety of antimicrobial agents has been tested in vitro, in animal models, and in clinical trials (see (96) for a recent in-depth review of clinical and animal trials). Despite

considerable effort, no fully effective therapy has been established yet. However, two drugs have emerged that show a consistent, although modest, benefit in placebo-controlled studies: paramomycin (97, 98) and, even more promising, nitazoxanide (99). The resistance of *C. parvum* against drugs that are highly effective against related apicomplexan parasites has puzzled and frustrated researchers and clinicians alike. Two general models can be developed to explain this resistance. The first model is the extracytoplasmatic model. It argues that the peculiar extracytoplasmatic subcellular localization of the parasite within its host cell severely limits access of drugs to the parasite. In addition, efflux pumps could further rid the parasite of toxic compounds protecting susceptible target enzymes. The second model, the metabolism model, proposes that the metabolism of *Cryptosporidium* differs from other apicomplexa much more than initially appreciated, and that drugs active against other apicomplexa fail because their targets are absent or divergent in *C. parvum*. Obviously, these models are not mutually exclusive and both mechanisms could act synergistically. It is of great practical value for the field, however, to address this issue, as it has important implications for the direction of future research. If the enzymatic targets evaluated so far are sensitive to inhibition but drug access is the limiting factor, screening for additional new targets might be futile. If the targets tested so far were metabolically inappropriate, however, targeting new enzymes in an empiric or directed fashion might very well lead to success.

2.1.2 Living on the Edge, *C. parvum* Is an Intracellular, but Extracytoplasmatic Parasite

Apicomplexans are obligate intracellular parasites. Within their host cell, they are contained within the parasitophorous vacuole that is established during host cell invasion (100). Experiments in *P. falciparum* and *T. gondii* have demonstrated that although a barrier to proteins and other macromolecules, the vacuolar membrane contains pores that will allow smaller molecules to exchange freely, thus providing the parasite access to a "sieved" host cell cytoplasm for its nutritional needs (101, 102). In *T. gondii*, this vacuole is surrounded by cytoplasm on all sites and is tightly associated with several host cell organelles ((103, 104) and Fig. 1a). In contrast, the *C. parvum* vacuole is restricted to a protrusion of the apical membrane of the infected cell. The parasite remains surrounded by a thin margin of host cell cytoplasm, but the area of contact with the bulk of the host cell cytoplasm is relatively limited. This point of contact with the host cell is where a number of unique structural modifications occur within the parasite and its host cell (see Figs. 1b and 3 for a schematic outline). During invasion, the parasite forms the feeder organelle (105), which is characterized by a set of folded and convoluted membranes. This organelle is further elaborated, increasing its surface along with the intracellular development of the parasite. There are several slightly diverging topological hypotheses to explain the genesis of the various membranes involved, but all predict a single unit membrane separating parasite and host cell cytoplasm at the end (106–109). As implied by its name, the organelle is believed to transport nutrients into the parasite (110). However, molecular data supporting this role are not yet available. The feeder organelle could also potentially pose an obstacle to molecular transport, effectively isolating the parasite from the host cell. Additional structures that potentially might interfere with transport are an electron-dense band separating the cytoplasm surrounding the parasite from the rest of the cell (Fig. 1b-6 (106–109)) and a host cell actin plaque, which is induced at the site of parasite invasion (111, 112).

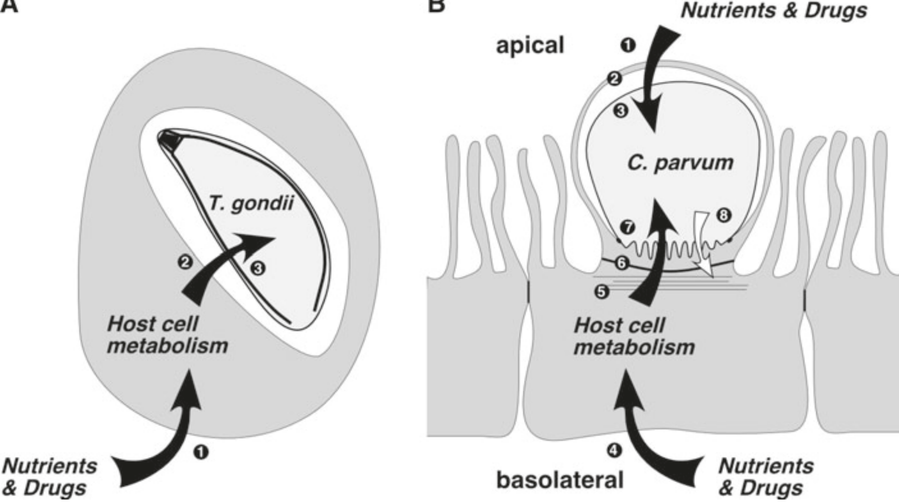

Fig. 1 The extracytoplasmatic hypothesis for *C. parvum* drug resistance. (**a**) *T. gondii* resides in a parasitophorous vacuole that is surrounded by cytoplasm on all sites. Nutrients and drugs pass the host cell plasmamembrane (1), pores in the parasitophorous vacuolar membrane (2), and the parasite's surface membrane (3). (**b**) *C. parvum* is confined to the apical membrane of its epithelial host cell. It has been hypothesized that drug uptake might be limited to the apical membrane (1–3). Alternatively, nutrients and drugs could enter through the basolateral membrane of the host cell, pass actin plaque (5), dense band (6), and feeder organelle (7). ABC transporters that have been localized to the boundary of parasite and host cell could actively export drugs (8)

The strongest support for the hypothesis, that the junction between parasite and host cell might be impermeable for drugs, stems from tissue culture studies with paromomycin. In a series of transwell experiments, Griffith and colleagues (113) showed that paromomycin was considerably more effective when administered in the apical than the basolateral medium compartment. This suggests that the drug travels through the apical membrane directly into the parasite (Fig. 1b 1–3) instead of traversing the host cell cytoplasm (Fig. 1b 4–7). A potential limitation of this assays lies in the extremely poor absorption of paromomycin into the cytoplasm of mammalian cells (114, 115), which on its own might explain why a parasite that directly underlies the apical membrane is more susceptible to apical inhibition. A second set of experiments shows that the related aminoglycoside geneticin (which is taken up much better) has parasite inhibitory effects in host cell lines expressing the resistance marker aminoglycoside phosphotransferase, which argues that drug import through the apical membrane is sufficient for killing (113). Unfortunately, geneticin kills the wild-type host cell as well as the parasite, making it hard to demonstrate in a positive assay that the drug traveling through cytoplasm would not be able to kill. Combining the geneticin experiment with the transwell assay might be a way to address this conundrum.

The potential restriction of molecular transport to the apical membrane would suggest that drugs are only taken up from intestinal lumen in vivo. The severe diarrhea that results from *C. parvum* infection generates a significant challenge to maintaining drug concentrations within the intestinal lumen at a therapeutic level. This decrease in drug availability is believed to account for the failure of paromomycin in the clinic (97, 113, 116–118). It will be important to establish how widely the paromomycin data can be generalized. A drug that would enter the parasite through the host cell could potentially be given intravenously, making it less sensitive to diarrhea, and thus may be more effective in the treatment of severe cryptosporidiosis in immunosuppressed patients.

2.1.3 Efflux Pumps Could Contribute to Drug Resistance

Active export of drugs by ATP-binding cassette (ABC) transporters could provide an additional model to explain *C. parvum* drug resistance (Fig. 1b-8). Such multidrug resistance (MDR) transporters can confer resistance to a range of structurally and mechanistically unrelated drugs. MDR proteins have been identified in *P. falciparum*, and were initially thought to be responsible for chloroquine resistance in malaria. Further extensive research has shown, however, that a membrane transporter unrelated to the ABC family is the main determinant for resistance to this drug (119–121). Four ABC transporters have been identified in *C. parvum* (96, 122–124); the complete genome sequence available now will undoubtedly dramatically extend this list. Sequence comparisons suggest that the transporters identified so far belong to the ABC transporter classes of MDR proteins (CpATP3), and MDR-associated proteins (MRP), CpATP1 and 2. Antibodies generated against CpATP1 and 2 showed relatively diffuse staining in extracellular sporozoites and labeled the surface of developing schizonts (122, 123). The staining seems to be more intense in the area of the feeder organelle. Whether or not these molecules are indeed secreted from the dense granules and subsequently locate to the parasite host boundary as suggested (123), remains to be validated by double labeling with antibodies to suitable markers and/or electron microscopic studies. At the moment, no data are available experimentally demonstrating a functional role of *C. parvum* ABC transporters in drug resistance. Pharmacological experiments, however, demonstrate that *C. parvum* growth in vitro is sensitive to several cyclosporine analogs that are potent modifiers of MDR proteins (125).

2.1.4 *C. parvum* Is a Highly Divergent Apicomplexan

The metabolic hypothesis put forward in this chapter explains *C. parvum's* resistance to typical anti-apicomplexan drugs as a reflection of its phylogenetic and metabolic uniqueness. Based on morphology and lifecycle, *C. parvum* was initially described as a typical coccidian parasite, most closely related to the genera *Eimeria* and *Toxoplasma*. However, more recent molecular and ultrastructural studies have revealed a surprising number of fundamental differences between *C. parvum* and Coccidia. Phylogenetic analysis based on small subunit ribosomal rDNA and β–tubulin assigns *C. parvum* to the base of the apicomplexan tree. *Cryptosporidium* could be a sister taxon to the gregarines, which are parasites of invertebrates and considered a primitive lineage of the Apicomplexa (92, 126–128). The relationship to gregarines is not fully resolved by the current dataset, but an ongoing EST sequencing effort for *Gregarina niphandrodes* might provide further insights.

One of the most striking differences between *C. parvum* and other apicomplexan pathogens is the absence of fully functional mitochondria and plastids (Fig. 2). An experimental study by Zhu and colleagues had predicted the absence of an apicoplast in *C. parvum* based on PCR and hybridization experiments, which failed to detect the presence of sequences conserved among all plastid genomes. Indeed, the completed *C. parvum* genome sequence does not contain either the organellar genome or the extensive set of nuclear encoded plastid targeted genes described for *P. falciparum* and *T. gondii* (64, 91, 129–131). The secondary endosymbiosis that let to the presence of the apicoplast is generally viewed as an early event in the evolution of Apicomplexa and Alveolata (132, 133) (196). The observation that several genes in the *C. parvum* genome show strong phylogenetic relationships to

Fig. 2 The metabolic hypothesis for *C. parvum* drug resistance. Schematic representation of some of the important mechanisms that shaped the genome and metabolism of *C. parvum*, and contributed to the pronounce differences between *C. parvum* and other apicomplexans

plants and algae supports this view, and suggests that *C. parvum* is derived from a lineage which once harbored an algal endosymbiont that was later lost (134–137). The lack of a plastid has important metabolic and pharmacological consequences. Like animals and fungi, *C. parvum* depends on a cytoplasmic type I fatty acid synthesis machinery instead of the plastid-associated prokaryotic type II system found in *P. falciparum* and *T. gondii* (129, 138). Loss of the organelle also explains *C. parvum's* resistance to macrolide antibiotics like clindamycin, which specifically target protein synthesis in the plastid and which are quite effective in *T. gondii* (60, 67).

C. parvum is also resistant to atovaquone (139), another drug with broad activity against apicomplexans. As described above, this drug targets the parasite mitochondrion. In *C. parvum*, however, electron microscopic studies have consistently failed to identify a classical mitochondrion, and experiments using dyes as probes for the mitochondrial membrane potential have arrived at conflicting results (87, 140–142). Despite these facts, the genome of *C. parvum* contains genes that show close similarity to nuclear-encoded mitochondrial genes in other organisms (142–144). One of these genes encodes a heat shock protein 60 homolog. Antibodies raised against this protein specifically label a membrane-bound organelle found close to the nucleus in *C. parvum* (145). The protein also carries a potential mitochondrial targeting motif on its N-terminus, and heterologous expression experiments in yeast and *T. gondii* have show that this sequence will target a reporter to the mitochondrion in these organisms (145–147). This finding of a relict mitochondrion, however, does not change the fact that *C. parvum* seems to lack an active Krebs cycle and oxidative phosphorylation, and seems to depend on anaerobic substrate phosphorylation (91, 142, 144, 148–150). So what does the relict mitochondrion do for *C. parvum*? One of the emerging functions of relict mitochondria in lower eukaryotes is the assembly of iron–sulfur clusters (151). Indeed, two key enzymes of this pathway, IscU and IscS, have now been identified in *C. parvum* (152). Both of these genes encode for proteins that contain an N-terminal targeting peptide, which confers import into the mitochondrion in a heterologous yeast assay (152).

2.2 Alternative Agents

2.2.1 The Emerging Genome Sequence Provides an Unprecedented View of *C. parvum*'s Metabolism

C. parvum is a challenging experimental system, and the lack of continuous culture models and transfection technology has posed limitations on the molecular analysis of this pathogen. However, providing *C. parvum* researchers with a rare treat, *C. parvum* has the Apicomplexa's most accessible genome. The genome is small (nine million base pairs) and introns are relatively rare, making gene prediction straightforward (153). This information can now be mined via the Internet through a convenient data base (154), (http://www.cryptoDB.org). The analysis of the genome sequence has just begun, but it has already unearthed a surprising number of metabolic differences between *C. parvum* and other apicomplexans. Due to space limitations, the remainder of this chapter will focus only on nucleotide biosynthesis, but a number of additional pathways have been shown to be highly divergent, and it will be exciting to establish a complete picture based on the soon-to-be fully annotated genome (or genomes) (91, 137, 144, 155–157).

Nucleotide biosynthesis has been a mainstay of antiprotozoal treatment. As described in the first part of this chapter, antifolates are highly active against *T. gondii* and *P. falciparum. C. parvum*, on the other hand, is resistant to pyrimethamine/sulfadiazine (158, 159). DHFR-TS is one of the few potential *C. parvum* drug targets that has been studied in detail. The gene for this enzyme has been cloned, and sequence analysis revealed several differences from the *P. falciparum* enzyme. Special attention has been devoted to Cys113, which was interpreted as analogous to *P. falciparum* Ile164 and as a potential determinant of pyrimethamine resistance (160). Transgenic yeast lines have been developed that depend on *C. parvum* DHFR-TS and these are highly pyrimethamine-resistant (161, 162) (Dr. Sibley, personal communication). Recently, the three-dimensional structure of *C. hominis* DHFR-TS has been solved. Structural as well as kinetic analysis suggests that the *Cryptosporidium* enzyme is quite different from previously characterized fused enzymes from kinetoplastids and apicomplexans (163, 164). Despite the fact that the TS domain is highly conserved, the *C. hominis* enzyme seems to be 10–40 times faster than the previously characterized enzymes. Furthermore, the kinetic analysis provided evidence against substrate channeling in

C. hominis. In contrast, the bifunctional DHFR-TS enzymes of *Leishmania* and *Toxoplasma* channel H_2 folate produced at TS directly to the DHFR active site, without equilibration in bulk solution (164–166). Presence or absence of channeling might affect the sensitivity of DHFR-TS to antifolates. Recombinant enzyme, transgenic yeast strains, and *C. parvum* tissue culture systems have been used to test a variety of alternative antifolates, and several compounds have shown promising initial activity (161, 162, 167).

2.2.2 Gene Loss and Horizontal Transfers Shape *C. parvum's* Nucleotide Pathway

In addition to differences in DHRF-TS, the general pattern of pyrimidine nucleotide synthesis and salvage could equally modulate the efficiency of antifolates. Recent genomic and experimental work has uncovered a surprising diversity of pyrimidine biosynthetic pathways within the Apicomplexa. *P. falciparum* is entirely dependent on *de novo* synthesis of pyrimidines, making DHRF-TS an essential enzyme (168, 169). *T. gondii* posses the ability to salvage uracil using uracil-phosphoribosyltransferase (170, 171). However, this salvage pathway is not sufficient to sustain the parasite in the absence of *de novo* synthesis. A targeted gene deletion of carbamoyl phosphate synthetase II (the first step in the *de novo* pathway) resulted in a severely attenuated parasite strain that was unable to produce an infection in a highly susceptible mouse model (172). *C. parvum*, finally, has lost all six genes for the enzymes in this pathway, indicating that it is unable to synthesize pyrimidines *de novo* (134). The parasite depends entirely on salvage, and three pyrimidine salvage enzymes have been identified; two of them, uridine kinase-uracil phosphoribosyltransferase (UK-UPRT) and thymidine kinase (TK), are not found in any other apicomplexan. Phylogenetic analysis suggests that both enzymes were obtained from other organisms by horizontal gene transfer – UK-UPRT from an algal endosymbiont that has since been lost (see plastid section above) and TK from a proteobacterium (134). The presence of TK in *C. parvum* provides an additional and potentially alternative source of *d*TMP for this parasite, and could reduce its sensitivity to inhibition of DHFR and the subsequent starvation of the thymidylate synthase reaction. Equally, the finding of UK-UPRT explains the difference in susceptibility to cytosine arabinoside between *C. parvum* and *T. gondii*. While *T. gondii* is highly resistant to this prodrug, which has to be activated by cytosine or uridine kinase, *C. parvum* was surprisingly susceptible (173, 174).

Both UK-UPRT and TK could be new targets to pursue for *C. parvum*. TK might especially hold promise, based on the successful exploitation of this target in the therapy of Herpes viruses (175). A large variety of compounds subverting the viral TK has been generated, and the relationship between enzyme structure and drug sensitivity and resistance is well understood (176–178). The divergent phylogenetic origin of the parasite enzyme from a proteobacterium might allow for the identification of compounds with selective specificity for the parasite versus the human enzyme. But will nucleoside analogs known to subvert this enzyme be able to reach their target within the parasites? Experiments using 5-deoxybromo-uridine in *C. parvum*-infected tissue cultures indeed suggest that this class of compounds gains access to the parasite (134).

Horizontal gene transfers into the *C. parvum* nucleotide metabolism are not limited to the pyrimidine pathway. *C. parvum* has obtained its inosine 5′-monophosphate dehydrogenase (IMPDH), an enzyme central to the purine salvage pathway, from an ε-proteobacterium, whereas *P. falciparum* and *T. gondii* harbor enzymes of clear eukaryotic phylogeny (169, 179) (197). As TK, IMPDH is a well-established target of antiviral and immunosuppressive therapy (180–182). Furthermore, prokaryotic and eukaryotic IMPDH differ in structure and mechanism, which should facilitate the identification of *C. parvum*-specific inhibitors (182–187). Kinetic analysis of recombinant *C. parvum* IMPDH has shown pronounced differences between the NAD binding sites of the parasite and the human enzymes (188, 198). This difference has been successfully exploited to identify parasite-specific inhibitors by high-throughput compound screening (199).

Genomic and experimental studies show a highly streamlined salvage pathway for *C. parvum* (see Fig. 3), which relies on adenosine as sole source of purine. Earlier biochemical studies on crude parasite lysates had also predicted adenine, hypoxanthine, xanthine, and guanine salvage; however, the genes for these enzymes seem not to be present in the genome (91, 134, 189). IMPDH is at the center of this streamlined pathway, and is an essential enzyme of the multistep conversion of AMP to GMP. Treatment of infected tissue cultures with the IMPDH inhibitors mycophenolic acid and ribavirin consequently results in dose-dependent inhibition of *C. parvum* development (134, 174).

One of these drugs, ribavirin, has also been tested in a neonatal mouse model of cryptosporidiosis, and treatment with 50 mg/kg for 1 week resulted in a 90% reduction of parasite load when compared to untreated controls (Mead & Striepen, unpublished). Interestingly, in these experiments the drug was injected into the peritoneum rather than given orally, suggesting that uptake from the intestinal lumen through the apical membrane of the host cell might not be necessary for this compound. Ribavirin, like the aforementioned drugs targeting TK, is a nucleoside analog. This class of drugs does not freely diffuse across membranes, but subverts the nucleoside transporters of their target cell to get access (190, 191). Nucleoside transporters are found on the

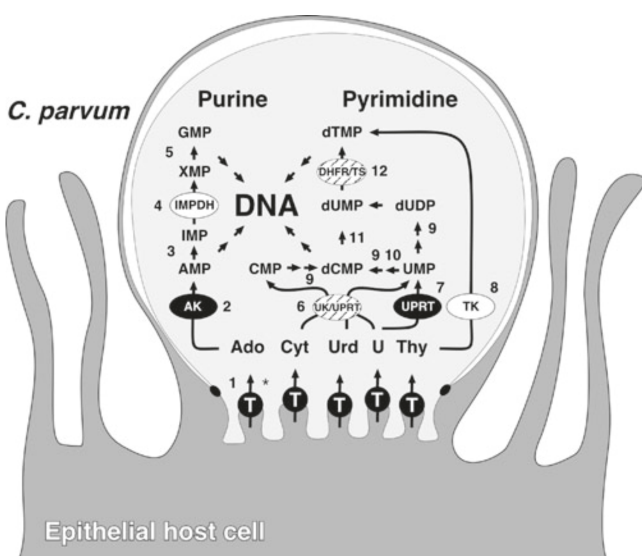

Fig. 3 The *C. parvum* nucleotide biosynthetic pathway is a phylogenetic mosaic. Enzymes labeled in white show strong phylogenetic association with eubacteria, hatched with plants and algae (see (134) for further detail). The membrane topology of the feeder organelle (110) has been schematized, and the localization of nucleoside transporters is entirely hypothetical; localization outside of the feeder organelle is equally possible. (1). adenosine transporter, (2). adenosine kinase, (3). adenosine monophosphate deaminase, (4). inosine monophosphate dedydrogenase, (5). guanosine monophosphate synthase, (6). uridine kinase-uracil phophoribosyltransferase, (7). uracil phosphoribosylltransferase, (8). thymidine kinase, (9). ribonucleotide diphosphate reductase, (10). cytosine triphosphate synthetase, (11). deoxycytosine monophosphate deaminase, (12). dihydrofolate reductase-thymidylate synthase. Reproduced with permission from (134)

apical as well as the basolateral membrane of epithelial cells (192). But where is the parasite nucleoside transporter localized? Several nucleoside transporters have been characterized in related intracellular parasites, and most seem to localized over the entire surface of the parasite (193, 194). A potential nucleoside transporter with similarity to the *T. gondii* adenosine transporter has been identified in *C. parvum*, and could provide an important molecular reagent to further address nutrient and drug transport in *C. parvum* (134, 195).

2.2.3 Conclusion

A series of recent genomic, biochemical, and cell biological studies has produced considerable support for the metabolism hypothesis for *C. parvum* drug resistance. The metabolism of *C. parvum* differs dramatically from its better-studied cousins *P. falciparum* and *T. gondii*. In all cases where the target of a widely used anti-apicomplexan drug has been characterized in molecular detail, it was either absent in *C. parvum* (e.g. clindamycin and atovaquone) or the enzyme was highly divergent and resistant (pyrimethamine). Limited drug access might still remain an important challenge to treatment, but the data warrants a fresh look at a new and metabolically more appropriate set of drugs and targets. The divergence of *C. parvum* from the generic eukaryotic metabolism might, after all, present an Achilles heel, and the presence of numerous bacterial enzymes provides an exciting set of candidate targets for parasite-specific inhibition. Transgenic models might provide urgently needed assay systems to validate targets identified by genome mining and screen compounds.

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Chapter 44
Drug Resistance in Nematodes

Roger Prichard

1 Introduction

Anthelmintic drugs remain the principal means of intervention for therapy and prophylaxis of nematode parasitic diseases in humans and animals. Other than improvements in sanitation, there are no effective alternatives to chemical control of parasitic nematodes. However, resistance to anthelmintics has become a major problem in veterinary medicine, threatens both agricultural production and animal welfare, and there is increasing concern that drug resistance could arise in nematode parasites in humans.

The use of effective pharmaceuticals to kill pathogens frequently leads to the organisms developing resistance, and we can lose our ability to treat or control that disease. This can also be true for parasitic nematodes. At introduction, an anthelmintic will be highly effective, and there may be a period of heavy promotion of use of the anthelmintic. However, unfortunately, biological reality eventually asserts itself, and we may be left contemplating the reappearance of a parasite disease problem many had thought solved forever. In the case of veterinary nematodes, widespread resistance to anthelmintics has left us searching for ways to maintain their effectiveness, even as we search for novel forms of control. We need to devise the best strategies for minimizing the impact of anthelmintic resistance. This requires a much better understanding of the basic biology of the parasites themselves, how they have become resistant to our current compounds, and what alternative means of control could be available.

For each chemical class of anthelmintics, resistance to one member usually confers resistance to the other members. It is possible, and increasingly common, to have multiple resistances where nematodes develop resistance sequentially and independently to several anthelmintic classes. Once resistance is present in a nematode population, reversion or loss of resistance occurs very slowly (1).

Despite intensive investigation, the molecular and biochemical bases of anthelmintic resistance are not very well understood, even for the benzimidazole anthelmintics in nematode parasites of animals. In humans, the lack of good animal hosts for some human nematodes, such as the filaria, limits our ability to adequately assess resistance development. The lack of reliable biological and molecular tests for drug resistance in nematodes means that we are not able to follow the emergence and spread of resistance alleles and clinical resistance, as well as we need. This chapter summarizes the extent of anthelmintic resistance in nematodes, recent findings on mechanisms of antinematode drug action, resistance mechanisms, factors that influence the rate of selection and spread of drug resistance in nematodes and recommendations to limit the impact of drug resistance, and alternative means of nematode parasite control.

2 Extent of the Resistance Problem in Nematode Parasites

Resistance in nematodes of livestock (sheep, goats, cattle, horses and, to a lesser extent, swine) to anthelmintics has become a serious problem in many parts of the world. In farm animals that graze pasture, veterinarians, animal health industry representatives, government, university and private farm management advisors have traditionally recommended that the whole herd/flock of animals be treated, at the same time, with a move of the animals to a pasture with low levels of the infective stage, so that the animals do not rapidly become reinfected from contaminated pastures. This has meant that the contribution of nematodes that

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survive the treatment (resistant worms) to subsequent generations of parasites has been relatively high, and resistance has resulted.

In sheep and goats, anthelmintic resistance in nematodes is already a serious problem (2, 3). In a number of countries, the prevalence and severity of resistance now threatens the profitability of the entire sheep industry (4–6). Resistance has arisen to all of the major families of broad-spectrum anthelmintics (7), the benzimidazoles (BZ), levamisole (LEV) and the other nicotinic agonists, and the avermectins and milbemycins (AM), which include ivermectin, doramectin, and moxidectin. Nematodes resistant to other narrow-spectrum anthelmintics such as closantel have also been reported (7). The situation in cattle is currently less severe, but there are now reports from New Zealand and South America of cattle nematodes resistant to multiple anthelmintic classes (8, 9), and the likelihood is that this will become more widespread. In horses, BZ-resistance is very widespread amongst the cyathostomins, but the AM are still very effective (10–12). This could change as they are used more frequently and selection pressure is increased. Anthelmintic resistance in nematodes of livestock is therefore a threat to agricultural incomes already under pressure in many parts of the world, and the increase in disease also poses a threat to animal welfare. The absence of viable alternative methods of worm control means that we must understand how resistance works in order to limit its impact as far as possible.

In the case of nematode parasites in humans, current control programs are focused on reducing infection load (worm burden) and transmission potential, to reduce morbidity associated with infection. Periodic treatment with broad-spectrum anthelminthics is the mainstay of soil-transmitted nematode (STN) control programs. The WHO advocated regular chemotherapy for at least 75% of all school-age children at risk of morbidity for soil-transmitted nematodes, by 2010. In the last few years, the Global Alliance for the Elimination of Lymphatic Filariasis (GAELF) and the Onchocerciasis Control programs have administered millions of tablets of albendazole (ABZ) and/or ivermectin (IVM), as well as diethylcarbamazine (DEC), by community and mass drug administration in endemic areas. This raises concerns that the frequent treatments used in chemotherapy-based programs to control human STN, onchocerciasis, and to eliminate lymphatic filariasis (LF) may select resistant worms that will impair the benefits of treatment at the individual and at the public health level.

However, there are as yet only few reports that suggest possible occurrence of resistance in soil-transmitted human nematodes (13–15). In 2001, the WHO (16) set the following minimal targets aimed at reducing morbidity due to soil-transmitted nematode infections by 80%, which can be achieved by all endemic countries, as an integral part of their health systems by:

1. Regular chemotherapy of at least 75% of all school-age children at risk of morbidity, by 2010;
2. Access to essential anthelminthic drugs by health services in endemic areas, down to the most peripheral level, for the treatment of symptomatic cases, as well as children, women, and other groups at risk of morbidity. This represents a proposed substantial increase in the use of chemotherapy against soil-transmitted nematodes in humans, and is likely to increase selection pressure for drug resistance in these nematodes.

In the case of filarial infections, the use of ivermectin for the reduction of morbidity associated with onchocerciasis and the reduction of transmission of *Onchocerca volvulus* is the most longstanding example of mass treatment of nematodes, with programs being introduced in West Africa under the Onchocerciasis Control Program (OCP) in the late 1980s and early 1990s; the African Program for Onchocerciasis Control (APOC), which has distributed ivermectin since 1995; and the Onchocerciasis Elimination Program in the Americas (OEPA), which started ivermectin distribution in 1993. Under OCP and its successor national programs, and under APOC, people living in onchocerciasis-endemic areas of Africa are being treated annually with IVM. The projected coverage is 65% of the eligible population, or better. Under OEPA, treatment is twice a year. More recently, the Program to Eliminate Lymphatic Filariasis (PELF) has been launched. This will involve annual treatment of people in areas endemic for lymphatic filariasis (LF) with a combination of ABZ and IVM where onchocerciasis exists in Africa, or of ABZ plus DEC in the rest of the world. The target for coverage in endemic areas is 85% of the eligible population. Hundreds of millions of doses of anti-nematode chemotherapy have already been distributed for the control of these filarial infections. Against filarial nematodes, the chemotherapy removes existing microfilaria and temporarily sterilizes the adult worms, reducing transmission. However, most adult worms are not killed by a single treatment of the anti-nematode drugs and can resume reproduction some months after single treatment, resulting in the skin (onchocerciasis) or blood (LF) being repopulated with microfilaria and transmission restarting. Thus, to suppress transmission, it is necessary to treat infected people at least annually. Adult worms should die of old age after about 12 years in the case of *O. volvulus* and 6 years in the case of *W. bancrofti* and the *Brugia* spp., the causative agents of LF. If chemoprophylaxis has suppressed transmission, then, in theory, infectious cases should diminish and the parasite population in areas under treatment should be greatly reduced or eliminated. In the case of onchocerciasis control in West Africa, some communities have had 14 or more rounds of IVM treatment.

The level of onchocerciasis has been dramatically reduced and is no longer a public health problem (17). However, *O. volvulus* has not been eliminated. There have recently been a few reports of sub-optimal responses to IVM in terms of microfilarial loads after many rounds of treatment (18–20), and there is evidence that IVM is causing genetic selection in *O. volvulus* (21–23). However, further work is required to determine whether these reports of a lack of good response to IVM in *O. volvulus* and of genetic changes associated with repeated IVM treatment are due to developing a resistance to IVM.

3 Measuring Resistance

Most surveys of clinical resistance involve treatment of infected hosts with a recommended dose of the drug, followed by a calculation of the effect of treatment by fecal egg count reduction compared with pre-treatment egg counts or those of untreated controls in the case of soil-transmitted nematodes, or of skin or blood microfilaria counts in the case of onchocerciasis and lymphatic filariasis, respectively. Reductions of less than 95% (based on group arithmetic means) are often treated as evidence of clinical resistance (24). Estimating treatment efficacy and the presence or absence of resistance is very difficult in the case of human filarial nematodes because the anthelmintic drugs are lethal to larval stages, while most adult worms survive but have their reproduction reversibly suppressed for prolonged periods. Therefore, resistance could be manifested in filarial worms as a failure to kill all microfilaria, a faster return of adult worms to fecundity, higher levels of fecundity after treatment, longer survival of adult worms despite repeated treatment, or a combination of these effects.

Some in vitro assays are available, such as larval development or larval motility tests for some nematodes for some anthelmintics. However, no universal, in vitro biological tests are available that work with all classes of anthelmintics on all important species of parasitic nematodes. Genetic tests detect the presence of specific resistance alleles in a population; such alleles must be present in the majority of cases of resistant worms. The application of genetic tests is limited by a lack of validated assays, and the development of such assays to measure gene frequency in populations is a major challenge.

4 Mechanisms of Resistance

Drug resistance can arise in a limited number of ways: a change in the drug receptor so that the drug no longer binds with high affinity and is thus ineffective at safe low concentrations; a change in metabolism that inactivates or removes the drug; or a change in the distribution of the drug in the target organism, which prevents it accessing its site of action.

4.1 Benzimidazoles

Benzimidazoles act by inhibiting the polymerization of tubulin to form microtubules, and it is clear that resistance is associated with mutations in β-tubulin genes that prevent the drugs binding to their target. However, several different polymorphisms of the β-tubulin genes have been correlated with BZ-resistance (25). The well-known Phe/Tyr polymorphism at codon 200 of β-tubulin isotype 1 was the first to be described, and it has frequently been considered the most important mutation conferring resistance to these compounds. However, even in the early studies, it was observed that highly resistant populations of *Haemonchus contortus* also showed selection on, or a deletion of, β-tubulin isotype 2 (26, 27). More recently, a second Phe/Tyr polymorphism, at codon 167 of β-tubulin isotype 1, was detected in BZ-resistant populations of *H. contortus*. Furthermore, it was reported that the same two polymorphisms also occur in the β-tubulin isotype 2 gene of *H. contortus*, and that they too can confer BZ resistance (25). Investigations on BZ-resistant trichostrongyle field populations revealed that the codon 167 polymorphism was also present in *Teladorsagia circumcincta*, but not in *Trichostrongylus colubriformis* (28). Mutations at codon 167, but not 200, were found in several highly BZ-resistant small strongyle species from horses (29). The significance of polymorphisms at codon 167 for different parasites has been only partially investigated, and current data indicate that there are differences between species. Benzimidazole binding studies with recombinant *H. contortus* β-tubulins indicated that mutations at codon 167 of isotype 1 or 2 confer resistance (25). On the other hand, genotyping two *H. contortus* field populations showed that Tyr at codon 200 was associated with BZ resistance in these isolates. The same study found that *T. circumcincta* homozygous for Phe at codon 200, but heterozygous or homozygous for Tyr at codon 167 survive BZ treatment (28). Interestingly, of *T. circumcincta* surviving BZ treatment, a similar proportion was heterozygous at codon 167 as was homozygous for Tyr. This implies that, unlike *H. contortus*, BZ resistance in *T. circumcincta* may not be recessive. Investigations using molecular techniques to evaluate resistance levels in the field will have to consider these data. In horse small strongyles, the β-tubulin isotype 1 codon 200 polymorphism is not the only (and probably not even the most important) mutation with respect to resistance (30–32). However, it remains to be seen to what extent codon 167 mutations contribute to resistance in these worms. The codon

200 polymorphism has been described in a cattle nematode, *Cooperia oncophora*; however, its relevance to resistance has not yet been investigated (33).

4.2 Levamisole

Levamisole is the most widely used cholinergic anthelmintic, acting as an agonist at nicotinic receptors at the nematode neuromuscular junction and causing a spastic paralysis in nematodes. Nematodes resistant to levamisole are also resistant to other nicotinic agonists such as morantel and pyrantel. In *C. elegans*, levamisole resistance results from the absence of levamisole receptors, which form one of the two populations of nicotinic acetylcholine receptors present at the neuromuscular junction (34); the second is preferentially activated by nicotine. It is presumably the presence of this levamisole-insensitive population that allows resistant worms to survive without functional levamisole receptors. The presence of two pharmacologically distinct nicotinic acetylcholine receptors at the neuromuscular junction, one of which is activated by levamisole, has been confirmed in *Ascaris suum* (35). Electrophysiological studies found fewer active levamisole receptors in muscle preparations of resistant *Oesophagostomum dentatum*, and those that were present possessed a different population of channel subtypes. Resistance resulted from a change in the averaged properties of the receptors, rather than their absence (36). Similar results were obtained using pyrantel-resistant nematodes (37). One explanation for these data is that different channel subtypes result from post-translational modifications of the receptors and that the changes seen in resistant nematodes are due to differences in post-translational modification; the use of protein kinase inhibitors has suggested that receptor phosphorylation does regulate levamisole sensitivity (38). However, the molecular basis for these physiological and pharmacological differences between levamisole-sensitive and levamisole-resistant worms remains obscure. Nematodes possess a very large family of nicotinic receptors, and molecular cloning efforts have so far failed to reveal any polymorphisms associated with resistance (7). There is genetic evidence that resistance is due to a single sex-linked gene in *T. colubriformis* and *O. dentatum*, but in *H. contortus* the evidence is inconclusive and some studies suggest that multiple genes could be involved. We need more information on parasite nAChRs before we can clearly define the molecular basis of levamisole/pyrantel resistance.

4.3 Avermectins and Milbemycins

With resistance to the *avermectin* and *milbemycin* (AM) anthelmintics, also collectively known as macrocyclic lactones, the picture is still confused. These drugs act on ligand-gated chloride channels, including glutamate-gated chloride channels (GluCl) and gamma-aminobutyrate-gated chloride channels (GABACl), a family of receptors widely distributed in nematodes, which regulate locomotion, feeding, and reproduction (39–41). The AM have effects on all of these functions, but it is likely that their relative importance in the overall anthelmintic activity varies between species, and thus mechanisms of resistance could also vary. Indeed, even different avermectin-resistant strains of the same species, *H. contortus*, have varying phenotypes (42). Parasites resistant to one AM, such as ivermectin, are generally resistant to the others, though there are suggestions that moxidectin might be more effective against some ivermectin-resistant isolates. Genetic studies have found that ivermectin resistance is dominant in *H. contortus*, perhaps reflecting a gain-of-function mutation, though it could be that true resistance is synthetic, resulting from the additive effects of a number of genetic changes. This is certainly true for high-level avermectin resistance in *C. elegans* (43), though the relevance of these studies to parasites could be questioned. Binding studies have failed to find any consistent changes in radiolabeled ivermectin binding to membranes from resistant *H. contortus* or *T. circumcincta* (44), suggesting that target-site mutations are not the mechanism of resistance in these species and consistent with suggestions that P-glycoproteins (Pgp) are involved in AM resistance (45, 46). The AM are excellent substrates for these pumps (47), and Pgp inhibitors such as verapamil enhance their activity (48). Binding studies did find an increase in the numbers of a low-affinity L-glutamate binding site in ivermectin-resistant isolates of both *H. contortus* and *T. circumcincta* (44, 49), but the nature of this site has not been investigated further. Population genetics studies in *H. contortus* have also found evidence for the association of Pgp and other ABC transport genes with AM selection (21–23, 50), but other population studies also found evidence for selection at a GluCl gene and a GABA receptor-related gene (51, 52). Ivermectin increased the response to GABA in cells transfected with an unselected wild-type allele of the *H. contortus* GABACl receptor subunit gene, whereas in the cells transfected with the AM selected GABACl allele, ivermectin attenuated the response to GABA (40). Recently, a polymorphism was found in a GluCl subunit from an ivermectin-resistant isolate of *C. oncophora* that caused the resultant channels to be less sensitive to both glutamate and ivermectin when expressed in vitro (53). An intriguing further observation is that the amphids (sensory structures in the head of nematodes) are altered in avermectin-resistant *H. contortus* (54). The amphids form a pathway from the environment into the interior of the worm, so defects could prevent drugs gaining access to their target sites. In nematodes, bundles of amphidial neurons extend from the nerve ring, which encircles the pharynx, and terminate near the amphidial openings. These neurons have extensive microtubule bundles that appear to be

shortened and deranged in IVM-resistant *H. contortus* (54). It is interesting that there is also selection for a particular allele of beta-tubulin in IVM-selected *O. volvulus* (23). At first sight, these data seem complicated, so what hypotheses can be formulated to explain them? A number of possibilities stand out. Resistance to the AM anthelmintics could be caused by a gain-of-function mutation in Pgp or ABC transporter genes, leading to more rapid removal of the drug from the worm. Such mutations could either cause increased expression of a pump capable of carrying the AM, or change the specificity to increase the affinity for these substrates. These genes are very polymorphic in nematodes that have been examined, and mutations that alter pump expression or activity, if present at a low frequency, could be selected very rapidly. Alternatively, parasites could become resistant by the accumulation of one or more mutations in GluCl or GABACl genes, which may affect the primary action of the AM. The role of beta-tubulin in AM resistance warrants further investigation, but mutations in this gene may affect the structure and uptake of AM via the amphids, or affect ligand-gated chloride channel function or transmission of neuromuscular signals, as microtubules are known to play a role in anchoring neurotransmitter channels at synapses. Such multigenic resistance would be slower to appear and the exact genes involved may vary between species, depending on their relative importance in drug action. As GluCls are expressed in amphid and extrapharyngeal neurons (55, 56), defects in these neurones could also cause the observed changes in morphology and response to AM. A more detailed comparison of the GluCl, GABACl, ABC transport, and beta-tubulin genes among many sensitive and resistant isolates is needed; the first step is to determine the number, sequence, and polymorphism of such genes in several parasite species.

5 Selection for Anthelmintic Resistance

When an anthelmintics class is first introduced, the frequency of resistance alleles is low, indicating that in the absence of anthelmintic treatment, resistance alleles confer a neutral or negative reproductive fitness. Resistance is not an inevitable consequence of drug use, and selection for resistance will depend on the relative reproductive fitness conferred by 'susceptibility' and 'resistance' alleles at the given level of drug usage. For some parasite/drug use situations, resistance may never develop. The presence of a resistance genotype always precedes observation of clinical resistance. As drugs are often used in the field at doses higher than the minimum required to kill or to inhibit reproduction in most worms, selection may produce a high frequency of 'resistance' alleles before clinical resistance is noticed.

Unfortunately, anthelmintic resistance in some nematode species is already a major problem, and one that we don't fully understand. The problem seems to be here to stay – there has been no evidence of reversion to anthelmintic susceptibility, even where the drug has been withdrawn (1), and it is likely to become more widespread and affect more nematode species parasitic in animal and human populations under drug treatment. In the short to medium term, there are no realistic alternatives to the continued use of current chemicals for parasite control – effective vaccines, new cost-effective compounds, and nonchemical means of control are all some distance in the future – so it is vital that we maintain the efficacy of existing anthelmintics for as long as possible. This will require good communication with the users of these products in order to reduce those practices that encourage the emergence and spread of resistance. Selection pressure for resistance is largely affected by the degree of *refugia* (57), that is, the proportion of the nematode population that is not exposed to the effects of the drug at the time of treatment, such as free-living egg and larval stages. Many animal health advisers have advocated that when farm animals are treated with anthelmintics, the whole herd should be treated at the same time, and the animals moved to an environment where they will not be readily re-infected. This includes treatment during periods of drought when few infective stages will be in the environment. While these practices will extend the time before animals become heavily re-infected, and possibly reduce the frequency of treatment, they result in treatment occurring when very few nematodes are *in refugia*, and result in high selection pressure for the development of resistance. Experimental and field studies have shown that selective treatment of infected individuals need not result in significant negative effects on morbidity or production yields in a population (58). However, if elimination is the goal, then the selective treatment of only individuals with moderate to high levels of infection will not allow elimination of the parasite in the population of hosts. It is widely believed that treatment frequency is a key factor in the selection of resistant nematode populations, so reducing treatment frequency may also be important to reduce selection pressure for resistance (59).

Selection pressure for anthelmintic resistance to develop will vary greatly between nematode species and populations. The initial frequency of resistance-associated alleles will affect the rate of selection for resistance. If resistance is recessive, as in the case of BZ resistance in a number of nematodes, the frequency of resistance alleles will need to be moderately high before a resistance problem is noted. If resistance is polygenic, as appears to be the case with AM anthelmintics, nematodes with resistance-associated alleles in two or more genes may be necessary before a resistance phenotype is seen. The use of combinations of anthelmintics with different mechanisms of resistance means that a resistance phenotype must be multigenic, and this can delay the appearance of resistant individual nematodes. The extent of genetic diversity in a nematode species and population will also affect

the likelihood of resistance developing rapidly. *Haemonchus contortus* is very polymorphic (25), and resistance to most antinematode drugs develops rapidly in this species.

The life history of the nematode will also greatly affect the likelihood of resistance developing. Nematodes that have large populations of free-living stages and that survive for long periods, such as *Ascaris lumbricoides*, are not likely to develop resistance rapidly. However, other nematodes in which almost all of the nematode population is in the host and host populations are subject to repeated high coverage treatment with the effects of each treatment being sustained for many months, such as the control or elimination programs for onchocerciasis and lymphatic filariasis, are likely to be under relatively high selection pressures, and resistance may develop, provided the alleles that can confer resistance are present in the nematode population. In the case of LF, anthelmintic combination treatment (ABZ + DEC or ABZ + IVM) is being used. This should help delay the onset of resistance.

6 Management of Nematode Infections in Endemic Regions

The aim of management is to make nematode parasite control sustainable. This implies the use of a range of control measures and a stabilization of resistance. At the same time, the morbidity due to nematode infection and the economic cost of parasitism (both by reducing health care costs and losses, and by minimizing productivity) must be kept down. Where feasible, public health and environmental hygiene are the best long-term solutions to nematode parasitism. In the short and medium term, and provided they still work, anthelmintics remain the most cost-effective method of control, and will therefore remain in use. There are few opportunities for synergizing chemical action (such as for insecticides), and therefore approaches focus on refining chemical usage, such as the use of combination therapy and targeting only individuals carrying high worm burdens, and augmenting this with local environmental hygiene measures and nutritional and immunological methods. The sustainability of any approach is difficult to ascertain, but knowledge of resistance mechanisms, selection factors, and mathematical modeling give us clues on how to design sustainable systems. To some extent, the better nematodes are controlled by drugs, and the greater the drug treatment coverage, the faster resistance can develop. What to advise is a complex matter. Where highly pathogenic species are prevalent, parasite control is critical, and there is less latitude for using nonchemical control.

Assuming drug use is required, treatment should be confined, wherever possible, to hosts suffering from parasitism; other hosts that can tolerate existing infections without significant impairment to health should be left untreated. Strategic treatment of parasites with anthelmintics provides efficient control, but often encourages resistance. Testing for infection and only treating when infections reach a threshold is 'curative' treatment. This might be on a herd basis (e.g., by egg counts on a subsample of a host population) or on an individual basis (e.g., the Famacha system (60) for anemia-producing parasites such as *H. contortus* in sheep, or other assessments of clinical condition of the host). These approaches mean the host suffers some parasitism, with some morbidity or loss of productivity. Testing for anthelmintic efficacy is essential in elucidating which drug classes are effective in a host population. Such efforts to reduce selection pressure in a particular population may be wasted if worms carrying resistance alleles are introduced to that population from infected migrant hosts. In farm situations, quarantine treatment of imported animals, usually with mixtures of several anthelmintic classes, is a possible way to exclude parasites.

Nematode parasites compete with the host for nutrients and, as a result, hosts may lose weight and their immune responses may become down-regulated. Energy, protein, vitamin, and mineral supplements may be useful in stimulating immunity. For farm animals, where additional pasture or other nematode-resistant livestock (such as older sheep, cattle, or horses, or alternative host species) are available, worms can be controlled by alternate grazing. For horse parasites, breaking the lifecycle is a possibility. Removal of feces from pastures removes all sources of re-infection, including resistant worms. In humans, the strict use of latrines and public health measures that stop environmental contamination, or transmission via vectors in the case of filarial worms, can break the lifecycle and remove the necessity for periodic drug treatment.

In conclusion, anthelmintic resistance in nematode parasites of farm animals is a major problem worldwide and may be developing in some species of nematodes of humans. We do not have the tools to reverse it, and so must take action to monitor for its development and take measures to reduce its selection. Until novel methods of nematode control are developed, we will need to implement strategies to maximize the effective lifetime of our current compounds. Such strategies will be based on a sound understanding of the nematode biology, of resistance mechanisms, and of epidemiological monitoring for resistance. More research in these areas is urgently needed.

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Chapter 45
Chemotherapy and Drug Resistance in Schistosomiasis, Fascioliasis and Tapeworm Infections

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1 Introduction

It is estimated that nearly 400 million humans are infected with trematode or cestode parasites (Table 1, (1)). Nearly half of the infections are caused by two species of schistosome (blood flukes), *Schistosoma mansoni* and *S. haematobium* and most of this chapter will therefore be concerned with these parasites, and praziquantel (PZQ), the principal drug being used against them. Brief mention will however also be made of other schistosomicidal drugs, treatment of infections caused by another trematode, the liver fluke *Fasciola hepatica*, and treatment of cestode (tapeworm) infections.

1.1 *Schistosomiasis*

Humans are infected mainly by three species of schistosome (2): *Schistosoma mansoni*, which is responsible for hepatic and intestinal disease and is found in Africa, the Caribbean and the north and northeast of South America; *S. haematobium*, the causative agent of urinary schistosomiasis and found also in many African as well as some Middle Eastern countries; and *S. japonicum*, named as a result of being discovered in Japan, but which has been virtually eradicated from that country. However, it still occurs in parts of China and some Pacific islands. Eighty-five per cent of the 200 or so million people infected with schistosomes live in Africa (3, 4), though a small number of tourists and other visitors to endemic areas who do not take care to avoid infection are also of course at risk (5).

Other schistosomes infect animals. *S. japonicum* infection is a zoonosis and in China farm animals are the main reservoir of infection and are treated with praziquantel to reduce transmission. *Schistosoma mattheei* and *Schistosoma bovis* are infections of cattle and small ruminants in Africa, but relatively little research has been undertaken on their chemotherapy.

Schistosomes are digenetic trematode (flatworm) parasites that require two hosts for completion of their complex life-cycles (Fig. 1): aquatic or amphibious snails, restricted to fresh water bodies in the tropics and serve as intermediate hosts in which asexual reproduction occurs, and vertebrate definitive hosts that allow sexual reproduction. Humans become infected when they come into contact with water in which patently-infected snails have recently released free-swimming larvae (cercariae). The cercariae penetrate and migrate through the skin and after 2–3 days they enter the blood stream via a capillary or lymphatic vessel (6, 7). In 4–5 weeks the parasites mature into sexually differentiated adults that live in their hosts' blood for the rest of their lives. The adult male and female worms of *S. japonicum* and *S. mansoni* live in mesenteric capillaries and their eggs leave the body in faeces. *S. haematobium* worms live in blood vessels of the urinogenital system and their eggs are passed out in urine. On contact with water ciliated miracidia hatch from the eggs: they seek and penetrate a new host snail in which again to reproduce asexually and thus complete the life cycle.

Like the diseases caused by many other helminths and protozoa, schistosomiasis is a chronic disease. Adult schistosome worms have been estimated to have half-lives of between 3 and 10 years, but instances of worms surviving for 3–4 decades have been recorded. In many endemic areas the heaviest infections are found in children and young adults. Infection intensities generally decline with increasing age, but prevalences tend to remain relatively high. As a consequence of these patterns of intensity and prevalence morbidity from schistosomiasis is most severe in younger age groups. In schistosomiasis, as in other parasitic diseases, the distribution of infection loads is thus highly aggregated (8) and many people will thus have infections of low intensity which are asymptomatic and not obvious enough

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Table 1 Principal trematode and cestode parasitic infections

Species	No	Distribution
Chlonorcis sinensis (t)	7.01	China, Korea, Taiwan, Vietnam
Diphyllobothrium latum (c)	9.00	Worldwide where fish is eaten
Echinococcus granulosus (c)	2.70	Worldwide: *E. multilocularis* in latitudes north of 30°
Echinococcus multilocularis (c)		
Echinostoma species (t)	0.15	Philippines, Thailand
Fasciola species (t)	2.40	China, Egypt, Europe, Iran, S. America
Fasciolopsis buski (t)	0.21	China, India, Bangladesh, S. E. Asian countries
Heterophyes species (t)	0.24	Egypt, Iran, Korea
Hymenolepis nana (c)	75.00	Americas, Australia, developing countries
Metagonimus species (t)	0.66	Korea and S. E. Asia
Opishthorcis species (t)	10.33	Kazakhstan, Laos, Thailand, Ukraine
Paragonimus westermani (t)	21.68	China, Ecuador, Korea, Laos, Peru
Schistosoma haematobium (t)	113.88	Africa, East Mediterranean
Schistosoma mansoni (t)	83.31	Africa, Caribbean, S. America
Schistosoma japonicum (t)	1.55	China, Western Pacific
Schistosoma mekongi (t)	0.91	Cambodia, Laos
Taenia saginata (c)	77.00	Worldwide, related to beef consumption
Taenia solium (c)	10.00	Worldwide, related to rearing pigs for consumption
Total	416.03	

t = trematode; c = cestode. Adapted from Crompton DWT, How much helminthiasis is there in the world? J Parasitol 1999; 85: 397-403.

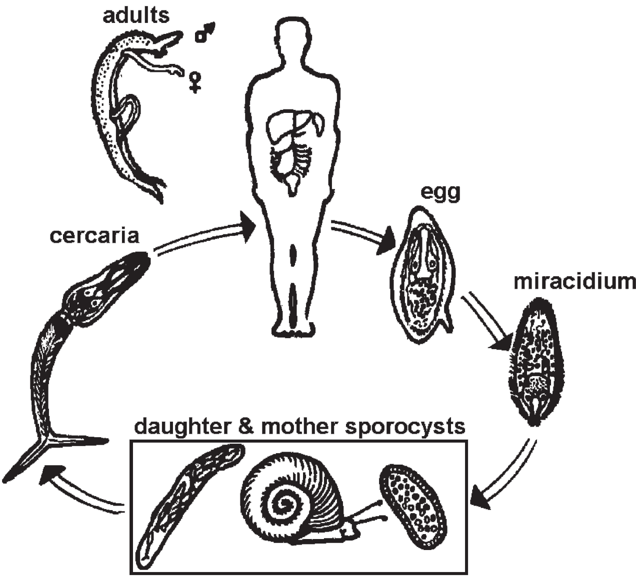

Fig. 1 The schistosome life cycle (simplified)

to warrant treatment. In sub-Saharan Africa the overall burden of disease from schistosome infection is, however, enormous: *S. haematobium* is estimated to cause haematuria in 70 million people, major bladder wall pathology in 18 million and hydronephrosis in 10 million. Annually 150 thousand die from non-functioning kidneys due to *S. haematobium* and 130 thousand from *S. mansoni*-induced portal hypertension (9).

Infections caused by all species of schistosome can be treated with the drug praziquantel (PZQ).

1.2 Fasciolosis

Fasciola hepatica in temperate regions and *F. gigantica* in the tropics are very important pathogens of sheep and cattle causing reduced growth due to liver damage and killing sheep with 'acute fluke disease'. In certain areas there are small foci of human infections as a result of ingestion of water plants contaminated with metacercariae.

1.3 Cestode (Tapeworm) Infections

Adult tapeworms do not usually cause significant pathology, so treatment is either for aesthetic purposes in pets, where owners do not like to see worm segments in the faeces, or to break the life cycle where larval stages are important. The larval (cyst) stages can cause considerable pathology, e.g. in man with *Echinococcus granulosus*, *Echinococcus multilocularis* and *Taenia solium* and chemotherapy of cysts is largely confined to these three species. Tapeworms in horses (primarily *Anoplocephala perfoliata*) can cause colic (10), so that treatment is recommended. Although a review of the literature (11) suggests that the tapeworm of sheep, *Moniezia expansa*, does not cause significant losses in production, in some areas it is considered an important pathogen, especially in parts of the former Soviet Union and southern Africa.

As mentioned, tapeworm larvae (cysts) can be a serious cause of disease in humans. Infections usually result from ingestion of oncospheres from the final host (e.g. canines with respect to *Echinococcus granulosus* and *E. multilocularis*, but which can include humans in a direct cycle of *Taenia solium*). Cysts of *T. solium,* the human pork tapeworm, are the commonest parasitic disease of the human nervous system and can result in seizures, epilepsy, hydrocephalus and dementia (12). This is one of the main causes of epileptic seizures in many less-developed countries (13). *E. granulosus* usually occurs in the liver and resembles a slow-growing tumour but

a small percentage of cysts can grow in the lungs or the nervous system. Rupture of a cyst in sensitized patients can lead to anaphylactic shock and even death. Control is most commonly by surgery. *E. multilocularis* normally lives in rodents and carnivores but when people become infected by ingestion of eggs the parasite grows in the liver and resembles a diffuse slow-growing tumour. It is regarded as the most dangerous human helminth infection with most untreated patients dying.

2 Treatment of Schistosomiasis with Praziquantel

The combination of excellent pharmacological properties against schistosomes and recent substantial reductions in price have meant that usage of PZQ is beginning to increase markedly (14). The majority of these infections however occur in people living in resource-poor conditions who do not have easy access to treatment. One response to this difficulty is the Schistosomiasis Control Initiative (SCI; http://www.schisto.org) funded by The Bill and Melinda Gates Foundation, which has the objective of reducing schistosome-induced morbidity in Africa by widespread treatment with PZQ, school children being targetted in particular (15).

A continuation of these recent trends will result in a massively greater rate of usage of PZQ than any other drug for treatment of trematode or cestode infections, with schistosomiasis being the principal target. This chapter will therefore deal mainly with PZQ in the context of its activity against schistosomes. After a brief description of the drug, its mode of use and its metabolism, the relatively limited knowledge available about resistance to PZQ in schistosomes will be discussed. Oxamniquine (OX) is another schistosomicidal drug, but it is effective only against one species, *S. mansoni*, and its rate of usage is diminishing. However, OX is given some attention below because it provides an interesting 'compare and contrast' exercise with PZQ.

For other recent reviews of the chemotherapy and drug resistance of schistosome infections the reader is referred to (16–21). For publications indicating that the rate of use of drugs for control of human schistosome and other helminth infections is set to increase markedly in the near future see (22–25).

2.1 History, Chemical Structure and Properties of Praziquantel

Early in the 1970s pyrazino-isoquinoline derivatives, initially synthesized and assayed for tranquilizer potential, were tested for anti-helminth activity (26). More than 400 compounds synthesized by E. Merck, Germany, were tested by Bayer (27) and one of the most effective during in vivo screening was PZQ, then identified as EMBAY 8440. Bayer initially marketed PZQ as Droncit for use as a veterinary cestocide, but in 1977 it was shown to be also effective against infections of different schistosome species in experimental animals (28) and performed satisfactorily in toxicological and pharmacological tests. The results of clinical trials performed jointly with WHO to test efficacy against *Schistosoma mansoni* (29), *S. haematobium* (30) and *S. japonicum* (31) were very positive and PZQ began to be marketed for human use as Biltricide. In 1983 the Korean company Shin Poong patented a new method for synthesizing PZQ and this initiated market competition that has resulted in marked price reductions. It is now produced by several generic manufacturers under a variety of brand names (e.g. Distocide, Bilharzid, Prazitel).

Praziquantel is 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino [2,1-a]isoquinoline-4-one and its chemical structure is illustrated in Fig. 2. It is a white crystalline powder and has a bitter taste, is stable under normal storage conditions, practically insoluble in water, but soluble in some organic solvents. The commercial preparation is a racemate composed of equal parts of 'laevo' and 'dextro' isomers, of which only the former has schistosomicidal activity either in vivo or in vitro (32–34).

Tablets of PZQ are usually oblong and contain 600 mg active ingredient. A syrup formulation containing 600 mg/5 mL suitable for small children is produced by some manufacturers: e.g. Epiquentel from EIPICO in Egypt. Recently over 30 samples of PZQ tablets from different producers were collected at user level and tested for quality (35, 36) and both brand and generic products complied well with industry standards. However, two samples from one manufacturer were counterfeit and did not contain any PZQ (37). PZQ can now be purchased for US$0.10/tablet or less. Thus in 2003 a normal treatment of 40 mg/kg body weight cost as little as US$0.20 per child and ~US$0.30 per adult (15), but the cost and degree of availability can vary from country to country.

Fig. 2 The structure of praziquantel

2.2 Efficacy of Praziquantel

The recommended treatment dose for schistosomiasis is 40–60 mg/kg bodyweight, the lower amounts being deemed suitable for *S. mansoni* and *S. haematobium*, and the higher dose (generally split in two administrations a few hours apart) for *S. japonicum* and *S. mekongi* (38). Bioavailability of PZQ is reported to increase with concomitant intake of food (39). Orally administered PZQ is rapidly absorbed and can be detected in the blood by 15 min after dosing (40), with peak levels normally occurring after 1–2 h (41). Individuals show widely varying peak plasma concentrations of 200–2,000 ng/mL (42).

A major asset of PZQ is its activity against a broad range of parasites (Table 1). Early experimental studies showed that it was more or less equally effective against all schistosome species (43) and this has been confirmed by a large data set accumulated from its use to treat endemic schistosomiasis throughout the world. Changes in patterns of schistosome egg excretion are mostly used to determine the drug's effectiveness, either in terms of a cure-rate (the number of patients who are not excreting eggs after treatment as a percentage of the number found excreting eggs before treatment) and/or the percentage reduction in the mean number of eggs excreted by the treated group. Earliest studies showed that PZQ always achieved cure-rates of 60% or greater, and often 85–90% (44). However, pertinent to a consideration of drug resistance, a 100% cure-rate has seldom, if ever, been recorded in an endemic area.

Also relevant to a discussion of drug resistance, and a potentially significant weakness of PZQ, is its relative lack of efficacy against juvenile schistosome worms in vivo and in vitro (28, 45–47). Schistosomes thus have an unusual biphasic sensitivity to PZQ and some other schistosomicidal drugs (45) whereby early migrating larval stages are susceptible, but susceptibility then decreases to low levels in 3–4 week-old infections and is only gradually regained. Worms of experimental infections are almost fully susceptible to PZQ when they are about 6–7 weeks old. The insusceptibility of immature worms may be a cause of some of the poor cure-rates and treatment failures observed in some patient groups, particularly those exposed to very high rates of transmission (see discussion below of the reasons for poor cure rates given by PZQ in Senegal.) A protocol involving administration of two courses of PZQ has been advocated for such situations (48) and adoption of this approach has indeed resulted in higher cumulative cure rates (49, 50).

Praziquantel is also effective against adult tapeworms and it is very widely used in pets, humans, sheep and most recently in horses [e.g. (51)]. It is also used for treatment of larval tapeworms. However, the dose used for adult tapeworms is considerably lower at 1–3 mg/kg than that for *Schistosoma* sp. (40 mg/kg). There are no published reports of resistance but failure of praziquantel to remove *M. expansa* from sheep has been found in New Zealand (52).

Early studies failed to show an effect of praziquantel on the surface of *F. hepatica* (53) and this lack of effect has been reflected in studies reporting low levels of activity [e.g. (54–56) but other reports suggest that it will work (57, 58)]. The reasons for the variation in response are not understood.

2.3 Metabolism, Toxicity and Side Effects of Praziquantel

PZQ is subjected to pronounced metabolism on first pass through the liver and it disappears relatively rapidly from the circulation with a half-life of 1–3 h. Elimination from the body is via urine and faeces and is 80% or more complete after 24 h (59). Cytochrome p450 enzymes are mainly responsible for metabolism of PZQ (60–62) and the bioavailability is thus increased by agents that inhibit cytochrome p450 activities (63). Subjects suffering from hepatic dysfunction, for example, because of severe schistosomal disease, metabolize PZQ more slowly (64). Metabolic derivatives of PZQ have not been fully analyzed for schistosomicidal activity.

In animal tests PZQ showed very low toxicity (65) and no genotoxic risks were detected in assays for mutagenicity (66). A review of existing data concluded that the few observations that suggested accumulation of potentially mutagenic metabolites may have been anomalies amongst a massive amount of evidence indicating PZQ is a safe drug (67). PZQ is tolerated and effective in patients of all ages and for treatment of the different forms of clinical schistosomiasis including cases of advanced hepatosplenic disease (68). Consistent with the conclusions that PZQ is a very safe drug, an *ad hoc* committee convened by WHO has recently recommended that it can be offered to pregnant and lactating women (69).

Side effects that are observed after treatment are generally relatively mild and transient, but affect as many as 30–60% of patients (70, 71). It is of interest that patients treated with the laevo form alone at half the dose of the racemate mixture had the same cure rates, but suffered fewer side effects (34). The frequency and intensity of side effects after normal treatment is correlated with the intensity of infection as measured by the number of eggs excreted before treatment, and the most severe side effects of bloody diarrhea or oedematous urticaria that are observed in areas with high intensities of infection (72) may thus be due to the release of the constituents of large numbers of dying worms and/or the host body's response to them.

2.4 Mechanisms of Action of Praziquantel

Some of the effects of PZQ on schistosome worms have been well-described, but the detailed molecular mechanisms of the drug's action are not yet known (19).

An obvious and rapid response of the worms after contact with PZQ is spastic paralysis of the musculature, which is accompanied – and perhaps caused – by a rapid influx of Ca^{2+} ions (73). Muscular contraction is not necessarily associated with worm death, however, since there are conditions of sublethal in vitro exposure (0.2–0.5 µg/mL for adults and 0.2–80 µg for immature worms) in which even a long-lasting paralysis is reversible after drug removal, with subsequent survival of the parasites (47). PZQ does not have the properties of an ionophore (73) and the ATP cases involved in pumping Ca^{2+} out of cells are apparently unaffected by PZQ (74).

Another early effect of the drug is a morphological alteration of the worm tegument consisting of vacuolization at the base of the tegumental syncytium and blebbing at the surface (53, 75). As a result of this damage antigen exposure is increased on the worm surface, particularly over the tubercles (76) and this, in turn, appears to render the worms more susceptible to attack by antibodies. Related to drug-induced antigen exposure, there is experimental evidence for a synergistic effect between drugs, including PZQ, and host antibodies in killing worms in vivo (77–81), but the efficacy of PZQ was not compromised in human subjects infected with HIV (82). These patients may however have become sensitized to schistosome antigens prior to becoming HIV-infected. Tegumental damage does not occur in the absence of Ca^{2+} ions or after exposure to the inactive dextro stereoisomer (83).

When experimental evidence was obtained showing that PZQ interacted with model membranes it was suggested that the drug altered the permeability or stability of schistosome surface bilayer membranes (84, 85). There have been no follow-up studies to confirm this hypothesis and it is argued against by the stereo-isomer selective schistosomicidal action of the drug.

Glutathione *S*-transferase has been suggested as a receptor for PZQ on the grounds that the 3-dimensional structure of this protein has a 'pocket' in which a molecule of the drug could fit (86), but the failure of PZQ to affect the activity of the enzyme tends to disprove this hypothesis (87).

Recent results have pointed to the beta sub-units of voltage-gated Ca^{2+} channels as targets of the schistosomicidal action of PZQ. The *S. mansoni* SmCa(v)βA and *S. japonicum* SjCa(v)β molecules have been shown to have structural motifs that differ from those found in other known beta subunits, and co-expression of these with a mammalian Ca^{2+} channel conferred sensitivity of the latter to PZQ (88). The Beta Interaction Domains (BIDS) of SmβA and Sjβ lack two conserved serines, each of which constitutes a consensus site for protein kinase C phosphorylation (89). It is the absence of these serines that appears to render schistosome cells sensitive to PZQ (89, 90).

3 Resistance to Praziquantel

3.1 Evidence of Resistance to Praziquantel in Schistosomes

There are three separate strands of evidence to indicate that *S. mansoni* can become resistant to PZQ. The first emanated from Senegal where the construction of a barrage dam to control the flow of the Senegal River in the mid-1980s resulted in an expansion of endemic schistosomiasis mansoni. By the early 1990s the rate of transmission had reached epidemic proportions in inland villages in Northern Senegal that had begun to benefit from agricultural irrigation projects along the dammed river (91, 92). When PZQ was used in an attempt to control the disease it gave cure rates of only 18–39% (93, 94), which were alarmingly low compared with the normally expected 60–90%. Increasing the dose of PZQ from 40 to 60 mg/kg body weight did not significantly improve cure rates (95). A more recent study involving treatment in the same area of Northern Senegal again resulted in relatively low cure rates (96). Two further observations indicated that *S. mansoni* in northern Senegal was responding aberrantly to PZQ: (a) a parasite line taken into laboratory passage from snails with patent infections collected in that area was found to have a decreased susceptibility to PZQ (97–99); (b) when the effect of oxamniquine (OX) was later tested in this area, the routine dose of 20 mg/kg gave a cure rate of 79%, compared with 36% in a simultaneously treated control group given 40 mg/kg PZQ (100).

Secondly, during the 1990s PZQ was also used widely in Egypt in a consolidated effort to control schistosomal disease (101) – it is estimated that between 1997 and 1999 alone some 60 million tablets were taken (35). During this period Ismail et al. (102) treated 1,607 *S. mansoni*-infected patients in the Nile delta region with 40 mg PZQ/kg body weight, and after an additional two treatments, the last at 60 mg/kg, 2.4% of the patients were still passing eggs. Laboratory life-cycles of several isolates were established from miracidia obtained from the eggs passed by uncured patients and the worms of these isolates were found to have two- to fivefold greater PZQ ED_{50}s after treatment in mice than isolates that had been established from eggs passed before treatment by patients who were easily cured (102). The in vivo results were confirmed by subsequent tests for responses to PZQ of individual worms in vitro (103).

Finally, in 1994 Fallon and Doenhoff (104) reported that resistance to PZQ could be selected for in laboratory-maintained *S. mansoni*. This was achieved by applying drug pressure to successive mouse passages of a 'hybrid' isolate that had earlier been raised from a pool of cercariae of four laboratory-maintained *S. mansoni* lines from different geographic areas. All the isolates contributing to the hybrid had been taken into laboratory passage before PZQ began to be used and the worms produced as a result of this selection pressure were less sensitive to praziquantel than controls not exposed to PZQ (99, 104).

In a recent collaborative series of experiments three laboratories in, respectively, Italy, Egypt and the UK, have used standardized protocols to estimate the ED_{50}s of *S. mansoni* isolates that were putatively resistant and sensitive to PZQ. Five representative resistant isolates, which originated from human treatment failures in Senegal and Egypt, or that were the result of laboratory-imposed drug pressure, were found to have a mean PZQ ED_{50} of 209 ± 48 mg/kg, which was approximately three times greater than the mean ED_{50} of 70 ± 7 mg/kg that was observed in four control *S. mansoni* isolates that had not ever knowingly been in contact with PZQ (105).

3.2 Mechanisms and Markers of Resistance to Praziquantel

In the absence of any firm knowledge of the mode of action of PZQ (see Sect. 2.4), hypotheses about mechanisms of resistance to this drug are bound to be highly speculative. Nevertheless, several genetically based differences between PZQ-resistant and sensitive isolates have been identified.

An analysis using a subtractive polymerase chain reaction (PCR) indicated that adult worms of a laboratory-selected PZQ-resistant isolate were expressing sub-unit 1 of the mitochondrial enzyme cytochrome C-oxidase at a five- to tenfold higher rate than worms of the parental hybrid isolate from which the former was derived (106). The actual activity of the enzyme, however, was unexpectedly fourfold lower in the resistant worms.

Use of a random amplified polymorphic DNA (RAPD) PCR showed that there was differential amplification of at least two major DNA nucleotide sequences between an Egyptian PZQ-resistant isolate and several PZQ-sensitive isolates from the same endemic area (107).

The discovery that a difference in the amino acid sequence of the beta sub-units of voltage-gated Ca^{2+} channels of schistosomes and other organisms may account for differential sensitivity to PZQ (see Sect. 2.4) stimulated an investigation to compare the sequence of these molecules in several PZQ-resistant and -sensitive isolates. No meaningful differences were found in cDNAs coding for either *Sm*$Ca_v\beta1$ or *Sm*$Ca_v\beta2$ that could account for differences in PZQ sensitivity between isolates (108). Furthermore, tests for differences in expression of β subunits between adults of different isolates or between adults and immature worms (see Sect. 2.2) gave negative results which were thus also not helpful in explaining drug resistance (108). This negative evidence does not however disprove the hypothesis that beta subunits of calcium channels may be involved in PZQ activity, since drug insusceptibility could arise from mechanisms other than modification of the drug's target; for example, a change in mechanisms of drug uptake and/or efflux.

It is important to note that thus far only a very limited number of putatively resistant and susceptible isolates have been compared with each other: extension of these comparative investigations is needed. The availability of isolates of *S. mansoni* from several different sources with confirmed differences in sensitivity to PZQ (105) should facilitate the search for the genetic and physiological mechanisms responsible for drug resistance.

Detection of PZQ-resistance currently has to rely mainly on either in vivo tests on infected mice or in vitro tests on adult worms derived from infected mice. Performance of these tests is therefore constrained to laboratories with the capacity to maintain and passage life-cycles and thus generally unsuitable for application in the field or clinic. In the immediate future it may therefore be necessary for tests to detect drug resistance to be performed 'centrally' in laboratories with appropriate facilities. Preliminary results indicate that the eggs and larvae (both miracidia and cercariae), as well as worms of resistant isolates, express phenotypic differences in terms of changes in survival or morphology that can be detected following exposure to PZQ in vitro (99). These assays could prove useful for detecting drug resistance in the field or clinic, but there is a need for markers that can be detected simply, for example, by PCR.

The immune-dependent action of PZQ and other schistosomicidal drugs has been noted (see Sect. 2.4). In the context of drug-resistance mechanisms, it was found that isolates deemed to be less sensitive to PZQ suffered a lesser degree of damage to their surface membranes than susceptible isolates after exposure to the same dose of drug in vitro (109) and in vivo (110). This factor may modulate the susceptibility of worms to immune attack in vivo, and thus confer a modicum of drug resistance that presently available in vivo assays for resistance would not be able to distinguish from, for example, the effects of a mutation in a specific drug receptor molecule. Changes in antigens, or antigen exposure cannot however alone account for PZQ-resistance in *S. mansoni* as worms from the laboratory-selected resistant isolates survived higher doses of PZQ in vitro than those of susceptible isolates (Pica-Mattoccia et al., unpublished results.)

Also as noted above, immature schistosome worms are relatively 'resistant' to schistosomicidal chemotherapy. It

has therefore been argued that the poor cure rates and treatment failures that have been observed in areas of high infection intensity and prevalence, which in turn result from high rates of transmission of the infection in such areas, are not due to a genetically driven difference in susceptibility to PZQ in adult worms, but instead to the presence of immature worms in the patients at the time they are treated (48, 111). This argument is supported by the higher cumulative cure rates that are achieved when two treatments are given a few weeks apart (49, 50). However, a *S. mansoni* isolate collected in patent snails (not from treated patients) before much PZQ had been used in northern Senegal was found to have a decreased susceptibility to the drug (97). This isolate did mature relatively slowly in mice, but nevertheless it was still less susceptible to PZQ than a similarly slow-maturing isolate from Kenya (98). Furthermore, oxamniquine is a drug that, like PZQ, is relatively ineffective against immature worms in experimental infections (45), but as mentioned above, when used at the normal dose rate in humans in Senegal it was more effective than PZQ (100).

In a meta-analysis that compared the data from Senegal with that from other areas, it was calculated that even after accounting for intensity of infection and sensitivity of diagnosis, Senegal remained atypical by showing cure rates significantly lower than expected (112). The authors of the latter study concluded that 'the suspicion of tolerance or resistance to PZQ.… cannot be ruled out.'

4 Alternative Agents for Schistosomiasis

4.1 Oxamniquine

Oxamniquine (OX) provides some interesting contrasts with PZQ, particularly with respect to the factors that affected its market potential and the amount of information we have about its mechanisms of action and of schistosome resistance to it. It was synthesized by Pfizer in the late 1960s and initial laboratory studies in mice and primates indicated that it was effective against *S. mansoni* (113). The first clinical trials were performed in Brazil, these showing it was safe and effective against *S. mansoni* (114).

Oxamniquine is 6-hydroxymethyl-2-isopropylaminomethyl-7-nitro-1,2,3,4,-tetrahydroquinolone. Its structure is illustrated in Fig. 3, as is that of hycanthone, a compound chemically related to OX and which also has activity against *S. mansoni*. Hycanthone had to be abandoned, however, because of suspected mutagenic, carcinogenic and teratogenic activity. Hycanthone has a 3-ring planar structure that is typical of DNA-intercalating agents, whereas OX has a simpler structure that has been shown to be devoid of intercalating activity (115).

Hycanthone Oxamniquine

Fig. 3 The structures of hycanthone and oxamniquine

In contrast to PZQ, OX is ineffective against *S. haematobium* or *S. japonicum*, the other two main species of schistosome infecting humans, and use of OX has been almost entirely restricted to Brazil and other South American countries. This factor, predicated mainly by its limited spectrum of anti-parasitic activity, imposed severe restraints on its marketability. Unlike PZQ, the price of OX has therefore remained practically unchanged and the latter is being replaced by PZQ now even in Brazil (116), the country that used OX for all its schistosome control activities throughout the 1980s and 1990s (117).

4.1.1 The Activity of Oxamniquine Against *S. mansoni*

When worms are exposed to OX in vitro no immediate adverse effects are noted: it is only 2–7 days (depending on the dose) after an initial 30-min exposure followed by culture in normal medium that the damage becomes noticeable, followed by the death of the worms some days later (16). A similar delay in schistosomicidal effect occurs in vivo (118). However, only short times of exposure to OX are required to cause worm death: 15 m in vitro or 2 h in vivo before transfer, respectively, to drug-free medium or untreated animals (119). OX is more effective against male worms than against female worms by a ratio of approximately 2:1, but like PZQ and some other schistosomicides, it is largely ineffective against immature worms (45).

Comparison of the efficacies of analogues of both OX and hycanthone indicated that the hydroxymethyl $-CH_2OH$ group (Fig. 3) was essential for activity. Replacement compounds carrying a methyl $-CH_3$ group instead were inactive in vitro or for parenteral administration, but were still active after oral intake because they were converted to hydroxymethyl metabolites by intestinal flora. Compounds with only a hydrogen atom in this position were completely inactive (120).

Nucleic acid synthesis is the first metabolic activity to be inhibited by OX: protein synthesis and all other metabolic pathways are affected only later (121). Experiments with tritiated OX indicated that the drug formed stable covalent bonds with worm DNA. Worms from *S. mansoni* isolates

that were resistant to OX failed to bind significant amounts of radioactive drug, as did *S. haematobium* and *S. japonicum* worms; i.e. species intrinsically insusceptible to OX (115).

4.1.2 Schistosome Resistance to Oxamniquine

Shistosomiasis mansoni in East Africa has been reported to be relatively less effectively treatable with OX than in Brazil (122), but the basis for this apparent difference has not been established. *S. mansoni* can otherwise become resistant to very high concentrations of OX (123). Genetic crosses between sensitive and resistant *S. mansoni* isolates, achieved by transplanting single male and female worms of opposite genotypes to new mouse hosts and testing F1 and F2 progeny for resistance, clearly indicated that resistance to OX is an autosomal recessive character (124). It was therefore predicted that OX per se is inactive as a schistosomicide, and it is activated by chemical transformation and OX-resistant schistosomes lacked a factor necessary for activation of the drug.

Extracts of drug-sensitive *S. mansoni* worms were shown to contain enzymatic activity capable of binding tritiated OX to DNA and other macromolecules, and this activity was absent in OX-resistant *S. mansoni*, in both *S. haematobium* and *S. japonicum*, and in representative human and rat cells (125, 126). It was also found that the drug-DNA binding activity was less abundant in female worms and scarcely present in immature schistosomes, in accordance with the relative drug-sensitivities of these organisms. Further work on characterization of the enzyme indicated that the enzyme that activated OX into a schistosomicidal derivative was a sulfotransferase (16). When two OX-resistant isolates from different geographic regions (Brazil and Puerto Rico) were crossed, the F1 progeny were resistant indicating that the same gene was responsible for resistance in the two independent isolates (121).

OX is therefore a pro-drug that a specific sulfotransferase enzyme activity in the parasite converts to a sulfate ester, which in turn undergoes spontaneous dissociation into an alkylating moiety that forms covalent bonds with schistosome macromolecules, notably DNA (127). Resistance to OX is thus due to the absence of the sulfotransferase activity.

Table 2 summarizes the main differences between praziquantel and oxamniquine.

Table 2 Comparison of praziquantel with oxamniquine

	Praziquantel	Oxamniquine
Effective against	All schistosome species	*S. mansoni* alone
Mechanism of action	Not known	DNA alkylation
Potential to develop resistance	Relatively low	Very high
Mechanism of resistance	Not known	Lack of drug activation
Price and usage	Cheap & extensive	Expensive & limited

4.2 *Artemisinin Derivatives*

Artemisinin is the active ingredient of the herb *Artemisia annua*. It is a sesquiterpine lactone which contains a peroxide bridge, from which synthetic derivatives have been synthesized including artemether and artesunate. Artemisinins are potent anti-malaria drugs and millions of doses have been administered for this purpose. Artemisinin activity against *Schistosoma japonicum* was discovered in the early 1980s (128) and effectiveness against other schistosome species confirmed subsequently (129). These compounds are well-tolerated and give only mild side effects, but their mode of action is not yet fully understood.

In contrast to the aforementioned drugs PZQ and OX, artemisinins are more active against immature than mature worms and it is in this context that artemether and artesunate have been used effectively in China as 'prophylactics' against *S. japonicum* infection during major floods (130). Chemoprophylactic effectiveness has also been demonstrated against both *S. mansoni* (131) and *S. haematobium* (132).

So far there are no reports of resistance to artemisinins in schistosomes, or in malaria against which it has been more widely used. Because of the poor cure rates given by PZQ in areas with high rates of infection transmission (which in turn may be due in part or wholly to the insensitivity of immature schistosomes to this drug) artemisinins may be of most use in these areas. Proposals for large-scale use of artemisinins in areas where *Plasmodium* spp. and schistosomes coexist, particularly in sub-Saharan Africa, will however naturally raise concerns about creation of conditions for inducing drug resistance in the former.

4.3 *Ro 15-5458*

With the marketing of praziquantel an interesting schistosomicide discovered by Hoffmann La-Roche, 10-(2--diethylamino)ethyl-9-acridanone(thiazolidin-2-ylidene) hydrazone, was not developed. Since the realisation that resistance to praziquantel could become a problem and discovery of novel schistosomicides is very difficult, there is renewed interest in the development of Ro 15-5458.

The first report of the activity of Ro 15-5458 against *S. mansoni* was in trials in vervet monkeys, where at 15 and 25 mg/kg worms were killed and tissue egg counts greatly reduced (133). At 25 mg/kg derivatives of 9-acridanone-hydrazone derivatives were fully active against *S. mansoni* in baboons (134). Ro 15-5458 killed almost all skin schistosomula in mice at 100 mg/kg and in Cebus monkeys it was fully effective at 25 mg/kg 7 days after infection (135). Against adult worms in mice a dose of 20 mg/kg removed 95% of *S. mansoni* and resulted in a disappearance of all immature

stages in worm eggs (136). If 4-week-old *S. haematobium* was treated in hamsters at 25 mg/kg Ro 15-5458 was more effective than praziquantel (total dose of 1,000 mg/kg) but at 8 and 12 weeks cure rates were similar (137). Combined one-third doses of Ro 15-5458 and praziquantel gave a 99.4% cure rate in susceptible CD mice (138). Since combination therapy is a well-known method of slowing the development of resistance, use of both products together would be beneficial but costs and the beginning of praziquantel resistance make it impractical.

Ro 15-5458 was rapidly absorbed in rabbits with a half life of about 6 h (139). From its effects on the worms it appears that the first target is RNA (140) with a resultant inhibition of parasite genes (141). Immunity to schistosomes can be produced by irradiated cercariae and treatment with Ro11-3128 which results in surface vesicles expressing surface antigens. Although Ro 15-5458 kills schistosomula in the skin, attenuation of infection at this stage produced a significantly lower level of resistance to reinfection than treatment with Ro 11-3128 and this was reflected by failure of serum from the treated mice to recognise Mr 28,000 and 32,000 antigens (142). The activity of Ro 15-5458 against differing ages of schistosomes and at least two species suggest that if the toxicity is acceptable and manufacturing costs are cheap enough, it could provide a useful alternative to praziquantel.

4.4 Ro 11-3128

In the 1970s, more than 400 benzodiazepines were screened for antischistosomal activity at Hoffmann-La Roche and some members of the group turned out to be quite active (143). Among them, the anticonvulsant clonazepam and its methyl derivative designated Ro 11-3128.

Ro 11-3128, given as a single oral dose of about 80 mg/kg, cured 90% of mice or hamsters infected with *S. mansoni* or *S. hematobium*, while *S. japonicum* was completely refractory to treatment. A lower dose (25 mg/kg) was curative in monkeys. Most important, the drug was active against immature stages. Initial toxicology and mutagenicity trials proved that the drug is well tolerated in animals (143). A clinical study in South Africa showed that a dose of 0.2–0.3 mg/kg was curative for most patients infected with either *S. mansoni* or *S. hematobium* (144). Unfortunately, the drug causes a severe and long-lasting sedation, accompanied by ataxia and muscle relaxation (145). To circumvent this problem, the benzodiazepine antagonist Ro 15-1788 could be administered without loss of antischistosomal activity (146), but the antagonistic effect of Ro 15-1788 was of short duration and repeated administration was required in order to counteract the long-lasting sedative action of Ro 11-3128. Due to these side effects, further development of the drug was abandoned.

Pax et al. (147) carried out extensive experimental work on Ro 11-3128, demonstrating that the drug has in vitro effects that are very similar to those of praziquantel (spastic paralysis, influx of calcium, tegumental vacuolization and blebbing). Benzodiazepine receptors in schistosomes have been the object of a study by Bennett (148) and renewed interest in the issue has appeared more recently (149).

It is unlikely that Ro 11-3128 may be used as a schistosomicide in human therapy, but the compound has similarities and differences with respect to praziquantel that might help interpret the mechanism of action of the latter drug (both compounds emerged from a screening of chemicals active on the central nervous system, both cause rapid Ca^{2+}-mediated contractions and tegument alterations, both display stereoselective activities).

4.5 Myrrh

There have been reports that a preparation of the plant extract myrrh has schistosomicidal activity in experimental animals (150) and humans (151). A recent evaluation of myrrh in experimental animals has however found no evidence of such activity (152).

5 Cross-Resistance and Spread of Resistance to Schistosomicides

So far the only noteworthy instance of cross-resistance in schistosomicidal drugs is between oxamniquine and hycanthone which is most likely due to a structural similarity in the two (see Sect. 4.1). However, in contrast to OX, development of hycanthone for treatment of schistosomiasis had to be abandoned because of its apparent carcinogenicity and mutagenic potential (16).

Several pieces of evidence indicate that there is no cross-resistance between OX and PZQ. Thus the resistance to each of these drugs that was selectively bred into two respective lines of laboratory-maintained *S. mansoni* was drug-specific (104) and OX gave normally expected cure rates against schistosomiasis mansoni in an area in northern Senegal in which PZQ had given poor cure rates (100). Additional evidence for an absence of cross-resistance is given by other clinical data (153, 154).

The evidence on PZQ accumulated so far only indicates that there is variation between schistosome isolates with respect to their sensitivity to the drug and the degree of variation uncovered is small – no more than three- to fivefold differences in the ED_{50}s of putatively resistant and sensitive control parasites. Fortunately as yet no case can be made for the occurrence of resistance against PZQ that is comparable

with the levels of resistance that have developed against drugs for treatment of many bacterial or protozoan infections, or even against the antischistosomal drug OX. However, there seems little doubt that in the near future schistosomes in many parts of Africa will be subjected to higher levels of drug pressure than in the past. The contribution that the offspring of worms that survive this pressure will make to the genetic constitution of an endemic population of schistosomes will depend on a number of factors, of which perhaps the two most important are the relative sizes of the 'refugia' into which they are entering and their relative 'biological fitness'.

5.1 Refugia

The concept of 'refugia' is assuming increasing importance in the analysis of the dynamics of drug resistance in helminths of sheep and cattle (155–157). Provided refugia populations remain large relative to the number of incoming offspring of drug-treated and uncured schistosomes the impact of the latter on the genetic constitution of the population as a whole will be small. Large refugia are likely to be found in human populations living in areas of high infection intensity and prevalence, and are subjected to chemotherapy only randomly or selectively. Similarly, infested environments in which intense transmission is occurring without interference from measures intended to control it (e.g. mollusciciding) are likely to provide relatively large refugia.

Human populations subjected to mass chemotherapy and/or endemic areas with low transmission rates will provide smaller refugia. Alternative scenarios that could enhance the impact of genetically drug-resistant organisms on the schistosome population as a whole can thus be envisaged: for example, if mass chemotherapy was performed at a time when an intermediate host snail population was re-establishing itself and was therefore largely uninfected, as might occur soon after flooding or application of molluscicide.

As with so much else with regard to our knowledge about PZQ, firm and consistent evidence about the relative biological fitness of putatively resistant and susceptible isolates is lacking. Six of the isolates established from uncured Egyptian patients have been studied through multiple successive passages in mice over a period of 5 years (158). In the absence of drug pressure three of these retained their initial levels of insusceptibility to PZQ, while two reverted to drug sensitivity that was no different from controls. The three isolates that had retained decreased sensitivity to PZQ showed some evidence of decreased cercarial production by infected snails (158), thus indicating there may be a cost of biological fitness in PZQ resistance.

When three *S. mansoni* lines that had been isolated from uncured Senegalese patients in the mid-1990s and subsequently passaged in laboratory mice without drug pressure for approximately 5–6 years were tested for susceptibility to PZQ they were found to be less susceptible than several control isolates (159). In addition, similar to the Egyptian isolates that remained resistant despite laboratory passage (158), the more recently performed tests on the three Senegalese isolates showed that they shed fewer cercariae per snail than other non-resistant non-Senegalese isolates, though the snails infected with the former survived longer (159).

6 Drugs for Liver Fluke Infections

Liver fluke infections can be treated with a number of fasciolicides (Table 3), some of which only kill adult fluke whilst others kill immature stages as well.

Given the importance of fasciolosis, it is surprising that there are no validated tests for resistance other than a controlled trial, that the molecular mechanisms of action remain unknown for all fasciolicides and that the mechanisms of resistance, where they occur, are not understood.

6.1 Triclabendazole

The most widely used drug is triclabendazole as it kills fluke from 1 week of age and older. It is technically a benzimidazole but must have a different mechanism of action as it does not act on nematodes and albendazole will kill adult triclabendazole-resistant *F. hepatica* (160). Furthermore, since all other fasciolicides also kill adult triclabendazole-resistant flukes they must all have a different mode of action to triclabendazole (160, 161). The exact mechanism of action of triclabendazole has not been established, but in common with some other fasciolicides one of the first signs of action is disruption of the tegument as well as secretory activity. This latter effect is compatible with disruption of microtubule function (162), but clearly cannot occur at the same site as other benzimidazoles. Triclabendazole sulphoxide also inhibits mitotic division of spermatogenic cells

Table 3 Fasciolicides licenced in the UK.

Action group	Name	Effective against immatures
Uncouplers	closantel	Yes
	nitroxynil	Yes
	oxyclosanide	No
Tubulin inhibitors	albendazole	No
	ricobendazole	No
Unknown	triclabendazole	Yes
Glycolysis inhibitor	clorsulon	No

(163) again suggesting disruption of microtubule function possibly by binding to a non-cholchicine binding site (164). In addition it seems that triclabendazole may inhibit protein synthesis (165).

6.2 Uncouplers

Rafoxanide has been withdrawn from sale in many countries, but the structurally related salicylanilide, closantel, is widely used for control of both fluke and *Haemonchus contortus*. Both closantel and the halogenated phenol, nitroxynil, have activity against immature fluke. The salicylanilide, oxyclosanide at normal doses only has activity against adult fluke. As all are uncouplers of energy formation in mammalian mitochondria, it has been suggested that they have a similar mechanism of action in fluke. However, nitroxynil might be active on ion permeability in muscle cells (166) rather than affecting mitochondria in what are largely anaerobic animals. Morphological and metabolic studies using the salicylanilides are compatible with effects on mitochondria (166). There is cross-resistance between rafoxanide, closantel and nitroxynil but apparently not to oxyclozanide. The difference with oxyclozanide could be related to blood profiles with a very short persistence for oxyclozanide but long half-lives for closantel (164). This is an area requiring further investigation as closantel and nitroxynil will become the major fasciolicides as resistance to triclabendazole spreads.

6.3 Benzimidazoles

Resistance has been selected in the laboratory to the experimental benzimidazole luxabendazole (164), but has not been reported for albendazole. Fluke eggs are prevented from embryonation by incubation in solutions of benzimidazoles (167) in a way similar to inhibition of embryonation in nematode eggs (168) suggesting a similar mechanism of action on β-tubulin. When an albendazole-resistant isolate is found it should be relatively easy to confirm this hypothesis as the sequence of *Fasciola* β-tubulin is already known (169).

6.4 Clorsulon

Present evidence suggests that clorsulon acts by inhibition of glycolysis leading to slow paralysis (164, 170, 171). There have been no reports of resistance to clorsulon so nothing is known about possible resistance mechanisms.

7 Other Drugs for Tapeworms

7.1 Pyrantel

Pyrantel is used exclusively for the treatment of *Anaplocephala perfoliata* in horses where a double dose (38 mg/kg as pyrantel embonate) is effective (172) and is not used in other species for control of tapeworms. It is assumed that the drug affects acetylcholine receptors as in nematodes, but the action of levamisole and pyrantel resistance has not yet been elucidated at the molecular level in nematodes. No resistance to pyrantel has been reported but a change in behaviour is widespread, with worms attaching to the caecal wall rather than the ileocaecal junction. It seems reasonable to assume pyrantel concentrations are less in the caecum than in the lower small intestine, so this change in attachment site may represent an adaptation to under-dosing with pyrantel which happens when the normal dose is used for treatment of nematodes in horses. *In vitro* tests suggest that the worms attaching to the caecal wall are not resistant (173).

7.2 Benzimidazoles

Modern benzimidazoles (e.g. fenbendazole, oxfendazole and albendazole) are used to treat *M. expansa* in sheep. The detailed mechanism of action is not known but presumably it acts similarly to its effect on nematodes where the drugs bind to β-tubulin preventing polymerisation to form microtubules. Reduced activity of benzimidazoles has been reported in sheep in New Zealand (52, 174) and in South Africa (cited in 157) but the mechanism of resistance is not known. It could occur by a point mutation in the β-tubulin as occurs, for example, in *H. contortus* (175). Multiple doses of albendazole and mebendazole are used to treat cysts of *E. granulosus* and the development of resistance has been found in laboratory studies (176), but again the mechanism of resistance has not been established.

7.3 Nitroscanate

This is used to treat tapeworms in dogs and cats. The detailed mechanism of action is not known and no cases of resistance have been reported.

7.4 Niclosamide

Reduced activity may have developed in sheep in South Africa (cited in 157), but there are no further details of the exact mechanism of action of this uncoupler on cestodes, or of the mechanism of resistance.

7.5 Treatment of Larval Tapeworms

7.5.1 Taenia solium

Infection in both humans and pigs can be reduced by treatment of people with praziquantel, but a dose of 10 mg/kg is recommended (177). Niclosamide (2 g per patient) has also been used to reduce prevalence (178). Since cysts in pigs are killed by treatment with benzimidazoles the infection can be controlled in both hosts. A single oral dose of 30 mg/kg of oxfendazole results in death of all cysts by 12 weeks (179), but single lower doses are not so effective (180). Both albendazole and praziquantel are used for treatment, though a definitive dose for treatment of people with neurocysticercosis has not been established (12) so the necessary minimum length of treatment and optimal doses are not known.

7.5.2 Echinococcus granulosus

Chemotherapy is with either mebendazole, or albendazole, or albendazole plus praziquantel, the combination appearing to be more effective than use of a single drug (181–183). Efficacy may be improved by use of cimetidine which increases benzimidazole serum concentrations (184, 185). Treatment with benzimidazoles is recommended prior to surgery (186). Different doses have been used in different trials but albendazole in the range 12–15 mg/kg per day over weeks or months may be used and the activity may be increased by using an emulsion formulation and with administration from 3 months > 1year it showed an efficacy of 98% and a cure rate of 75% (187).

7.5.3 Echinococcus multilocularis

Treatment is with long-term courses of benzimidazoles (mebendazole or albendazole). Both anthelmintics produced an overall success rate of 97%. Since albendazole was less expensive and easier to take it may be the preferred drug (188). Experimental murine studies suggest that using nitazoxanide with albendazole may improve antiparasitic activity (189).

8 Concluding Remarks

For the foreseeable future praziquantel will be overwhelmingly the drug used for treatment of trematode and cestode infections in humans, particularly schistosomiasis and except for treatment of fasciolosis. The large reductions in its price that started in the 1990s will lead to a much greater rate of usage under the auspices of national and multinational control programmes such as The Schistosomiasis Control Initiative.

There is currently much debate whether PZQ is destined to suffer the fate of becoming less useful because of drug resistance, as has been the case with very many other anti-infection drugs. Some of the recent discussion has been concerned with whether or not PZQ-*resistant* schistosomes already exist, but it is now at least clear that variation in susceptibility to PZQ does exist in *Schistosoma mansoni* (105) in so far as isolates of this species that have been exposed to praziquantel either in the field or in the laboratory have a decreased drug sensitivity in comparison with isolates that have never been exposed to the drug, either because the latter were established before the advent of praziquantel or because they came from patients that were later successfully treated. The ED_{50} differences are relatively small, but generally reproducible, and no 'super-resistant' isolates have been encountered so far even after continuous application of drug pressure in the laboratory (Doenhoff et al, unpublished results).

The degree of variation in susceptibility found so far in *S. mansoni* may not pose a real problem for human chemotherapy, especially since the doses routinely employed in clinical practice – at least theoretically – eliminate the large majority of parasites. Continued monitoring seems necessary, however, particularly during the course of prolonged chemotherapy-based control programmes, since we could be party to only the first step of an escalation to resistance.

Much of the recent debate on resistance to PZQ has to a large extent obscured the fact that when this drug is used according to recommended schedules it can result in relatively low cure rates, for example, in Senegal (93, 94) and Uganda (183). One obvious remedial strategy – increasing the dose – unfortunately did not appear to improve cure rates (95). Adoption of protocols involving two successive closely spaced treatments with the same drug (48, 111, 190) or treatment of initial therapeutic failures with a different drug (146) may be effective, but such strategies will of course be more expensive. Combination chemotherapy is currently not used for schistosomiasis, but the prospects for this have recently been reviewed (191).

Situations now unquestionably exist or will develop where schistosomiasis is not treatable very effectively with PZQ and this will most likely be in part due to the intrinsic limitations of the drug when dealing with recent infections. In spite of its enormous usefulness, PZQ is therefore not *the* perfect drug. Unfortunately, with the possible exception of artemisinin derivatives (yet to be developed into front-line anti-schistosome drugs), the last schistosomicides were introduced in the 1970s, since no systematic attempt has been made to discover new drugs apart from a small investment recently made by WHO/TDR to promote compound screening. Pharmaceutical companies have not been at all motivated to

invest in research for antischistosomal drugs, since the prospects of economic returns are far from realistic.

Similarly the cost of developing new drugs for the control of fasciolosis of domestic animals is such that there is little interest in searching for new alternatives. When resistance occurs to both triclabendazole and closantel/nitroxynil only products for treating adult fluke will be available which will restrict effective disease control. Before that situation develops, validated tests for resistance are urgently required so that meaningful management systems can be put in place to try to limit the development and spread of fasciolicide resistance. Similarly validated tests are required for the detection of resistance in adult tapeworms.

Due to lack of commercial interest public institutions, international organizations and charitable foundations will need to continue leading at least in the initial stages of drug-discovery. It is also important that an effort is made to create and sustain research environments that will attract the best minds to tackle the infectious diseases that affect overwhelming numbers of people and animals in resource-poor countries.

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Chapter 46
Drug Resistance in Ectoparasites of Medical and Veterinary Importance

Kathryn Stafford and Gerald Coles

1 Introduction

Early insect control on animals relied on a combination of husbandry methods coupled with the use of mineral oils and tar distillates such as kerosene, phenols and cresol. While these proved to be highly effective insecticides, they were also comparatively toxic to the host. A number of inorganic compounds based on arsenic, boron and fluoride have also been used in insect control and the first reports of resistance were to these compounds. The development of DDT (dichlorodiphenyltrichloroethane) as an insecticide in 1939 led to its use worldwide for insect control. This was most evident in the control of the malaria and typhus vectors during World War II where large amounts were used. Concerns over toxicity to both humans and wildlife, coupled with reports of product failure led to its widespread withdrawal during the 1970s. Since then, a number of new insecticides have been marketed, but as with any drug, regular and indiscriminate use has led to the development of resistance to the majority of these products.

2 Background

The first reported case of control failure was to lime sulphur in 1914 by the San Jose scale, *Aspidiotus perniciosus*. Early reports of resistance were in agricultural crop pests; however, by 1946 resistance had been recorded in the cattle tick, *Boophilus microplus* and the blue tick *Boophilus decoloratus,* in both cases to sodium arsenite dips. These early reports of resistance received very little attention until 1946 when DDT reportedly failed to control the house fly *Musca domestica* in both Sweden and Denmark. In 1947, failure of DDT to control the bedbug *Cimex lectularius* was reported from Hawaii and in 1951 the human body louse *Pediculus corporis* in Korea and Japan.

Owing to these early reports of DDT failure, the product has been withdrawn from sale in the majority of countries because of its toxicity and has been replaced by newer generations of insecticides. The 1950s saw the development of the carbamate insecticides which are similar to organophosphates (OPs) in that they exert an anticholinesterase action on the insect's nervous system by inhibiting cholinesterase enzymes. These were followed by the pyrethrins and pyrethroids, which due to their relative low toxicity to mammals are used widely in the control of fleas on pets and in the treatment of the human head louse. Resistance to the synthetic pyrethroid and permethrin has been recorded in both the species (25, 47).

Later classes of insecticides include the insect growth regulators which are effective where an immediate kill is not essential since they act on the developmental stage of the insect larvae; the macrocyclic lactones such as ivermectin, the arylheterocycles phenylpyrazoles such as fipronil and the chloronicotinyl nitroguanidines such as imidacloprid.

3 Definition of Resistance

Resistance is defined by the World Health Organisation as 'development of an ability in a strain of some organism to tolerate doses of a toxicant that would prove lethal to a majority of individuals in a normal population of the same species'. It has been suggested that a better definition would be 'a response of an organism or a population to a toxicant that enables the organism or population to withstand future toxicant exposures better, because gene amplification which may confer resistance does not require selection (23) and other individual responses to sub-lethal exposures are included' (9).

Whatever the definition of resistance used, monitoring field-collected isolates for the presence of resistance is necessary for maintaining the efficacy of insecticides through resistance management. A simple test used for monitoring resistance is the contact test. Groups of insects are held, for established time periods, in tubes containing insecticide-impregnated papers. At the termination of the given time period, the number of live and dead individuals are counted. From this data, the establishment of LD (lethal dose) values can be calculated. These are useful in monitoring resistance once it has reached high levels in any given population, but are limited in detecting the emergence of resistance (74). The introduction of the discriminating dose (34)

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to distinguish between resistant and susceptible individuals has proved more efficient than estimating resistance using regression lines (74). The discriminating dose is defined as the dose which just kills 100% of susceptible test insects within a given population. Any individuals from the field-collected isolates which survive at this dose are by definition resistant.

4 Mode of Action and Mechanisms of Resistance

There are a variety of neural transmitters and neural modulators present within insects. Neurotransmitters are chemical messengers released into the synaptic cleft where they have a temporary effect on the electrical potential of the postsynaptic membrane. Neuromodulators are released into the vicinity of the synapse where they modify synaptic transmission. A number of these, acetylcholine (Ach), γ-aminobutyric acid (GABA), glutamate, serotonin and octopamine, have been examined in detail. The neurotransmitter acetylcholine has been found to be the most important excitatory neurotransmitter present in the insect central nervous system. The receptors occur in at least two forms, nicotinic Ach and muscarinic Ach (79, 89). Glutamate is the principle excitatory transmitter found at the junctions of nerves and muscles while GABA is the principle inhibitory neurotransmitter at the nerve/muscle junction as well as the central nervous system.

4.1 The Organophosphates

Organophosphates target the enzyme acetylcholinesterase (AchE), a key enzyme in the nervous system. This enzyme is a glycosylated dimer which is attached to a membrane via a glycolipid anchor (16, 31). In insects, it terminates nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine. When targeted by organophosphates (OP), enzyme activity is inhibited by the serine residue within the active site gorge being covalently phosphorylated (21). Resistance to OPs-has been correlated with the overexpression of AchE (31), but is more usually associated with a point mutation which is frequently accompanied by a modification of the kinetic parameters of acetylcholine hydrolysis (24, 45, 64, 91, 99). Resistance-associated mutations involving substitutions at key sites located within the active site gorge of the enzyme have a steric effect or alter the orientation of the active site residues (90). However, the isolation of AchE sequences from *Boophilus microplus*, indicate that an insensitive AchE phenotype does not result from an amino acid substitution in the AchE protein itself which would suggest the involvement of another mechanism (4).

4.2 The Carbamates

Carbamate insecticides target the same site as OPs (the enzyme acetylcholinesterase), and inhibit the enzyme activity by carbamylating the serine residue within the active site gorge (21). They are derivatives of carbamic acid and are relatively unstable compounds which break down in the environment within weeks. Hinkle et al. (38) observed higher AchE activity in two strains of the cat flea *Ctenocephalides felis*, one resistant to organophosphates while the second strain was resistant to the carbamate, propoxur, and suggests that the same mechanism of resistance is shared by the two insecticides.

4.3 The Pyrethroids

The pyrethroid insecticides are classified into type 1 and type 2 compounds. Type 1 compounds include DDT plus analogues and all pyrethroids containing descyano-3-phenoxybenzyl or other alcohols (8). They cause a repetitive discharge at the presynaptic nerve end. Type 2 compounds contain an α-cyano-3-phenoxybenzyl alcohol and cause a toxic release of transmitter, indicative of membrane depolarisation (8). Under normal conditions, the sodium current activates and deactivates within a few milliseconds but in the presence of pyrethroids this state is altered. Pyrethroids delay the deactivation of the sodium channel prolonging the open state and thus allowing a persistent inward current which results in repetitive firing and depolarisation of the nerve membrane.

There are two major mechanisms of pyrethroid resistance in insects. One is associated with an increase in the rate of metabolic detoxification of the insecticide while the other is associated with changes in target site sensitivity.

4.4 The Insect Growth Regulators

As the name implies, the insect growth regulators cause a disruption in the moulting process. Insect growth regulators (IGRs) such as methoprene act as juvenile hormone mimics preventing the process of ecdysis while ones such as diflubenzuron inhibit the synthesis of chitin (27). Since they act on the immature stage of the insect, they are widely used in environmental control but are of little use when the immediate control of adult insects is necessary. However, lufeneron is used to prevent viable egg production in the cat flea.

4.5 The Macrocyclic Lactones

The avermectins and milbemycins are a group of related macrocyclic lactones isolated from *Streptomyces* microorganisms. They block electrical activity in the nerve and muscle preparations by increasing the membrane conductance to chloride ions causing ataxia and paralysis. Blocking occurs at the α-subunit of the glutamated-gated chloride channels in invertebrates. Work undertaken with the nematode *Caenorhabditis elegans* suggests that in order for resistance to occur there must be simultaneous mutations of several GluClα genes. Early reports of resistance were recorded in the house fly, *Musca domestica* (75) and the cockroach, *Blattella germanica* (20).

4.6 The Arylheterocycles Phenylpyrazoles

Phenylpyrazole insecticides such as fipronil disrupt normal nerve functions by blocking the GABA-gated chloride channels of neurons in the central nervous system. The GABA receptors are responsible for inhibition of normal neural activity by preventing excessive stimulation of the nerves. Blocking of these receptors results in neural excitation and ultimately death. *Anopheles gambiae*, resistant to dieldrin, were also found to be resistant to fipronil (11), but there have been no recorded incidences of resistance in other insects of medical and veterinary importance.

4.7 The Chloronicotinyl Nitroguanidines

Imidacloprid, thiacloprid and nitenpyram belong to this group of insecticides and are used as crop and structural pest insecticides and for flea control treatment. Imidacloprid binds to the nicotinergic acetylcholine receptors present on the post-synaptic membrane in the nervous system. This induces a slow depolarization in motorneuron cells from cockroach nerve cord preparations (2). To date, there have been no reported cases of resistance in ectoparasites to this insecticide.

5 The Development of Resistance in Individual Genera of Insects

5.1 Arachnida

5.1.1 Acari (Mites)

The acari are a subclass of the Arachnida containing two superorders, the anactinotrichida which contain the orders astigmata, prostigmata and oribatida and the actinotrichida which contains four orders, two of which are of medical importance, the mesostigmata and ixodida. The order astigmata contain the medically and veterinary important *Sarcoptes* and *Psoroptes* mites which cause scabies in humans and mange or scab in domestic animals in addition to various other allergic responses. The larval stages of three families of mites can act as vectors of scrub typhus, a rickettsial disease affecting humans while other families cause dermatitis in both humans and domestic animals. Treatment is traditionally with either topical applications of lindane or permethrin; however, recently ivermectin has proved effective. Resistance has been described in Norwegian scabies to lindane (67), and other failures, e.g. to permethrin, have been reported (summarised by (36)).

The order Prostigmata contain the *Demodex* mites of which *D. canis* can cause demodectic mange in dogs whilst related species infect domesticated farm animals. The order Mesostigmata includes the suborder Dermanyssina which is of veterinary importance since this includes the chicken red mite *Dermanyssus gallinae*. Resistance has been recorded in *D. gallinae* to DDT, organophosphates and pyrethroid insecticides (5, 19, 30, 98).

Early reports of resistance in *Psoroptes ovis* came from South America to the insecticide hexachlorociclohexane (HCH) (1) and lindane plus diazinon (71). Following UK deregulation of sheep scab, resistance has been reported to the synthetic pyrethroid, flumethrin (86) and the organophosphate, propetamphos (18).

5.1.2 Ixodida (Ticks)

The ixodida ticks can be divided into two groups, the argasidae or soft ticks and the ixodidae or hard ticks. Both feed on vertebrate blood and are major vectors in the spread of disease.

Resistance was first recorded in *B. microplus to* arsenic prior to 1940 and to DDT and other chlorinated hydrocarbons by the early 1950s (92). By the late 1960s, resistance to the organophosphorus acaricides and carbamates had also been reported (82). Resistance to chlorvenphinphos, amitraz and cypemethrin has been reported in *Boophilus decoloratus* in South Africa (61). Significant cross-resistance has been shown between carbaryl and the organophosphates, coumaphos and diazinon (51). In South America, *B. microplus* resistance has been documented to organophosphates, synthetic pyrethroids and amitraz (60). Martins and Furlong (59) documented the first failure of injectable avermectin in the control of *B. microplus*. Amitraz resistance has been reported in Mexico (70). Its toxicity was significantly synergised by both triphenylphosphate and piperonyl butoxide (50). From crossing experiments, it appears that more than one gene is involved and resistance is inherited as an incomplete recessive trait with a strong maternal effect on the larval progeny (52).

Li et al. (49) reported resistance in *B. microplus* to the acaricides, coumaphos and diazinon with a significant cross-resistance pattern between the two. Their results suggest that an enhanced cytochrome P450 monooxygenase (cytP450)-mediated detoxification mechanism may exist in resistant strains in addition to the cytP450-mediated metabolic pathway that activates coumaphos. The failure of piperonyl butoxide (PBO) to synergise diazinon suggests a specific cytP450 involved in detoxification (49). They concluded that resistance to coumaphos was likely to be conferred by a cytP450-mediated detoxification mechanism in addition to the mechanism of insensitive acetylcholinesterases. A recent finding from resistant populations in Yucatan, Mexico involved a sodium channel mutation (isoleucine substituted for phenylalanine) in the S6 transmembrane segment of domain III (72).

5.2 Insecta

5.2.1 Diptera (Flies)

A number of diptera families contain species of medical and veterinary importance. These range from the nuisance bite of midges and stable flies through the transmission of diseases

and various forms of myiasis. Myiasis is defined as the invasion of living tissue of animals by larvae of Diptera (43).

5.2.2 *Ceratopogonidae* (Biting Midges)

The *Ceratopogonidae* is a large family containing more than 60 genera and nearly 4,000 species (95). Most of the females within this family require a protein meal for maturation of the ovaries and this is only acquired from a blood meal taken from warm-blooded animals in four genera. The genera of *Culicoides* is the largest and is of veterinary importance in horses since the bite of *Culicoides* can lead to the development of culicoides hypersensitivity, more commonly referred to as sweet itch. They are also important as vectors of arboviruses, blood-dwelling protozoa and filarial worms. Since people entering into areas of midges are more sensitive to their bite, control has been essential in certain parts of the world before an area can be opened up for tourism. Despite this, no reports of resistance have been recorded.

Psychodidae–Phlebotominae (Sandflies)

Around 700 species of phlebotominae have been described, found mainly in warmer climates. They are the intermediate host of leishmaniases and vectors of *Bartonella* and the viral disease, papatasi fever. Since their distribution is patchy and they are rarely present in sufficient numbers to reach pest proportions, control is conducted by house spraying, usually as part of malaria control. This could account for resistance to DDT in *Phlebotomus argentipes* in Bihar, India (44). Evidence for elevated esterases and altered acetylcholinesterase has been found in flies trapped in southern India (85). Deltamethrin-impregnated collars for dogs have recently been introduced to areas where canine leishmaniasis is widespread (58).

Simuliidae (Blackflies)

There are 24 genera of simuliidae of which four are of economic importance. The females of most simuliids require a blood meal for egg development to occur and they will feed on a variety of mammals including man. Eggs are laid in slow running water and therefore control is targeted towards this stage of the insect life cycle. Simuliidae are important as vectors in the spread of the filarial worm, *Onchocerca volvulus* which causes onchocerciosis or river blindness. In 1994 Davies (22) reviewed onchocerciasis control and described cases of resistance including resistance to DDT, temephos and chlorphoxim which spread very widely once it occurred in migratory species. Resistance has been recorded in the larva of simuliidae to the insecticide permethrin (39). Montagna et al. (62) reported on the mechanisms of both pyrethroid and DDT resistance in populations of Patagonian *Simulium* where resistance was attributed to agricultural insecticide exposure.

Tabanidae (Horseflies, Deer Flies and Clegs)

There are more than 400 species of tabanidae organised into four subfamilies, three of which are of economic importance since they are the vectors of three species of filarial worm and various viral diseases. In large numbers, tabanids worry stock leading to a loss of production. While little effort has been made to control tabanidae species (48), describes the use of lambda-cyhalothrin-impregnated ear tags for the control of *Tabanus fuscicostatus*.

Glossinidae (Tsetse Flies)

Tsetse flies are the biological vectors of pathogenic trypanosomes which cause sleeping sickness in humans and nagana in cattle. The breeding and feeding habits of tsetse make chemical control difficult and therefore most control is via visual- and odour-baited traps (87). As a result, the development of resistance has not been reported.

Muscidae and Fanniidae (Houseflies and Stableflies)

Musca domestica, the house fly, has a worldwide distribution and is important since it has been found to harbour over 100 different pathogens from helminths to viruses. Their role in the spread of disease is unclear. Greenberg (33) found a reduction in diarrhoeal infections due to *Shigella* but those due to *Salmonella* were unaffected following spraying with DDT. However, because of their close contact with man chemical control has been extensively used. One of the earliest recorded incidence of DDT failure was to the house fly in Sweden and Denmark and to OPs occurred in Australia (35).

The stomoxyinae are haematophagous insects found worldwide. Both *Stomoxys calcitrans* and *Haematobia irritans* are of economic importance since their bite causes worry in livestock which can lead to reductions in milk yield and loss of condition. The control of *H. irritans* in the United States of America has primarily been based on the use of insecticides and therefore widespread resistance has resulted. The first reported case of resistance was to the OP fenchlorphos in 1963 (13); however, there had been unconfirmed reports of DDT failure as early as 1959 (14). This was followed by reports of resistance to tetrachlorvinphos which was the first insecticide used in impregnated ear tags (83). The late 1970s saw the use of pyrethroids for *H. irritans* control, but reports of resistance soon followed (73). Recent studies have shown resistance to diazinon, fenthion, ethion, pirimiphos-methyl and tetrachlorvinphos (3).

Calliphoridae (Blowflies)

The calliphoridae is a large family of over 1,000 species. Two families are of medical and veterinary importance, the Chrysomyinae and Calliphorinae. The Calliphorinae are important since they are the agents of myiasis or the invasion of living tissue. *Cochliomyia hominivorax* is an obligatory agent of myia-

sis (43) and will attach to both animals and humans. Because of their life cycle, they are ideally suited to control by non-chemical methods and therefore in the USA and Central America the screwworm fly has been eradicated through a controlled program of sterile male release. Flies in the genus *Lucilia* and *Calliphora* are facultative agents of myiasis which can cause considerable economic loss as well as being a major welfare issue. Control is essential in the large sheep-producing countries. Early control methods relied on plunge dip formulations of organochlorine compounds. Resistance was first recorded in Australia to the organophosphate insecticide in 1965 (81), to the carbamate group of insecticides in 1973 (76) and malathion in 1984 (40). McKenzie (56, 57) reported resistance to dieldrin, diazinon and malathion, all primarily due to allelic substitutions at a single genetic loci. Bioassays of field and laboratory populations (78) indicated no resistance to the pyrethroid, deltamethrin, despite its widespread use for lice control. Because of the welfare issues involved with myiasis and the development of resistance, alternative methods of control are being evaluated using traps and targets.

Oestridae (Gad Flies, Warble Flies and Stomach Bots)

The Oestridae are divided into four groups or subfamilies – Oestrinae, Hypodermatinae, Gastrophilinae and Cuterebrinae. The Oestrinae develop in the nasopharyngeal cavity of sheep, goats, equids and camels. The Hypodermatinae and Cuterebrinae are dermal parasites of cattle, rabbits and rodents, while the Gasterophilinae parasitise the alimentary tract of equids. Rich (69) described the infestation of sheep with *Oestrus ovis* as a relatively benign disease. There are no current reported cases of resistance. The warble flies *Hypoderma bovis* and *H. lineatum* are parasites of cattle which cause the phenomenon called 'gadding' and result in reduced weight gain and reduced milk production. *Gasterophilus* are stomach bots of equids that cause swelling around the point of attachment which in heavy infestations can cause chronic gastritis, loss of condition and in rare cases, perforation and death (7). As with the Oestrinae, there are no current reports of resistance.

5.2.3 Hemiptera (Bugs)

While a number of Hemiptera are bloodsucking, only two families are of medical importance, the Cimicidae and Triatominae. Both are temporary ectoparasites of birds and mammals.

The two species of *Cimex* of medical importance are the bedbugs *C. lectularius* and *C. hemipterus*. Both parasitise on humans and chickens while *C. lectularius* also parasitises domestic animals. *C. lectularius* is distributed throughout both temperate and subtropical regions while *C. hemipterus* occurs in warmer tropical regions.

While many have been implicated in the spread of disease, there is no scientific data to support this; however, Hepatitis B antigens have been found to persist for up to 6 weeks and are present throughout this period in the faeces (94). Lyons et al. (55) recorded the presence of HIV in *C. lectularius* for up to 1 h allowing for the possibility of mechanical transmission, but (53) showed that bedbugs were not major routes for the distribution of Hepatitis virus and were therefore much less likely to transmit HIV since this is a much less virulent virus.

The medical importance of bedbugs is because of their irritating bite which can cause sleeplessness.

By far the more important biting bugs medically are the triatominae as they act as vectors of Chagas disease caused by *Trypanosoma cruzi*. The mature trypanosomes are found in the faeces of the bugs which are deposited on the skin while the bug feeds. Once triatominae are infected with *T. cruzi*, they remain so throughout their life which can extend for several years.

Venezuelan *Rhodnius prolixus* was found to be resistant to all pyrethroids tested; but Brazilian *Triatoma infestans* remained susceptible to β-cypermthrin and lamda-cypermthrin (88). *T. infestans*, highly resistant to pyrethroids, have been reported from northern Argentina (66). Gonzalez et al. (32) suggest that degradative esterases and cytochrome P450 may be involved in *T. infestans* resistance to pyrethroids. *Cimex* was reported to be resistant to DDT following anti-malaria house spraying (15, 54). The introduction of pyrethroid-impregnated curtains for the control of these bugs (37) could result in its development. The use of pyrethroid-treated bednets in Tanzanian villages resulted in the disappearance of bedbugs for a period of 6 years (65).

5.2.4 Phthiraptera (Lice)

The phithiraptera comprise four groups. The Anoplura and Rhynchophthirina groups are bloodsucking lice of mammals, while the Amblycera and Ischnocera groups are chewing lice which live on skin debris. Within the Anoplura group are the medically important *Pediculus capitis, P. humanus* and *Pthirus pubis* (the human head, body and pubic lice). *P. humanus* is important for its role as the vector in the spread of epidemic typhus (*Rickettisia prowazekii*) and relapsing fever (*Borrelia recurrentis*). The medical importance of *P. capitis* and *P. pubis* is due to the development of secondary infections following scratching.

Early reports of insecticide failure were in *P. humanus* to DDT following its extensive use in World War II. Resistance has been widely reported in *P. capitis* to synthetic pyrethroids (17, 25, 63) and the beginning of resistance to the insecticide, carbaryl has been reported in the UK (26). Resistance has also been recorded in the sheep louse (*Bovicola ovis*) in Southern Australia to synthetic pyrethroids (42). Lee et al. (46) showed that the mechanism primarily involved in pyrethroid resistance in head lice is due to the two point mutations in the para-orthologous sodium channel α-subunit possibly supplemented by oxidative metabolism as shown by synergism with PBO.

5.2.5 Siphonaptera (Fleas)

The order Siphonaptera is a large one comprising over 2,000 species and subspecies. They are laterally compressed, hematophagous insects with a worldwide distribution. Two of

the most important species are *C. felis* and *C. canis*, the cat and dog flea (77) since both have a low host specificity and will parasitise both animals and man. Both species have been reported to be the intermediate host of *Dipylidium caninum* the dog tapeworm (96) and (97) which is an occasional parasite of man (80). More importantly, for medical reasons they are reported to be the transmitter of Friend Leucemia Virus (68), *Rickettsia typhi* (29) and *Yersinia pestis* (6, 41, 93). In addition to their importance in the spread of diseases, the bite of the flea can cause the condition flea allergy dermatitis in domestic pets.

Early reports of resistant fleas occurred in 1952 from the southern United States, and by 1971 resistance was being recorded to the insecticides chlordane, dieldrin and hexachlorocyclohexane (HCH) (12). Since then, resistance has been recorded to bendiocarb, carbaryl, diazinon, malathion, propetamphos and propoxur (28) plus, cypermethrin, D-phenotrin, fenvalerate, permethrin and resmethrin (47). The later generation of insecticides used for flea control such as the arylheterocycles, phenylpyrazoles have not yet been implicated in the development of resistance, but it is probably just a matter of time before they are.

6 Cross-Resistance

Cross-resistance is where an insect with resistance to one insecticide is able to survive exposure to a related insecticide. Examples of this are where insects display resistance to both lindane and dieldrin or parathion and malathion because of a common mode of insecticide action such as inhibition of acetylcholineasterase. If the same mechanism of resistance does not occur, then multiple resistance is developed and not cross-resistance. While it is understandable that cross-resistance can develop between insecticides in the same class, cross-resistance has also been recorded between different groups e.g. the pyrethroid, deltamethrin and the organophosphate, fenthion, in *Aedes aegypti* (10) and organophosphate resistance plus butacarb resistance in *Lucilia cuprina* (76).

7 Conclusion

There is clearly a lack of knowledge regarding mechanisms of resistance in many ectoparasite species, the genetics of resistance and possible changes in the biology associated with the development of resistance. There is an almost total lack of knowledge on the epidemiology of insecticide resistance even in such important insects as the cat flea.

Despite this lack of knowledge, experience in the control of ectoparasites demonstrates that any widespread use of insecticides is very likely to result in the development of resistance. To combat this, treatments should either give 100% control or substantial populations of ectoparasites need to be left untreated to ensure that parasites surviving treatment make only a small contribution to the next generation. Where ectoparasites are permanent residents on the host, this latter strategy will obviously not be acceptable on the grounds of welfare.

Therefore, monitoring for insecticide resistance is vital using tests based on discriminating doses or, where available, biochemical and/or molecular based tests. Where resistance is found, alternative insecticides must be used or alternative non-insecticidal treatments must be developed. These may include the development of vaccines, use of target traps, application of repellents and non-chemical control agents such as emulsions of oils to prevent insect respiration, fine silica dust to disturb water balance or changes in lighting patterns to disrupt feeding behaviour e.g. (84). Ideally, integrated pest management will evolve using a variety of strategies to control ectoparasites so that total reliance on chemical control is not necessary thereby reducing the risk of resistance developing.

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